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Section: Microbiology, Mycotoxins and Food Safety

Elimination of post-harvest and pre-harvest aflatoxin contamination

Bhatnagar, D.*#
USDA/ARS/SRRC, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124, USA.
Email: Deepak.Bhatnagar@ARS.USDA.GOV

* Corresponding author
# Presenting author

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Abstract

Aflatoxins are extremely carcinogenic secondary metabolites produced by *Aspergillus flavus* when this fungus invades crops such as maize, cotton, tree nuts and peanuts prior to harvest, and a host of other commodities after harvest (during storage). Adverse health effects from the ingestion of these toxins have caused regulatory agencies throughout the world to limit the amount of aflatoxins that are permitted in food or feed that is available for sale. This results in undue economic burden on the grower. Strategies to address the food safety and economic issues employ both pre-harvest and post-harvest measures to reduce the risk of aflatoxin contamination in food and feed. Post-harvest measures, such as adequate storage, detection and decontamination or disposal, as well as continuous monitoring of potential contamination during processing and marketing of agricultural commodities, have proved to be crucial and indispensable in ensuring food and feed safety; however, these measures do not address the issue fundamentally. The post-harvest contamination is usually the result of preharvest presence of fungal contamination. Therefore, research focus in the past decades has shifted from post-harvest control to a more preventive approach employing various pre-harvest control measures. Pre-harvest control includes good cultural practices such as insect control, irrigation during drought conditions, planting and harvesting dates. In addition, new biotechnologies such as, 1) the use of non-toxigenic biocompetitive strains of *A. flavus* for biocontrol of aflatoxin contamination, and 2) identification of plant constituents that disrupt aflatoxin biosynthesis or fungal growth and their use in new biochemical marker-based breeding strategies to enhance resistance in crops to aflatoxin, could potentially save the agricultural industry in the U.S. alone hundreds of millions of dollars. Study of the genetics of the aflatoxin biosynthetic pathway for understanding how and why this fungus makes aflatoxins has enabled scientists to examine strategies to interrupt aflatoxin synthesis, thereby preventing aflatoxin contamination of crops. The fungal genome of *A. flavus* has been sequenced to understand the regulation of aflatoxin formation by environmental factors. This information is being used to assist in the development of host-resistance against aflatoxin contamination by studying the effects of various physiological parameters, *e.g.*, drought stress on gene expression in toxigenic fungi.

Aspects of the methodology validation for light filth in fruit pulp

Dimov, M.N.*#, Atui, M.B., Silva, A.M., Marciano, M.A.M., Franco, V.P.A., Chasin, L.B., Silva, L.A., Fioravanti, M.I.A., Oliveira, M.M.L.
Adolfo Lutz Institute, Food and Chemistry Division, Food Microscopy Section - Av. Dr. Arnaldo, 355 – Cerqueira César - SP/Brazil – CEP: 01246-902. E-mail: dimonog@usp.br

* Corresponding author
# Presenting author

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Abstract

Methodology validation is an important tool to support the activities of metrology in the quantification analysis. Extraneous materials may be present in the pulps due to the conditions or the practices of production, storage and distribution. Since there is no specific methodology for fruit pulp light filth quantification a research has been carried out to test two methods for isolation of light filth in mango, strawberry, tomato and guava pulps. For guava, mango and tomato pulps the AOAC Official Method 964.23 was used, and for strawberry pulp AOAC Official Method 950.89, with adaptations. The micro-analytic standard for insect fragment and rodent hair was prepared in the laboratory. The study has been conducted on 63 samples of 100 g of pulp analyzed in duplicate after contamination with 5, 15, 30 insect fragments (IF) per 100 g and 5, 10, 15 rodent hair (RH) per 100 g, with blank samples as a control. The validation parameters used were precision and accuracy. Recovery of insect fragment was considered satisfactory in mango, tomato, guava pulps, and the average recovery ranged from 87 to 96%. For strawberry pulp, the recovery rate ranged from 68 to 80%. For rodent hairs, the best recovery rate was observed in mango pulp ranging from 73 to 81%, followed by guava pulp ranging from 65 to 76%, tomato pulp ranging from 50 to 67%, and strawberry pulp ranging from 33 to 35%. It was concluded that the methodologies used to detect light filth in guava, mango, tomato and strawberry pulps can be adopted in the monitoring routine of fruit pulp extraneous material contamination even though they were found not very efficient for rodent hairs.

Keywords: Insect fragment, Rodent hair, AOAC determination method, Recovery rate, Fruit pulp.

1. Introduction

The validation of the analytical method is an important requirement to support the activities of metrology in the measurement and analysis to show that the method is suitable for its intended use. A validation process has to be well defined and documented. In microscopic analysis the micro-analytical methods used for light filth isolation can be found among the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, 2005).

Food contaminated with light filth looks normal, not allowing the consumer to identify it by naked eye. Examples of light filth are insect fragments, animal hairs, bird barbules, mites, among others. Analytical methods require a large number of steps to prepare the sample for the final extraction. Light filth has a specific affinity for oil and can be trapped off in an oil layer, filtered and examined microscopically.

The fruit pulp is the product obtained by crushing the edible parts of fleshy fruits through appropriate processing. To meet the regulation governing hygienic and sanitary condition of fruit pulp, the product should be prepared with healthy fruit, clean, without parasites and other animal or plant waste and without inedible parties (Brazil, 2003).

A growing number of products derived from fruit that has been developed and launched in the domestic market and for exportation demand that require the compliance with satisfactory quality and safety standards. Extraneous materials may be present in the pulp due to poor conditions or practices of manufacturing, storage and distribution which include decayed organic materials, miscellaneous materials such as soil, sand, glass and others.

Since there is no specific methodology for fruit pulp in the AOAC Official Methods considering that they are Brazilian products, a study to evaluate the performance of two methodologies (pasta and fruit jelly) for isolation of light filth in mango, strawberry, tomato and guava pulps has been carried out.

2. Materials and Methods

2.1. Material

Specifically for this study the fruits that were used for fruit pulp processing that are guava, mango, tomato and strawberry have been grown in the city of Matão-SP, Brazil.

2.2. Methods

The experiment took place from May to December 2009, in the Microscope Food Section at Adolfo Lutz Institute. To detect light filth in guava, mango and tomato pulps we used the AOAC Official Method 964.23 (16.10.05), Chapter 16, pp. 29, 2005, with the following adaptations:

- The sample was weighed in the 2 L Wildman trap flask.
- The agitation was limited to 15 min per sample.
- Heptanes was used as flotation oil.
- Before the first trapping sequence, the waiting time was 20 min before trap off.
- Then, before the second trap off the waiting time was fixed at 10 min.
- During extractions, the rod and neck of the bottle trap were washed with hot water.

To search for light filth in strawberry pulp, AOAC Official Methods 950.89 (16.10.06, Chapter 16, pp. 30, 2005) was used with the following adaptations:

- The sample was weighed in 1L capacity Wildman trap flask.

During the extraction process:

- The magnetic stirring was fixed at 5 min.
- After filling the flask with water, stirring was done every 5 min on a period of 20 min.
- The waiting time was fixed at 10 min.
- The rod and the neck of the bottle trap were washed with hot water.

2.3. Sample Contamination

Micro-analytical standards for insect fragments (Order: Dyctioptera; Family: Blatellidae) and rodent hair (Mus musculus L.) were prepared in the laboratory using the technique described by Brickey et al. (1968). Contamination was made using the following spike levels: 5, 15, 30 for insect fragments (IF) per 100 g and 5, 10, 15 for rodent hairs per 100 g.

2.4. Collaborative Study

The study has been performed by 9 analysts during 4 months. Sixty-three samples of 100 g of pulp were analyzed in duplicate totaling 126 determinations contaminated with 5, 15, 30 insect fragments (IF) per 100 g and 5, 10, 15 rodent hairs (RH) per 100 g, with blind samples as a control.

3. Results and discussion

The presence of insects and rodent hairs count method was used to evaluate the sanitary conditions of the fruit pulps during their production, storage and distribution. Insect fragments and rodent hairs were chosen based on the Brazilian legislation (Brazil, 2003), which enforces a strict regulation on the presence of extraneous materials harmful for human consumption. The validation parameters used were precision and accuracy.

Recovery of insect fragment was considered satisfactory in mango, tomato, guava pulps, for which the average recovery rate remained in the range of 87 to 96%, and for strawberry pulp for which the range was 68 to 80%, as shown in Table 1. For recovery of rodent hairs, the best recovery rate was observed with mango pulp ranging from 73 to 81%, followed by guava pulp ranging from 65 to 76%, tomato pulp ranging from 50 to 67%, and strawberry pulp ranging from 33 to 35%, as mentioned in Table 2.

Table 1 Average recovery rate of insect fragments (IF) in mango, guava, tomato and strawberry pulps contaminated by different numbers of IF (5, 15 and 30 IF/100 g).

	Recovery Rate (%) Insect Fragments Added (#/100 g)		
Plant	**5**	**15**	**30**
Mango**	93.94	95.94	92.76
Guava**	93.33	89.26	90.37
Tomato**	87.41	90.58	87.12
Strawberry*	79.68	67.96	71.18

*AOAC Method No. 950.89 (16.10.06); ** AOAC Method No. 964.23 (16.10.05)

Table 2 Average recovery rate of rodent hair (RH) in mango, guava, tomato and strawberry pulps at different contamination levels (5, 10 and 15 RH / 100 g).

	Recovery Rate (%) Rodent Hair (RH)		
Plant	**5**	**10**	**15**
Mango**	73,45	72,54	80,85
Guava**	75,56	65,00	70,74
Tomato**	57,22	66,82	50,41
Strawberry *	33,43	33,58	35,22

*AOAC Method No. 950.89 (16.10.06); ** AOAC Method No. 964.23 (16.10.05)

Strawberry pulp was tested using the same methodology for other pulps, but the results were not acceptable due to large amount of residue in the extracted material. Then, another methodology was tried (AOAC Official Method 950.89). Strawberry pulp has its own characteristics, like great quantity of fruit (akene) and hairs. The large amount of residues in strawberry pulp might have influenced the recovering of insect fragments and rodent hairs. It was believed that the rodent hair was stuck to strawberry hair, style, stigma that remained at the bottom of the Wildman trap flask. Although its recovery rate was lower than at other pulps, this data could not be ignored and should be improved in future studies. According to Dimov et al., (2004), recovering of rodent hair could be a problem linked with the stirring conducted by the analyst. Brickey (1968) also reported that the stirring method plays an important role in recovering this kind of contaminant and depends on the formation of a vortex that barely touches the stir bar and no audible or visible splashing occurs. The recovery of rodent hair when compared to insect fragment was poor in all pulps, showing that the techniques used were more effective for insect fragments.

Figures 1 and 2 showed the data obtained by analysts for both contaminants in tomato pulp. It was observed more precision for insect fragments which average recovery rate remained in the range of 80 to 100% in the 3 contamination levels. For the rodent hair, average recovery rate remained in the range of 20 to 100%. The variability among analysts and contamination level was high, showing more spread results than for insect fragments recovery.

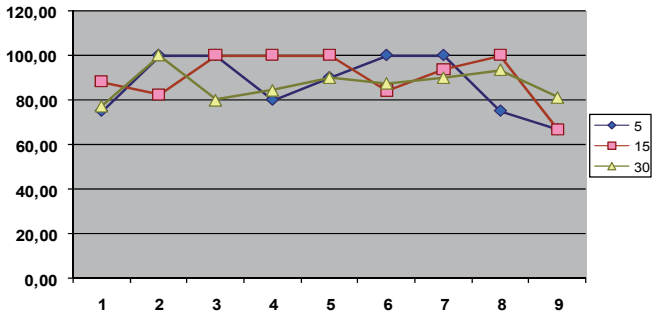

Figure 1 Variability of the recovery rate of insect fragment (IF) in mango, guava, tomato and strawberry pulps by different laboratory assistants (IF contamination levels: 5, 15 and 30 IF/100 g).

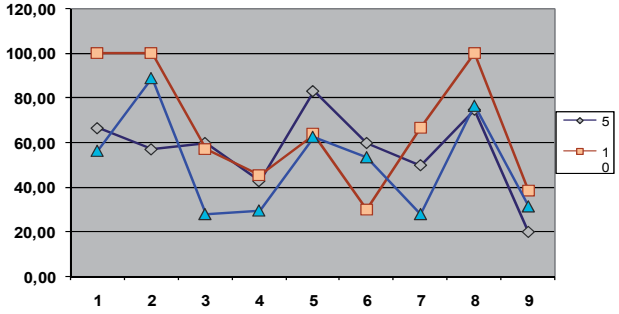

Figure 2 Variability in the recovery rate of rodent hair (RH) in mango, guava, tomato and strawberry pulps by different laboratory assistants (contamination levels 5, 10 and 15 RH/100 g).

4. Conclusion

Based on the results obtained, it was concluded that the methodologies applied in this validation study can be used for the isolation of light filth in guava, mango, tomato and strawberry pulp, and might be adopted in routine monitoring due to the lack of appropriate techniques until now. The methodology used for the recovery of rodent hairs may underestimate the actual amount of this contaminant. More studies are needed to improve the techniques for routine use development.

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Evaluation of contamination for extraneous materials in "sun meat" sold in the "houses of the north" in the municipality of Diadema (SP, Brazil)

Mennuci, T.A.[1]*, Marciano, M.A.M.[1], Atui, M.B.#[2], Poli Neto, A.[1], Germano, P.M.L.[1]
[1] Coordinator for Health Surveillance – Surveillance Service for Diadema – Diadema – SP - CEP: 09911-160. Email: tatifisvet@bol.com.br
[2] Adolfo Lutz Institute, Food and Chemistry Division, Food Microscopy Section - Av. Dr. Arnaldo, 355 – Cerqueira César - SP/Brazil – CEP: 01246-902. Email: marcatui@terra.com.br

* Corresponding author
# Presenting author

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Abstract

The "sun meat" is a handmade product, combining surface techniques of salting and dehydration, commonly used by people from the North and Northeast regions of Brazil. The sun meat cooking process lacks in sophisticated technology and official standards of procedure and quality. Thus, production, sale and distribution under unsatisfactory sanitary conditions are risky to the consumers' health. This paper is aimed at evaluating the sanitary conditions of "sun meat" sold at the "houses of the north" for the presence of extraneous materials. Analysis of 44 samples of "sun meat" from 22 "houses of the north" located in "Diadema District" (SP, Brazil) had been carried out. The product conditions of exposure and sale in the "houses of the north" were evaluated and provided additional information to assess the degree of food safety of this product. In 44 samples were found various types of extraneous materials such as whole insects and debris, larvae, exuvia, mites, rodent hairs, bird feather pieces, fungi, and sharp objects. Mechanical vectors were observed at 11 locations of the sale area. The results indicated that 90.9% of the "sun meat" has unsatisfactory sanitary conditions, caused by the presence of physical hazards of contamination. These results, associated with conditions found in the "houses of the north", indicated that these products can put the health of consumers at risk.

Keywords: Sun meat, Sanitary Conditions, Food security.

1. Introduction

The sun meat is obtained from the combination of salting techniques and partial dehydration of the meat, being widely consumed by people from some regions of Brazil, mainly North and Northeast (Costa et al., 1999). The sun meat can be confused with jerked beef, an industrial product (Lira et al., 1998). Due to bad refrigeration conditions this food came up in the Northeast region as an alternative to preserve bovine meat in excess. Inhabitants had a very low buying power and could not acquire refrigerators (Souza, 2005). However, excellent weather all along the year with huge amounts of marine salt existing in those geographical areas allowed the product to be conserved by salting and dehydration processes. This handmade technique became popular, but the sun meat production presented unsatisfactory hygienical-sanitary condition resulting in a product with short on shelf life (Costa et al., 2001). In the beginning, the sun meat was destined in supplying protein needs to the local, neighbor and regional populations (Nobrega et al., 1983). Today, this characteristic of regionally consumed food and ingredient required in some recipes has been changed to a product that increased its consumption area being now appreciated in the whole national territory (SIC, 2007).

Although linked to the Brazilian culture history and rooted in the food habits of the population, mainly the northeastern one, the sun meat sanitary issues have been object of few researches. It should be regarded a highly popular product since it does not require sophisticated technology to be made, nor official standards of specification and quality requirements. These facts allowed the product to be homemade, but under inadequate sanitary conditions. Together with the absence of regulations concerning production, its sale has been facilitated by little conservation requirements, with no need of packing, nor storage under refrigeration. Almost the whole production of sun meat comes from small stores specifically dedicated to this business, or from retailers whose clients appreciate this product.

Despite the conditions mentioned above, literature about sun meat storage and sanitary quality issues, though scarce, shows the product can contain extraneous substances harmful to the health. A study

carried out by Santos and Rodrigues (1991) to appraise the degree of contamination of sun meat sold in the city of São Paulo, came to the conclusion that, even in the presence of crystal sodium chloride, eggs and larvae of insects had developed.

The evaluation of the sun meat quality is relevant mainly when it concerns meat distributed for consumption that scarcely comply with minimum standards of sanitary quality, becoming an agent of dissemination of pathogens, putting the consumer health at risk. Being so, this work was aimed at: i/ macro and microscopically evaluating this product, focusing on the presence of extraneous materials, harmful or not to health; ii/ comparing macro and microscopical evaluation results with parameters read in the Brazilian legislation that rules similar products; iii/ observing procedures taken by the Houses of the North and the sun meat pieces displayed for consumer sale as to its aspects favorable to physical contamination of the product and; iv/ joining the observed stores conditions to the results obtained in this analysis.

2. Materials and methods

2.1. Material

Forty-four samples of sun meat were analyzed, weighing between 150 and 400 g each, bought in the retailer market, specifically in the Houses of North located in the city of Diadema, state of São Paulo. Samples had been acquired at random, in 22 stores, in duplicate. Each unit sample of sun meat was picked up between July and November, 2008 and were examined by the Laboratory of Alimentary Microscopy of the Institute Adolfo Lutz of São Paulo.

2.2. Analysis methods

The macro and microscopical research of extraneous materials followed the recommendations of both Food and Drug Administration and *Codex Alimentarius* and has been carried out based on the methodology used by Santos and Rodrigues (1991). Each unit sample was macroscopically examined through direct observation by naked eye in order to identify the presence of extraneous materials at the surface of the product. Thus, the entire sample was spread over a plastic tray for a thorough visual inspection. Next, they were cut with the aid of instruments in order to check existing extraneous materials in the internal parts of the product. All the extraneous materials found were kept apart and had been placed in a Petri dish for identification by the stereoscopic microscope.

After macroscopic analysis, with extraneous materials found or not by naked eye, the analytical units had been washed with filtered water and this content was filtered to hold other materials not identified macroscopically. This filter paper had been sent for examination by the stereoscopic microscope at a magnification 10 to 30 times. The isolated extraneous materials which were identified as whole insect and/or fragments were kept in 70% alcohol and sent, for further entomologic classification, to the Entomology Laboratory of Oscar Freire Institute of São Paulo University. The same procedure was adopted for identification of materials such as animal hair that had been previously fixed on a glass slide to help identification.

2.3. Comments on products and stores

During the sun meat purchasing activity at sun meat stores or sale shops, an inspection of the sanitary condition of 2 sun meat stores was also carried out.

3. Results and discussion

The extraneous isolated materials found in samples by macroscopic and microscopic methods was recorded and gathered as shown in Table 1. Table 2 and 3 refer to the results obtained from local observations at sun meat stores, which focused on the display of sun meat and vector presence. The general analysis of Table 1 informed that identified and isolated materials had been found in all stores. Most materials were classified as harmful to health, according to the Resolution RDC 175/2003. In the examined samples extraneous materials, harmful to health or not, had been identified as: whole insect or insect fragments, larva, rodent hair, exuvia, mite, bird feather or fragment, hair of unidentified animal, fungi and other materials such as plastic fragment, string, wood piece, carbonized substance, and bone fragment. These materials can point out that good procedures of manufacturing, storage and distribution normally required to control food contamination had not been taken (Brazil, 1997; Atui et al., 1999)

Table 1 Results of macro and microscopic analysis of sun meat showing number and percentage of unacceptable samples (2 samples/store a, b) from Houses of the North, stores listed by number and extraneous materials found, Diadema, SP, Brazil. 2008.

	Extraneous Material Types										
	Harmful to health as per Resolution RDC 175/2003					Not mentioned in Resolution RDC 175/2003					
Store number	Whole insects	Fragments of insects	Larvae	Rodent hair	Other extraneuous materials	Exuvia	Mites	Unidentified Animals Hair	Birds Feather	Filamentosos Fungi	Other extraneous materials
1	-	-	-	1[b]	1[a]	-	-	1[a]	-	-	-
2	-	1[a]	-	-	-	-	-	-	-	1[a]	-
3	-	1[b]	-	-	-	-	-	-	1[b]	-	-
4	-	1[a] ;1[b]	-	1[a]	-	1[b]	-	-	-	-	-
5	1[b]	1[a] ;1[b]	-	-	-	-	-	1[a]	-	-	1[b]
6	1[b]	1[b]	-	-	-	-	-	-	-	-	-
7	-	1[a]	-	-	1[b***]	-	-	-	-	-	1[a]
8	-	1[a]	1[a]	-	-	-	1[a];1[b****]	1[b]	-	1[a]	-
9	1[b]	1[a];1[b]	-	1[a]	-	-	-	1[a]	1[b]	-	-
10	-	1[b]	-	-	-	-	-	1[a]	-	-	-
11	-	-	-	-	-	-	-	1[a];1[b]	-	-	-
12	1[a]	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	1[b]	-	-	-	-	-	-
14	-	1[a];1[b]	1[a];1[b]	-	-	-	-	-	-	-	-
15	1[a]	1[a];1[b]	-	-	-	-	-	1[b]	-	-	-
16	-	1[a];1[b]	-	-	-	-	-	1[a]	-	1[b]	1[b]
17	-	1[a];1[b]	-	-	1[a];1[b]	-	-	1[a]	1[a]	1[a]	-
18	-	-	-	-	-	-	-	1[a]	-	-	-
19	-	1[b]	1[a]	-	1[a];1[b]	-	-	1[b]	-	-	-
20	-	-	-	-	1[b]	-	-	-	-	1[b]	-
21	-	1[a];1[b]	-	-	-	-	-	-	-	-	1[b]
22	-	1[a];1[b]	-	-	-	-	-	-	-	1[a]	-
Partial Total = 32 samples (a+b)						Partial total = 23 samples (a + b)					
T 44	5	25	4	3	8	1	2	12	3	6	4
% 100.0	11.4	56.8	9.1	6.8	18.2	2.3	4.5	27	6.8	13.6	9.1

T = Absolute Totals; % = Relative Totals. *fragments of plastic, bones and wood; ** string and carbonized material; *** Greasy cyst in sample; ****Live and dead mites in sample; - Absence of extraneous materials

Extraneous materials not regarded as health harmful by the Resolution RDC 175/2003 was identified in 23 samples, while extraneous substances mentioned by this Resolution as health harmful were found in 32 samples. Samples from stores 8 and 17 presented the largest quantity and diversity of extraneous material.

Other inert materials such as plastic, wood and bone fragments, found in 18.2% of the samples are considered as physical hazards and harmful to human health. The insects (complete individual or fragment), as well as larvae and rodent hairs can carry infectious agents to the food, causing harm to the human being health (Brazilian National Health Vigilance Agency (ANVISA), 2003). The presence of whole insects was observed in 11.4% of the samples and insect fragments were isolated in 56.8% of the samples. Insect larvae were found in 9.1% of the samples. Santos and Rodrigues (1991) examined jerked beef and "feijoada" ingredients and found 28.8% of samples containing whole live and dead insects, or their fragments, besides larvae and mites. The presence of such materials can be connected with the display of sun meat during the processes of salting and drying and in the sale points sanitation. In general, species found belonged to the order Diptera, mostly being domestic flies. The Resolution RDC 175/03 regards this product as improper for consumption (ANVISA, 2003).

Mites have been identified in 4.5% of the examined samples. According to Gorham (1987), the presence of mites in food is caused by inadequate storage. Consequently, the consumption of products affected by those agents represents a serious threat to human health, and may cause intestinal disorders with or without fever and pain. Fungi were isolated in 13.6% of the samples. According to Baglioni et al. (1999), some species of fungi are heat-resistant and are part of the deterioration process of the food by causing chemical degradation and changes in the components as well as the production of metabolites, acting in the nutritional parameters and sensory characteristics of the product (only in high r.h. conditions and activity of water over 0.75 in the food product). Bird feather or feather fragments, were seen in 6.8% of

the samples and suggested a factor of physical and microbiological contamination, since pathogens like bacteria, and ectoparasites as mites can be transported by birds. (Costa et al., 2002; Tucci et al., 2005). Rodent hairs were found in 6.8% of the samples, evidencing the presence of some or large number of rodents in the sun meat store, and a possible contact of the meat with the animal or its excrement. Rodent hairs are eliminated via its excrement and their presence is a serious hazard for consumer health. The rodents are important carriers of *Salmonella* spp (Veiga et al., 1978; Carter et al., 1991; Acha et al., 2003), spreading this pathogenic bacteria in its environment together with its excrement. The presence of unidentified animal hair was observed in 27.3% of the samples and exuvia in 2.3% of the samples. Although they are not regarded as extraneous material harmful to health, their presence in food sold to consumers was found repugnant.

Concerning the set of extraneous isolated substances and following the Resolution RDC 175/03, the samples of insects or products derived from its metabolism, not recognized as mechanical vectors such as *Dyctioptera* and *Diptera* orders species or other bio-contaminants like mites, fungi, and other extraneous materials were considered satisfactory. Only the stores 11 and 18 did not present extraneous materials that are health harmful. Then, out of 22 stores among 20 (90.9%) presented, at least, one sun meat was not fit for consumption due to the presence of extraneous materials which were health harmful.

Regarding the display of sun meat in the Houses of North (Table 2) it has been observed that 90.9% of the stores kept the sun meat over a surface similar to a sale counter, and 54.5% located near the entrance door, facilitating the storage of several types of dirtiness contamination existing in and around the stores, such as car smoke, dust in the air left by cars while passing the public street. Samples from stores 16 and 22, hung near the entrance door, were subjected to weather changes, facilitating the adherence of very small burnt parts, contamination and growth of fungi.

Table 2 Results of observations of Houses of the North, sun meat supplier, regarding product display, Diadema, SP, Brazil, 2008

	Types of display of Sun Meat					
Store Number	**Hanging on**		**Over surface (counter)**		**Near the door**	
1	X		X		X	
2	X		X		X	
3	-		X		X	
4	X		X		X	
5	-		X		-	
6	-		X		-	
7	-		X		-	
8	-		X		-	
9	-		X		-	
10	-		X		-	
11	-		X		-	
12	X		X		-	
13	-		X		-	
14	-		X		X	
15	-		X		X	
16	X		X		X	
17	-		X		X	
18	-		X		X	
19	-		X		X	
20	X		-		X	
21**	-		-		-	
22	-		X		X	
	X	-	X	-	X	-
T	6	16	20	2	12	10
%	27.3	72.3	90.9	9.1	54.5	45.4

Notes: Yes = X ; No = -; T = Absolute Totals; % Relative Totals; * Multiple answers;
** Product displayed in plastic box covered with film paper

The study of vector animals and/or urban pests existing in the stores is shown in Table 3. There were mechanical vectors in 50% of the stores. Most belonged to the order Diptera (mainly domestic flies) flying over the sale area or over products displayed. When comparing Table 3 and Table 1, only the samples from stores 1 and 11 did not show isolated whole insects or fragments during the macro and microscopic evaluations. In all other places where flies were present, insects were always found in the analyzed samples. The issue regarding mechanical vectors is that these animals have potential to disseminate pathogens. According to Thyssen et al. (2004), one of the reasons why the Diptera presents this potential is because it is in very close contact with man and his environment.

In store 17, there were live birds in cages hanging over the sun meat displayed over a surface. Table 1 shows that one sample from this store presented bird feathers, signaling physical contamination of the product for consumer sale that most likely came from the birds kept in the store.

Table 3 shows that 32 analyzed samples were improper for consumption because containing extraneous materials harmful to health. The conclusion reached is that the absence of both packing and good manufacturing practices connected with integrated control of plagues facilitated this situation.

Table 3 Results of observations of Houses of the North, meat suppliers, concerning vectors presence and urban pests, Diadema, SP, Brazil, 2008.

	Vectors and Pests		
Store Number	**Presence**	**Observed animal(s)**	**Where**
1	X	Pigeons and flies	Entrance Door
2	-	-	-
3	-	-	-
4	X	Domestic Fly	Inside the store
5	X	Domestic Fly	Inside the store
6	X	Domestic Fly	Inside the store
7	X	Domestic Fly	Inside the store
8	-	-	-
9	-	-	-
10	-	-	-
11	X	Domestic Fly	Inside the store
12	X	Domestic Fly	Inside the store
13	-	-	-
14	-	-	-
15	-	-	-
16	X	Domestic Fly	Over products
17*	X	Birds in Cage	Over products
18	-	-	-
19	-	-	-
20	-	-	-
21	X	Domestic Fly	Inside the store
22	X	Domestic Fly	Over products
	X -		
T	11 11		
%	50.0% 50.0%		

Notes: Yes = X ; No = -;T = Absolute Total; % = Relative total;
*Store that sold live birds and sun meat sharing the same physical area or close

The results shown in this research allowed the conclusion that the sun meat sold in 20 among 22 (90.9%). Houses of the North were improper for consumption. Materials found in samples together with sale procedures taken by the Houses of North indicate that these products can put the consumer health at risk.

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Assessment of hygienic conditions of ground pepper (*Piper nigrum L.*) on the market in São Paulo city, by means of two methodologies for detecting the light filth

Atui, M.B. *#[1], Castejon, M.J.[2], Yamashiro, R.[2], De Lucca, T.[2], Flinn, P.W.[3]
[1] Adolfo Lutz Institute, Food and Chemistry Division, Food Microscopy Section - Av. Dr. Arnaldo, 355 – Cerqueira César - SP/Brazil – CEP: 01246-902. Email: marcatui@terra.com.br
[2] Instituto Adolfo Lutz, Department of Serology, HIV Laboratory, Av. Dr. Arnaldo, 355 – Cerqueira César - SP/Brazil
[3] Grain Marketing and Production Research Center, Kansas, USA.

* Corresponding author
# Presenting author

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Abstract

Pepper should to be collected, processed, and packed under optimum conditions to avoid the presence of foreign matter. The hygienic conditions of ground pepper marketted in São Paulo city were assessed in determining the presence of foreign matter by means of two extraction methodologies. This study was carried out during a six-month time period from May to September 2006. The occurrence of light impurities was determined either by the flotation technique following the methodology recommended by AOAC or by enzyme – linked immunosorbent assay (ELISA). It was observed that 100% of the examined samples contained insect fragments, and many samples were housing more than one type of foreign matter. Twenty-two percent of samples were unqualified for consumption owing to the occurrence of rodent hairs. For the calibration of ELISA test for quantification of insect contamination level in pepper samples, a range of standard-infested samples was prepared in adding 1, 2, 4, 8 and 10 insects in a control sample to estimate the number of insects in the analyzed samples by measuring optical densities (OD) values with a spectrophotometer. Among the 22 samples, 36.4% of samples presented OD values close to that corresponding to the standard infested with eight insects, 40.9% of samples were comparable to OD of the standard infested with four insects, 18.2% comparable to standard with 10 insects**,** and 4.5% to the standard with two insects. According to the results observed in the present study, the technique described in AOAC official methods manual was found more suitable for detecting not only the insects but also the additional impurities in analyzed samples, while ELISA is specific to detect myosin from the insect muscle, which undergoes serious degradation with time.

Keywords: Pepper, ELISA test , Light filth, AOAC official method.

1. Introduction

Brazil is a remarkable black pepper producer and its large use as a condiment may lead to a health hazard, since it can be directly added to food without any procedure to neutralize eventual contaminants. Black pepper of good quality should be harvested, processed, packed, delivered and stored under conditions that avoid the development and/or addition of physical, chemical or biological substances potentially harmful for the consumer's health (Brasil, 2005). In tropical and subtropical regions, weather changes and the natural drying processes increase the chance of spices to be contaminated by microorganisms and insects. These, in turn, facilitate the growth of fungi due to their metabolic activity that increases the humidity and environment temperature (Correia et al., 2000). The study of extraneous materials in food (black pepper) has been of fundamental importance in order to maintain the physical, sanitary and nutritional qualities of the product. The Association of Official Analytical Chemists (AOAC) defines as extraneous materials any different materials which do not take part of the existing food, resulting from inadequate practices during production, storage or distribution. Light filth such as whole insect and insect fragments, mites, animal hair and bird feathers are some of the extraneous materials among many others (AOAC, 2005). Detection and quantification of these contaminants in dry foodstuffs represent a constant challenge for industry, and require periodical control analyses of the filth contamination of stored products either in the raw or processed condition.

The Consumer Rights Code, effective since 1991 in Brazil, has induced significant changes on the relationship between consumers and manufacturers in the Brazilian food-processing industries. The current legislations that regulate microscopic extraneous matter in food are Resolution RDC no. 175/MS

and Regulation no. 326/MS which describe the general steps of the procedure for assessing the presence of extraneous material that may be harmful to human health in packed food. These regulations establish the general hygienic requirements and the good manufacturing practices that are convenient as well as for raw agricultural product and for finished food ready for consumption, respectively (Brasil, 1990, 1997, 2003)..Flotation methodology for the light filth extraction from black pepper has been regulated by the AOAC and it has long been used by official laboratories. Today, an immunoenzymatic assay (enzyme-linked immunosorbent assay– ELISA) has been developed for the detection of insect fragments in food samples via the reactivity of antibodies to insect myosin – the protein found in insect muscles. In comparing this technique with Flotation Method, ELISA may be considered as a rapid method, with a relative low cost and allowing the analysis of a large number of samples in a test run of a few hours. Myosin is found in large quantities in both larvae and adult insects and can be easily extracted. Some technical variations of the original extraction method were performed according to the species of insects in study (Kitto, 1991, Bair and Kitto, 1992). Thus, a comparative study evaluated the hygienic-sanitary conditions of black pepper in powder available in various sale points in the city of São Paulo, by using the flotation technique (AOAC) and enzyme-immunoassay – ELISA.

2. Materials and methods

From May to September 2006, 22 samples of ground black pepper were collected and analyzed for their light filth content. These samples from different trademarks, batches numbers and consumption deadline time delay were bought in local markets of São Paulo city. The study was carried out in two laboratories of Instituto Adolfo Lutz-Central Laboratory, being Food Microscopy Laboratory and Laboratory of HIVAids that are, Laboratório de Microscopia Alimentar e HIV/Aids do Instituto Adolpho Lutz – Laboratório Central (HIV/AIDS Laboratory of Adolpho Lutz Institute) and in the laboratory of the Grain Marketing and Production Research Center of the United States Department of Agriculture, KS, USA. For extraction of light impurities in ground black pepper the Flotation Method no. 972-40A described in the Association of Official Analytical Chemists (AOAC) International manual (AOAC, 2005) was used as a reference method. In comparison, the immunoenzyme assay – ELISA commercial kit (Biotec - Austin, TX, USA) was used for insect myosin detection using polyclonal antibodies, and following the procedures recommended by the manufacturer (AOAC, 2005). Samples were processed in duplicate, and results were expressed in arithmetic average.

3. Results and discussion

Tables 1 and 2 show the results found in ground back pepper samples analyzed by means of flotation methodology. In Tables 1 and 2, respectively, 100% (22/22) of black pepper samples were housing insect fragments, and several kinds of extraneous material were found in more than one sample. The origin of the contamination cannot be determined and may be from either. The pepper tree plantation or from the storage and processing operations. Although these kinds of insects do not cause any harm to consumer's health, industries are reluctant to process dirty, dusty or spoiled raw products, and are compelled to follow suitable procedures and to observe good manufacturing, storage, and distribution practices (Brasil, 1997, 2003).

Table 1 Number and percentage of samples with light filth in 22 samples of Ground Black Pepper purchased in the city of São Paulo in 2008.

	Presence		Absence	
Black Pepper	**No.**	**%**	**No.**	**%**
Insect	4	18.2	18	81.8
Insect Fragment	22	100	0	0
Larvae	3	13.6	19	86.4
Larvae Fragment	4	18.2	18	81.8
Mites	9	41	13	59
Rodent Har	5	22.7	17	77
Fragment of bird feather	1	4.5	21	95.5
Unidentified animal Hair	1	4.5	21	95.5

No. = Number of samples

Table 2 Various types of extraneous material found in ground black pepper samples purchased on São Paulo city markets, 2008.

Sample Number	Fragment of Insect	Larvae	Fragment of bird feather	Insect	Fragment of larvae	Mites	Rodent Hair	Unidentified Animal Hair
1	3.5	1	0	0	0	0	0	0
2	34	0	0	0	0	0	0	0
3	22.5	0	0	0	0	0	0	0
4	7	0	0	1	0	0	0	0
5	11	1	0	1	5	1	0	0
6	6.5	0	0	0	0	0	0	0
7	17	0	0	0	0	0	0	0
8	49.5	0	0	0	0	1	0	0
9	293	1	0	0	1	1	0	1
10	67.5	0	0	0	0	1	0	0
11	40.5	0	0	3	1	2	1	0
13	352	0	0	0	0	0	2	0
14	65	0	0	0	0	0	0	0
15	10.5	0	0	1	0	1	0	0
16	19	0	0	0	0	0	1	0
17	56.5	0	0	0	0	0	2	0
18	12.5	0	0	0	0	1	0	0
19	6	0	0	0	0	1	0	0
20	466	0	1	0	1	9	1	0
21	23	0	0	0	0	0	0	0
22	14	0	0	0	0	0	0	0
23	55	0	0	0	0	0	0	0

Insect fragments are composed of chitin. When eaten by man, chitin is not metabolized due to the lack of the chitinase enzyme in the human digestive system. Nevertheless, even if there is no nutritional hazard associated to the ingestion of insect fragments, it may cause harm to the intestinal mucous (Gorham, 1981). The raw agricultural product may be attacked and harmed by insects which grow in the crop and harvest areas. The most common insects in pepper plantations belong to the following Orders: Lepidoptera, Coleoptera, Hymenoptera, Homoptera and Hemiptera. During pepper storage, the most common insects to attack the product are of the Coleoptera and Lepidoptera. Many of the insects found in the ground black pepper samples are homopteran, which are phytophagous: many species appear as plagues in cultivated plants (FDA, 1982; Wirtz, 1991). In the present study, 41% (9/22) of samples were contaminated with mites which may carry bacteria, yeast and fungi (Franzolin et al., 1999). The small part of excrement from mites contains allergenic substances that can cause reactions in human or animals, and may induce acute enteritis and also macrotis lesions in intestinal mucous; as in case of a massive mould mite pollution on humid food material (Gorham, 1981). Mites found in this study belonged to Mesostigmata and Cryptostigmata, mostly from Oribatidae family which are ground mites (Baggio and Franzolin, 1991; Fletchman, 1986).

Gecan et al. (1986) reported a study on various types of condiments sold in the retailer market, carried out to determine their sanitary conditions. As far as black pepper is concerned, authors verify that of 1523 analyzed samples, 98.4% held insect fragments, 46% were contamined with mites and 20% contained rodent hairs. These results are similar to those data found in the present study. Graciano et al. (2006) examined ground black pepper and found 98.5% of samples contaminated with fragments of insects; 24.6% with mites and 23.2% with rodent hairs. Samples housing rodent hairs are improper for human consumption, because: 1 of the presence of extraneous materials (e.g., pathogenic bacteria) regarded as harmful for human health, and 2 considering that rodents are potential carriers of several diseases, such as leptospirosis, salmonellosis, plague (bubonic plague, pneumonia, septicemia) (Gecan et al., 1986; Carvalho Neto, 1987).

Finally, in comparison with data found in a similar study carried out in year 1999-2000, it was found that the quality control of manufacturers and black pepper packing industries in the state of São Paulo was not improving during this period. The totality of analyzed samples (22/22) do not comply with the current lawful requirements for insects and fragments, mites, unidentified animals hair, food products contamination. These findings indicate the non-adoption and/or lack of maintenance of Good Manufacturing Practices (Brasil, 1997). According to Domestic Regulation RDC 175/2003 which regulates the extraneous macro- and microscopic materials, harmful to health, only the samples contaminated with rodent hairs (22.7%) can be considered as improper to consumption and should be withdraw from market places (Brasil, 2003; FDA, 1982).

The series of results obtained through enzyme-immunoassay – ELISA are shown in Fig. 1. In this figure, the extracts from samples showing a similar OD than the standards spiked with one, two, four, eight insects before grinding were associated in order to estimate the "quantity" of insects present on raw material in the 22 samples by optical density (OD) values. According to the above mentioned pairing, 36.4% (8/22) of samples presented OD values close to "pattern four insects"; 18.2% (4/22) close to "pattern 10 insects"; and 4.5% (1/22) similar to "pattern two insects". The number of additional insects that may be estimated by the ELISA test was found at 0.5 insect per 50 g sample. Samples with contamination higher than 10 insects per 50 g sample were at the value of the highest contamination rate of 10 insects per 50 g sample. Regarding to the accuracy and limit of detection of the AOAC recommended method, the ELISA myosin antibody test was unable to quantify the level of pollution of pepper by insect fragments, even when insects occur in large number in the raw material.

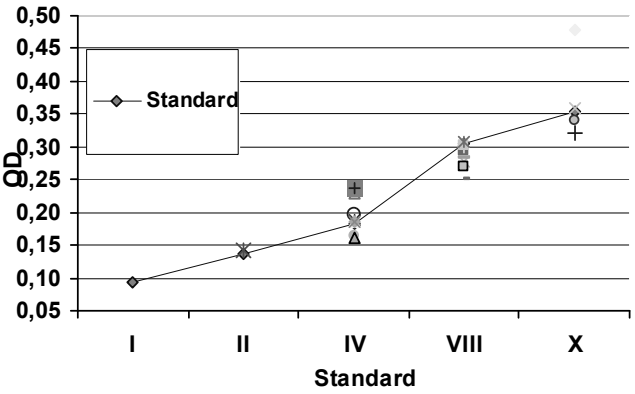

Figure 1 Results (OD) of samples and Standards using enzyme – linked immunosorbent assay (ELISA) to quantify insect fragment contamination level in ground pepper samples

Following the literature, the polyclonal antibody used in ELISA reacts with the myosin from various species that may infest ground material. The Immuno-sorbent assays based on a colorimetric reaction give consistent quantitative answer only in the case of raw product infestation with a large quantity of insects (Chen and Kitto, 1993). This study used ELISA kits containing polyclonal antibodies and the patterns are specific for *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). For samples infested with other insect species, the myosin antibody reaction values may be lower than those found in our study. Most insects found in these black pepper samples were not identified and those identified as homopteran (field pests). Thus, the quantity of insect myosin may be underestimated considering that the test was developed for coleopteran insects (beetles) belonging to the (Atui, 2002).

In the study reported by Kitto (1991), ELISA tests showed an excellent correlation between the obtained coloured reaction and the number of *Sitophilus granarius* added to the samples of clean wheat. The intensity of developed color is proportional to the quantity (in percentage) of myosin present and to be evaluated, which indicates a very good correlation with muscle mass of insect present in wheat or flour sample, and that may be considered as an extrapolation from the numbers of insects in the sample (Kitto, 1991).

In the study performed by Atui (2002) it was reported that myosin present in flour (after grinding grain) was degraded after 2 wk. The same situation may occur with the concentration of myosin in black pepper purchased from different market sale points in São Paulo city, because the date of pepper batches grinding date and delay before purchase of ground material was unknown.

The standard samples, infested by known numbers of insects, have high co-relation with optical density on ELISA, as reported in several studies. In this investigation, there was no correlation between data for the flotation method or those observed with ELISA tests, since there was no previous knowledge on contamination of black pepper samples (Atui, 2002; Kitto et al., 1992; Kitto et al., 1996). The immuno-enzymatic assay – ELISA is specific to detect insect myosin. Hence, it can detect insect fragments only. The flotation method can determine which species is infesting a sample by identification of insect recovered fragments. So, the immune-enzymatic ELISA assay is not able to determine the species of insect at the origin of the contamination.

Myosin concentration may serve to estimate the number of insects and fragments. ELISA is a rapid, sensitive, cheap and easy technique. In can be used for assessing insect presence in freshly processed products susceptible to infestation, but it is unsuitable for analyzing samples sold on the market.

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Toxigenic fungi in corn (maize) stored in hermetic plastic bags

Castellari, C.[1], Marcos Valle, F.[1], Mutti, J.[1], Cardoso, L.[2], Bartosik, R.*#[2]
[1] Facultad de Ciencias Agrarias, UNMdP;
[2] EEA INTA Balcarce, Ruta 226 km 73,5, Balcarce (7620), Provincia de Buenos Aires, Argentina.
Email: rbartosik@balcarce.inta.gov.ar

* Corresponding author
# Presenting author

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Abstract

In Argentina, 35 million tonnes are stored in hermetic plastic bags. Inside the bags, the modified atmosphere has an effect on stored grains, insects and fungi. Fungi taxa and species typically isolated from stored grains consist of species of the genera *Aspergillus*, *Penicillium*, *Fusarium* and xerophilic fungi, some of which are potential producers of mycotoxins. The province of Entre Rios, Argentina, is an important area of study because much of the production of maize stored in bags is supplying the poultry industry demand. The objective of this study was to identify mycotoxigenic fungi species in maize stored in hermetic plastic bags, located in three Departments of Entre Rios province. A total of 176 samples of maize were analyzed, stored in 23 bags located in the Departments of Paraná (west region), La Paz (northern region) and Tala (central region). Two potential producers of aflatoxins (*A. flavus* and *A. parasiticus*) and a potential producer of fumonisins (*F. verticillioides*) were identified in all the plastics bags evaluated. In La Paz Department, *Aspergillus* spp. and *F. verticillioides* were detected in 66.7% and 54% of samples, respectively. In Tala Department, *Aspergillus* spp. were detected in 63.3% and *F. verticillioides* in 43.6%, while in Parana, *Aspergillus* spp. was detected in 88.2% of the evaluated samples and *F. verticillioides* in 41.4% of the samples. The results revealed that mycotoxigenic fungi can develop in maize stored in hermetic plastic bags. This implies a potential risk of contamination with aflatoxins and /or fumonisins in the grain lots stored inside these bags.

Keywords: Mycotoxigenic fungi, Grain, Plastic silo-bag, Grain quality, Fungi spoilage

1. Introduction

The total storage capacity of Argentina is estimated 73 million tonnes, which represents about 75% of the total production. As a result, the efficiency of the postharvest system is largely compromised. To overcome these unfavorable circumstances, a new storage technique has gained popularity among farmers to store dry grains (wheat, maize, soybean, etc) in hermetic plastic bags. These bags can hold approximately 200 tonnes of grains, and since plastic enclosure material are 230 µm thick, they are airtight to water and gasses (O_2, CO_2 and water vapor). The respiration of grain, fungi, insects and other live organisms consumes the oxygen and generates carbon dioxide. This modified atmosphere has effects on the seeds, insects and fungi (Cardoso et al., 2008; Rodriguez et al., 2008).

The fungi genera typically found in stored grains are *Aspergillus*, *Penicillium*, *Fusarium* and some xerophilic species, several of them with capabilities of producing toxins (Christensen, 1987; Lacey, 1989). The development of these fungi can be affected by moisture content of the product (Giorni et al., 2009; Hell et al., 2000), temperature, storage time, degree of fungal contamination rate prior to storage and insect and mite activity that might facilitate fungi dissemination. Those facts lead to the importance of identifying the fungi species in stored grain, with a special consideration for micotoxigenic ones, since they can be a potential threat for people and animal health. The goal of this study was to characterize the fungi species found in maize grain stored in hermetic plastic bags. This maize is to be used by the poultry industry in Entre Rios province, Argentina. This province concentrates 47% of the poultry and eggs industry in the country. The results of this study aim at helping farmers and the industry to better produce and store properly their quality grains, especially in terms of good hygiene practices and sanitation.

2. Materials and methods

2.1. Geographical situation of maize production area in Argentina

This study was carried out from maize samples collected in hermetic plastic bags located in the Departments of Parana (West), La Paz (North) and Tala (Center) in Entre Ríos province, Argentina. Entre Rios province limits to the North with Corrientes province, to the West, separated by the Paraná River, with the Santa-Fe province, to the South with Buenos Aires province, and to the East, separated by the Uruguay River, it limits with the Uruguay Republic.

2.2. Grain Sampling

The maize harvest was in April, and the grain sampling was done at the end of July, after 3 to 4 months of storage.

One sample was collected every 10 m along the bag (in general the plastic bags are 60 m long) with a compartmented sampling spear probe of 1.8 m long which allows to take samples from the entire vertical profile of the grain mass. The collected grains were separated according their location in the profile of the grain mass: i/ upper layer (0-0.1 m) and ii/ middle and lower layers (0.1-1.8 m).

Grain temperature and moisture content were also recorded for each grain layer and sampling location. Temperature was determined with a portable temperature sensor that can be inserted in the grain mass and measure grain temperature at 0.1; 0.7 and 1.6 m from the top of the bag. Grain moisture content was determined with a moisture meter (Dickey-John, GAC 2100,).

The collected maize samples were placed in plastic bags with hermetic sealing and shipped to the Microbiology Laboratory of the Balcarce Integrated Unit (INTA-Agronomy College of Mar del Plata University), and stored at 4°C until processed.

2.3. Fungi identification

2.3.1. Fungi isolation

The presence of filamentous fungi was determined implementing the direct plate technique. In a laminar flow cabinet, 110 kernels of each sample were selected from each grain sample and placed in a Petri dish (10 kernels per dish) on agar culture medium supplemented with 18% of glycerol (DG18 medium). The DG18 medium has a water activity of 0.95 that allows the growth of non xerophylic species (*Penicillum, Aspergillus*), as well as xerophylic fungi (*Eurotium* spp.) or yeast (Pitt and Hocking, 1997). The remaining 10 kernels were placed in a Petri dish on dichloran chloramphenicol peptone agar (DCPA), in order to detect *Fusarium* spp.

The Petri dishes were incubated at 25°C during 7 days. Then, the percentage of fungi contaminated kernels was determined for each sample, and all the colonies with a visual difference in morphology were removed in sterile conditions to be deposited in Petri dishes with DG18 and DCPA culture media. The isolated fungi strains were incubated at 25°C during 7 days to obtain a pure monospecific isolated culture.

2.3.2. Fungus species identification

The isolated fungi of the genus *Penicillium*, *Aspergillus* and *Eurotium* were identified using the taxonomic key of Pitt and Hocking (1997), based on morphological and biochemical characteristics. For the species belonging to *Fusarium* genus, in addition to the Pitt and Hocking key, the key of Samson et al. (1995) and the *Fusarium* genus atlas (Gerlach and Nirenberg, 1982) were also used. For conducting these identification procedures in rigorous conditions of comparison, the pure cultures were all grown on both DCPA and potato dextrose agar (PDA) medium (Pitt and Hocking, 1997).

2.4. Determination of the Isolation Frequency

The isolation frequency of the fungi genus and of the potentially aflatoxigenic species in each plastic bag was determined as the ratio between the number of samples with positive isolation and the total number of samples analyzed from each bag. The frequency of isolation for each one of the identified species was also determined as the ratio between the number of samples with positive isolation for each species and the total number of samples analyzed from each Department.

3. Results

A total of 176 samples of maize collected from 23 different hermetic plastic bags were analyzed. The genera *Penicillium*, *Aspergillus*, *Fusarium* and *Eurotium* were isolated and identified from each bag in this study. Even though *Penicillium* and *Aspergillus* had the higher proportion of positive isolation, only two species of *Aspergillus* genus (*A. flavus* and *A. parasiticus*) and one of *Fusarium* genus (*F. verticillioides*) were identified in the literature as potentially mycotoxigenic. Therefore, those are considered of importance for the human and animal health. It was also determined than 90% of *Aspergillus* spp. isolation cases corresponded to *A. flavus*, a potentially mycotoxigenic species.

Table 1 shows the isolation frequency of the potentially mycotoxigenic species the most important for stored maize, aggregated by Department.

Table 1 Isolation frequency (in percentage of analyzed samples), of potentially mycotoxigenic species from three Departments of Entre Rios province, Argentina.

Department	**Frequency (%)**		
	Fusarium verticillioides	***Aspergillus* spp.(#)**	***F. verticilloides* and *Aspergillus* spp.(#)**
La Paz (North)	54.0	66.7	52.4
Paraná (West)	41.4	88.2	36.4
Tala (Central)	43.6	63.3	ND

(#) Aspergillus flavus; Aspergillus parasiticus; ND: not determined

The location of the grain in the profile of the grain mass (in upper or inner layer of the storage enclosure) did not have influence in the type of fungi species identified, since in the two layers evaluated (upper and middle-bottom) the same species were identified, even though the isolation frequency was different.

Mycotoxigenic species of the *Aspergillus* and *Fusarium* genus were isolated from all the bags. Grain samples coming from the Paraná Department had the higher percentage of isolation of afatoxigenic fungal species, while samples from La Paz Department had the higher proportion of *F. verticillioides* (fumonisin producer). In this Department 52.4% of the samples were contaminated with the three potentially mycotoxigenic species (*A. flavus*, *A. parasiticus* and *F. verticillioides*). It was also observed that in samples in which *A. flavus* and *F. verticilloides* were present, the proportion of *F. verticillioides* was always higher (data not presented).

4. Discussion

Storing grain in hermetic plastic bags requires frequent monitoring in order to early detect grain spoilage and/or mycotoxigenic fungi development. In this study some potentially mycotoxigenic species of the *Aspergillus* and *Fusarium* genus were identified in grain samples, in addition to other species from the *Eurotium* and *Penicillium* genus. *A. flavus* and *F. verticilloides* were the most important species due to their capacity to produce mycotoxins (aflatoxins and fumonisins, respectively) and to contaminate the grain (Lino et al., 2007; Logrieco et al., 2007).

Previous studies identified *Aspergillus* and *Fusarium* mycotoxigenic species in stored grains, as well as their mycotoxins, aflatoxins and fumonisins, in different concentrations (Pacin et al., 2009; Moreno Cunha et al., 2009). There is a general trend to increase the consumption of cereal grains and cereal products. On the other hand, the consumption of contaminated grain with mycotoxins causes different problems, including death (Lerda et al., 2005; Voss et al., 2007). There is a general agreement that the consumption of contaminated grains with mycotoxins is a risk for the animal and human health, and may lead to an important economic problem in the near future.

The study of the conditions that lead to the development of fungi during storage and the production of mycotoxins indicated that the grain moisture content is one of the most important factors (Hell et al., 2000; Giorni et al., 2009). In maize, for instance, it was determined that a storage moisture content of 13% is sufficiently low to prevent fungus development and mycotoxin production (water activity below 0.65). In this study, all the grain samples presented moisture content above 13%, reaching values as high as 25.1% in samples from La Paz Department (Northern region). This unsafe storage conditions would indicate that the grain stored in plastic bags can be contaminated with different levels of mycotoxins. In fact, *Fusarium*, which is a genus that typically affects the grain in the field, was found with a frequency

of 40% in the samples collected from the bags, most likely due to fungal activity during storage of extremely high moisture content grain.

In Argentina, storing grain in hermetic plastic bags is a common practice. It became an important tool for agriculture, both from the logistic and the economical point of view, since it is a simple and inexpensive storage system. However, proper storage conditions should be considered and evaluated, especially those affecting the grain sanitary and hygienic quality, such as moisture content, temperature and storage time. These seem to be the most important factors conditioning the development of mycotoxigenic fungi during storage, which are of importance for the management of hazards to human and animal health they represent.

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Monitoring carbon dioxide concentration for early detection of spoilage in stored grain

Maier, D.E.*, Channaiah, L.H.#, Martinez-Kawas, A., Lawrence, J.S.[1], Chaves, E.V., Coradi, P.C., Fromme, G.A.[2]
Department of Grain Science and Industry, Kansas State University, Manhattan, Kansas 66506, U.S.A..
Email: dmaier@ksu.edu, kantha@ksu.edu
[1] Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, Indiana 47907, U.S.A.,
[2] BinTech, Orchard Drive, Louisville, Denver, Colorado 80027, U.S.A.

* Corresponding author
# Presenting author

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Abstract

Field experiments were conducted in storage silos to evaluate carbon dioxide sensors to monitor spoilage in grain prior to spoilage detection by traditional methods such as visual inspections and temperature cables. Carbon dioxide concentrations in the storage silo were monitored up to eight months and correlated to the presence of stored-product insects, molds and mycotoxin levels in the stored grain. The data showed that safe grain storage was observed at CO_2 concentrations of 400 to 500 ppm. Higher concentrations of CO_2 clearly showed mold spoilage or insect activity inside the grain storage silo. Carbon dioxide concentrations of 500 to 1200 ppm indicated onset of mold infection where as CO_2 concentrations of 1500 to 4000 ppm and beyond clearly indicated severe mold infection or stored-product insects infestation. The percent kernel infection was in the range of 30% for CO_2 concentrations of 500 to 1000 ppm to 90% for CO_2 concentrations of 9000 ppm. Fungal concentrations were in the range of 2.0×10^2 colony forming units per gram (cfu/g) at 500 ppm CO_2 concentration to 6.5×10^7 cfu/g at 9000 ppm CO_2 concentration. Fungi of genera *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. were isolated from spoiled grain. High concentration of fungi and presence of mycotoxins (aflatoxin: 2 ppb and Deoxynivalenol (DON): 1 ppm) were correlated with high CO_2 concentration in the silos. The findings from this research will be helpful in providing more timely information regarding safe storage limits, aeration requirements and costs of spoilage mitigation measures such as turning, aerating and fumigating grain. Additionally, it will provide information on preventive stored grain quality management practices that should reduce residue levels of mycotoxins, pesticides and other foreign material in our food supply. The CO_2 monitoring technology will increase the quality and quantity of stored grain, while saving the U.S. and global grain production, handling and processing industry millions of dollars annually.

Keywords**:** Carbon dioxide, Grain storage, Stored-product insects, Mold and mycotoxin

1. Introduction

Temperature, relative humidity and moisture content (m.c.) of the stored grain are the most important factors that influence stored-product insect activity, mold growth and subsequent production of mycotoxins in storage. Maintaining optimum temperature, relative humidity and proper moisture content are the challenges faced because of the seasonal and daily climate fluctuations, the economics of drying grain, and the need to process grain at higher moistures. The optimum temperature range for mold growth is 25-30°C, and temperatures above 15°C are ideal for insect growth and reproduction. Insect metabolic activity in dry commodities (below 15% m.c.) can result in heating up to 42°C (Mills 1989). A major contributor to the spoilage of grain is growth of various mold species, including several that produce mycotoxins. Mycotoxins are natural chemicals produced by fungi that are detrimental to the health of both animals and humans. The U.S. Food and Drug Administration has placed an action level for mycotoxins in stored grain and other food products including milk. As a result, millions of dollars are spent each year to screen food that includes stored grain, processed food, milk and animal feed. The earlier methodologies such as human sensory exposure and temperature cables have their own limitations and drawbacks in monitoring grain spoilage during storage. New management practices are needed that will allow grain processors to maintain high quality grain free of stored-product insects, fungi and mycotoxins. Previous studies have shown that CO_2 sensors can be effectively used to monitor early detection of spoilage during storage (Zagrebenyev et al., 2001; Maier et al., 2002; Bhat et al., 2003;

Maier et al., 2006; Bartosik et al., 2008). The goal of this project was to refine the existing CO_2 based technology for its accuracy and consistency in real time monitoring of grain spoilage prior to detection by traditional methods such as visual, smell and temperature sensors.

2. Materials and methods

2.1. Site selection and installation of CO_2 sensors

To monitor CO_2 concentration for early detection of spoilage due to mold and stored-product insects, we selected a corrugated steel bin containing 254 tons of maize located near Manhattan, Kansas. The CO_2 sensor box (BinTech Company, Denver, CO, USA) box was installed on the roof of the silo close to a vent by cutting a 10.7 cm diameter hole using a metal cutter. Care was taken to seal the gaps around the sensor box to protect water infiltration and to avoid CO_2 leakage. Once the sensor box was installed the CO_2 sensor was released inside the silo by maintaining roughly one meter distance above the stored grain. The control box comprising the battery and display board was mounted on the outside wall of the silo about 1.50 m above ground. The CO_2 sensor and the control boxes were connected and tested for wireless telephone signals and its connectivity to the main server. During grain storage, the CO_2 data were transmitted to the main server (BinTech Company) in the form of digital codes using the wireless telephone network.

2.2. Monitoring changes in CO_2 concentrations

Carbon dioxide concentrations in all storage bins were monitored from February to August 2009. The maize samples were collected and analyzed for grain quality parameters, stored-product insect incidence, presence of molds, and mycotoxin contamination. A log book was maintained to document all grain storage activities including information on battery change and dates of sample collection. Furthermore, details of the storage silo including size, number of fans, pesticide usage, storage start date and contact details of the cooperator were recorded. The silo was inspected and maize samples were collected based on high CO_2 concentration readings and correlated to mold spoilage or stored-product insect activity in the silo. Two sets of grain samples in replicates were collected by probing with a grain sampler. One set was used to analyze molds, mycotoxins and insects in the Grain and Feed Microbiology and Toxicology Laboratory (Department of Grain Science and Industry, Kansas State University, Manhattan, KS, USA) while the second set of grain samples was sent to the Kansas Grain Inspection Service Lab (Topeka, KS, USA) to determine grain quality parameters such as moisture content, dockage and damaged kernels.

2.3. Isolation, enumeration and identification of insects and molds

Grain samples collected during this study were immediately brought to the lab and sieved (480-µm openings) to separate all live insects. These insects were identified, counted and expressed as number of insects per kilogram (kg) of grain. For isolation of molds from grain samples we followed the procedure described by Samson et al. (1996). Twenty five grams of representative sample was soaked in 250 mL of sterile peptone (0.1%) water for 30 min before stomaching for two mins. One mL of the sample, serially diluted in 9 mL of peptone water and a 100-µL sample from serial dilutions, was drop-plated on Dichloron Glycerol-18 (DG-18) agar medium (Oxoid Chemicals, Hampshire, UK) and incubated at 30°C for 4-5 d in an upright position. After incubation, the colony forming units were recorded to determine the number of molds per gram of grain (cfu/g). To confirm the species level, the isolates were observed under microscope for the morphology of spores and mycelia. The observations were recorded and matched with descriptions given by Samson et al. (1996) to confirm genus and species.

2.4. Detection of mycotoxins using ELISA

The levels of aflatoxin, fumonisin, and Deoxynivalenol (DON) in maize grain samples were quantified using the AOAC International, Gaithersburg, MD, USA) approved method based on an Enzyme Linked Immmunosorbent Assay (ELISA) (AgraQuant® Mycotoxin ELISA Test Kits, Romer Labs Inc., Union, MO, USA). Twenty grams of representative sample was grounded and extracted using 70/30 (v/v) methanol/water. For DON analysis the grains were extracted with 100 mL water. The extract was mixed and added to the antibody-coated microwell. Mycotoxins in samples and control standards were allowed to compete with enzyme-conjugated mycotoxins for the antibody binding sites. After the washing step, an enzyme substrate was added for color (blue) development. A stop solution was added to stop the reaction which changed the color from blue to yellow which was measured optically (450 nm)

using a microplate reader (Stat Fax® 303+ Microstrip Reader, Awareness Technology, Inc., Palm City, FL, USA) to determine the concentration of mycotoxins in a sample which was expressed in ppb or ppm.

3. Results

The storage silo selected for this study had spoilage before the CO_2 sensor was installed. It can be observed in Fig. 1 that high CO_2 readings (>3000 ppm) were detected by the sensors in March of 2009. We inspected the bin and found an inch thick of spoiled grain on the surface. We took samples and brought this to the attention of the cooperator and recommended the removal of the moldy grain. As time elapsed, CO_2 readings remained stable until late May of 2009. As ambient and headspace temperatures increased, CO_2 readings in June rose to around 1000 ppm and above, which clearly indicated mold or insect activity. In early July, CO_2 readings began to increase up to 5000 ppm and on inspection of the silo, mold development and spoiled grain on the surface layer were noticed. A sudden drop in concentration of CO_2 on 8 July, 2009 was the result of an attempt to further clean out the top layer of spoiled grain. This did not work because CO_2 readings shot up even higher than before to 7000 ppm and above. The bin was finally emptied in the middle of August. Samples were analyzed and as expected the maize grain was heavily damaged due to mold. We observed a strong correlation between the rise in headspace CO_2 concentrations verses mold and stored-product insects activities in stored maize. Analysis showed a high concentration of mold (6.5×10^7 cfu/g) per gram of maize (Table 1). The percent kernel infections assay showed that 90.0% of the maize kernels were infested by molds at CO_2 concentrations of 9000 ppm and above.

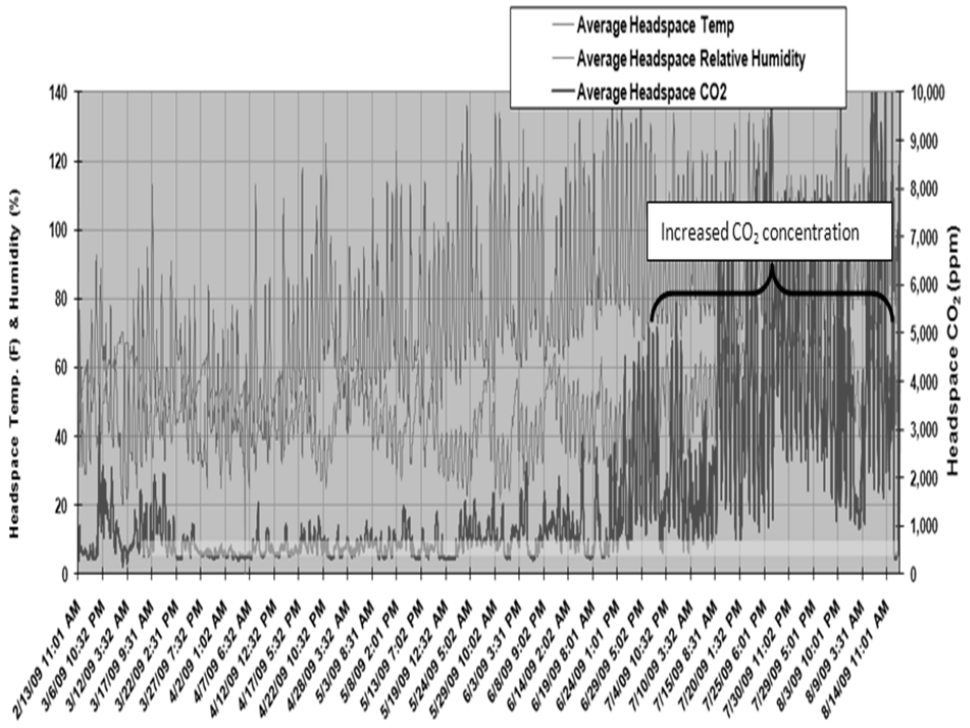

Figure 1 Change in headspace CO_2 concentration, relative humidity and temperature during grain storage.

Table 1 Grain quality parameters and incidences of stored-product insects, molds and mycotoxins in maize during storage

Time (2009)	Grain Moisture (%)	Air Temperature (ºC)	Relative Humidity (%)	Stored-product Insects (No. insects/kg)	Percent Infections (%)	Kernel cfu/g	Molds Total aflatoxins (ppb)	Mycotoxins Fumonisins (ppm)	Mycotoxins Deoxynivalenol (ppm)
February	14.5	16	60	0	30	$2.0 \pm 0.2 \times 10^2$	0	0	0
March	NA	26	60	1	70	$5.0 \pm 0.1 \times 10^6$	0	2	0
April	NA	26	50	2	NA	$2.2 \pm 0.0 \times 10^2$	0	0	0
May	NA	33	50	4	NA	$2.5 \pm 0.2 \times 10^3$	0	0	0
June	NA	43	52	10	NA	NA	0	0	0
July	13.5	50	59	18	80	$4.2 \pm 0.3 \times 10^6$	1	0	0
August	13.7	49	62	27	90	$6.5 \pm 0.3 \times 10^7$	2	0	1

NA: Data not available

We detected 2 ppb of aflatoxins and 1 ppm of DON in the unloaded maize (ending) sample. Mold concentration in the maize correlated with high CO_2 readings in the silo. Also, heavy stored-product insect infestation was noticed. These insects were identified as flat grain beetle*, Cryptolestes pusillus* (Schönherr) (Coleloptera: Laemophoeidae) which is a mold feeder, and maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) which feed on the maize. Upon enumeration we noticed 27 live insects per kg of maize (Table 1). Nearly 100 tones (40% of stored maize) of spoiled and damaged maize was separated and used for animal feed.

4. Discussion

It is essential for the grain storage industry to have effective management programs to protect against economic loss due to contamination from stored-product insects, molds and mycotoxins. Manual grain inspection (human sensory exposure) and measuring grain temperature are the main tools used by the farmers and the grain industry for monitoring proper storage conditions (Bartosik et al., 2008). Human sensory exposure literally means having personnel "walk" the grain mass, smell the grain, smell the aeration discharge stream and look at the grain. Human sensory exposure for mold spoilage and other quality parameters could be biased and it varies from person to person. Temperature cables are routinely placed in modern grain bins. Unfortunately, a temperature cable will not detect the fungal growth several feet away from the cable until the size of the spoiling grain mass is large enough to raise the temperature around the volume of the temperature cable. These limitations are overcome with the CO_2 sensors. Fungi and their related mycotoxin contamination problem is one that is not easily resolved by the food, feed and grain processing industry (CAST, 1989; Miller and Trenholm, 1994). Only organic acids are available for controlling fungal growth. Unfortunately, these acids are not suitable for many situations because they severely limit the number of end uses available for the treated grain. Our experiment clearly demonstrated that CO_2 sensors can be effectively used to detect stored-product insects infestation and grain spoilage due to mold infections well before spoilage detection by traditional methods such as visual inspections, smell and temperature cables. Production of carbon dioxide has been a method used for many years to predict the storability of grains under laboratory and field conditions (Stroshine and Yang, 1990; Maier et al., 2006). In this study, we successfully used the CO_2 sensors under field conditions for early detection of spoilage in maize due to molds and stored-product insects. Further, in this study we refined the CO_2 sensor technology that provides accurate and consistent results.

Carbon-dioxide-based, spoilage-detection devices are expected to save grain producing, handling and processing industry millions of dollars annually. Reducing spoilage would lower residue levels of mycotoxins, pesticides and other foreign materials in our food supply, and maintain the quality and quantity of stored grain, while minimizing storage and handling costs. Such an early warning system would provide more timely information to farmers to make the correct management decision to avoid the costs of spoilage mitigation measures such as turning, aeration, and fumigation. This would help in continuing to store grain or market it early to avoid further quality deterioration.

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Aspergillus flavus infection and aflatoxin contamination in peanuts stored at wholesale and retail levels in Bandung, Bogor and Jakarta (West Java, Indonesia)

Dharmaputra, O.S.*#[1], Retnowati, I.[2], Ambarwati, S.[2]
[1] SEAMEO BIOTROP, Jl. Raya Tajur Km. 6, PO Box 116, Bogor 16134, Indonesia. Email: okky@biotrop.org; okky_sd@yahoo.com
[2] Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Jl. Raya Pajajaran, Bogor, Indonesia

* Corresponding author
# Presenting author

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Abstract

The objective of the study was to obtain information on the percentage of kernels infected by *Aspergillus flavus* and aflatoxin contents in peanuts stored at wholesale and retail levels (traditional market and supermarket) in three cities (Bandung, Bogor and Jakarta). The moisture content of the kernels were also investigated. The three cities were selected because they are different in terms of their elevations and rainfalls. A total of 105, 101, 87, 99, 104 and 98 peanut kernels were collected in March, June, September, December 2005; in March and June 2006, respectively. The moisture content of peanut kernels either collected at wholesaler or retailer in the three cities (Bandung, Bogor and Jakarta) fluctuated during sampling (between 6 – 8%). The moisture content of kernels either at the wholesaler or retailer in Bogor had the same pattern with those in Jakarta, while those in Bandung had different patterns. The moisture content of kernels either at wholesale or retail levels in the three cities were relatively similar, although the relative humidites and temperatures of the storages at wholesale and retail levels in Bandung were relatively lower than those in Bogor and Jakarta. The percentage of kernels infected by *A. flavus* in peanuts collected from wholesaler had the same pattern with those collected from retailer. The highest percentage of kernels infected by *A. flavus* either collected from wholesaler or retailer was in June 2005, while the lowest was in September 2005. The median and the highest range of percentage of kernels infected by *A. flavus* were in peanuts collected from Bandung, while the lowest were those collected from Bogor. The median and the range of percentage of kernels infected by *A. flavus* collected from wholesaler were higher than those collected from retailer. The median of aflatoxin B_1 content in peanut kernels either collected from wholesaler or retailer during sampling were relatively similar (< 20 ppb), nevertheless their broadest range was recorded in June 2005. They were correlated with the percentage of kernels infected by *A. flavus*. The highest median (± 20% of 204 samples) and the broadest range (10 – 60%) of frequency of samples contaminated with aflatoxin B_1 > 15 ppb were in samples collected from Bandung, followed by samples from Jakarta (the median ± 20% of 181 samples, the range 10 – 40%), and samples collected from Bogor (the median ± 15% of 180 samples, the range 10 – 40%). The frequency of peanut samples contaminated with aflatoxin B_1 > 15 ppb collected from retailer was higher (the median 26% of 390 samples) than those collected from wholesaler (the median 18% of 175 samples), although the median and the range of percentage of kernels infected by *A. flavus* collected from wholesaler were higher than those collected from retailer.

Keywords: *Aspergillus flavus*, Aflatoxin, Peanuts, Storage, Wholesale and retail levels.

1. Introduction

Surveys of aflatoxin B_1 contents in two peanut producing regions in Central Java (Pati and Wonogiri), and Cianjur region in West Java concluded that the majority of aflatoxin contamination of local peanuts occurred in the wholesale and retail levels, and especially in traditional markets selling raw kernels (Dharmaputra, 2003a, 2005, 2007a), despite their moisture contents being less than 8%. In Indonesia in 2009 the total production of peanuts was 785,151 Tonnes (BPS, 2009). However, Indonesia imports peanuts mostly from China, India and Vietnam, and sometimes from Thailand and Africa. BPS (2009) reported that in Indonesia in 2009, as much as 177,030 tonne of peanuts were imported from several countries. According to Dharmaputra et al. (2007b) the majority of aflatoxin contamination of imported peanuts also occurred in the wholesale and retail levels, although in general their moisture contents were also less than 8%. Moisture content of the substrate and temperature are the mains factors affecting *A. flavus* growth and aflatoxin formation. Moisture content is always in equilibrium with the

relative humidity of the storage. According to Diener and Davis (1969), and Heathcote and Hibbert (1978) the minimum and optimum moisture contents of peanuts for aflatoxin production at 30°C are 9-10% and 25%, respectively.

The objectives of the study were:

1. To obtain information on aflatoxin contamination in peanuts stored at wholesale and retail levels (traditional market and supermarket) in three cities (Bandung, Bogor and Jakarta). The moisture contents of peanut kernels and the incidence of *A. flavus* were also determined.
2. To develop an enhanced understanding of factors leading to the post-harvest build up of aflatoxin in peanuts during storage.

2. Materials and methods

2.1. Time and location of surveys

Surveys were conducted from mid-March 2005 until mid-June 2006 at selected wholesalers and retailers in Bandung, Bogor and Jakarta, West Java. The three cities were selected, because they are different in terms of their elevations and rainfalls (Table 1). The retailers included outlets at traditional markets and supermarkets.

Table 1 The elevations and rain-falls of Bandung, Bogor and Jakarta in 2003

City	Height from the sea level (m)	Rain-fall per year (mm)
Bandung	791	2200.6
Bogor	250	2387.1
Jakarta	30	1903.8

(Source: BPS 2006)

2.2. Sampling methods

Random sampling of peanut kernels consisted of either local or imported peanuts. The number of samples collected from each peanut delivery chain located in each city is presented in Table 2. A total of 105, 101, 87, 99, 104 and 98 peanut kernels were collected in March, June, September, December 2005; in March and June 2006, respectively.

Table 2 The number of peanut samples collected from each delivery chain located in each city.

Month and year of sampling	City	Number of samples			
		Wholesale	Retail at traditional market	Supermarket	Total
March 2005	Bandung	10	24	2	36
	Bogor	10	21	2	33
	Jakarta	10	24	2	36
					105
June 2005	Bandung	10	24	2	36
	Bogor	10	21	1	32
	Jakarta	10	21	2	33
					101
September 2005	Bandung	10	24	2	36
	Bogor	10	24	2	36
	Jakarta	5	9	1	15
					87
December 2005	Bandung	10	24	0	34
	Bogor	10	18	1	29
	Jakarta	10	24	2	36
					99
March 2006	Bandung	10	24	2	36
	Bogor	10	21	1	32
	Jakarta	10	24	2	36
					104
June 2006	Bandung	10	24	2	36
	Bogor	10	15	1	26
	Jakarta	10	24	2	36
					98

Each peanut sample (2 kg kernels per sample) collected from each wholesaler could be derived from one or more stacks, depending on the number of stacks available at the time of sampling. The number of bags (sacks) from a stack where the peanut samples were taken depended on the total number of bags in the stack.

Peanut samples collected from each retailer at traditional markets consisted of two qualities if possible (3 samples per quality, 2 kg kernels per sample), and those collected from each supermarket consisted of two brands if possible (2 kg kernels per brand) with the peanuts being packed in polyethylene bags. Peanut samples collected from each wholesaler, retailer and supermarket were divided three times using a box divider to obtain working samples for various analyses.

2.3. Moisture content, A. flavus and aflatoxin determination

The relative humidities and the temperatures of the storage at wholesalers and retailers at traditional markets were monitored using tiny tag data loggers at hourly intervals. Moisture contents of kernels (based on a wet basis) were analyzed using a SINAR TM AP 6060 Moisture Analyzer at the time of sampling. Two replicates were used from each sample.

The percentage of kernels infected by *A. flavus* was determined using a plating method (100 kernels per sample) on Aspergillus Flavus and Parasiticus Agar (AFPA) (Pitt et al. 1983). Aflatoxin B_1 content was determined because it is the most dangerous toxin. Aflatoxin B_1 contents in the kernels was determined using the ELISA method (Lee and Kennedy, 2002). Two replicates were used from each sample.

3. Results

3.1. Moisture content

The moisture content of peanut kernels either collected at wholesaler or retailer outlets in the three cities (Bandung, Bogor and Jakarta) fluctuated during sampling and were between 6 – 8% (Fig. 1). The moisture content of kernels either at wholesaler or retailer in Bogor had the same pattern with those in Jakarta, while those in Bandung had different patterns (Fig. 1). The moisture content of kernels at wholesaler and retailer in the three cities decreased in September 2005, except at the wholesaler in Bandung.

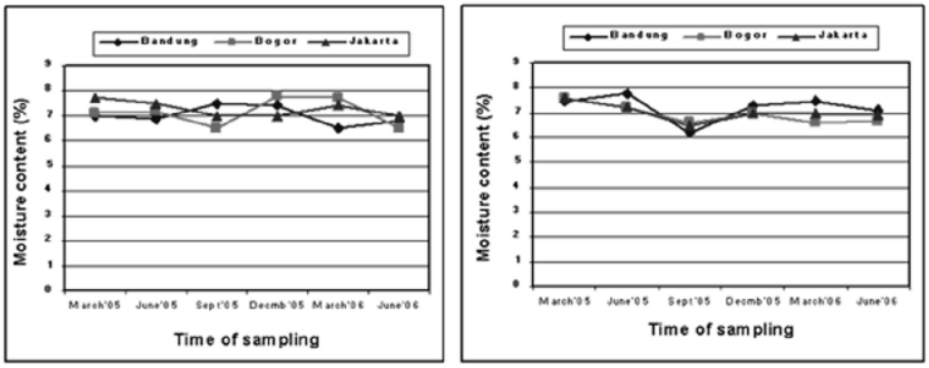

Figure 1 Moisture contents of peanut samples collected from wholesaler (a) and retailer (b) in Bandung, Bogor and Jakarta in March 2005 – June 2006

The moisture content of kernels either at wholesaler or retailer levels in the three cities were relatively similar, although the relative humidites and temperatures of the storages at wholesaler and retailer levels in Bandung were relatively lower than those in Bogor and Jakarta (Fig. 2 and 3). In general, the patterns of kernel moisture contents were relatively similar with those of relative humidites.

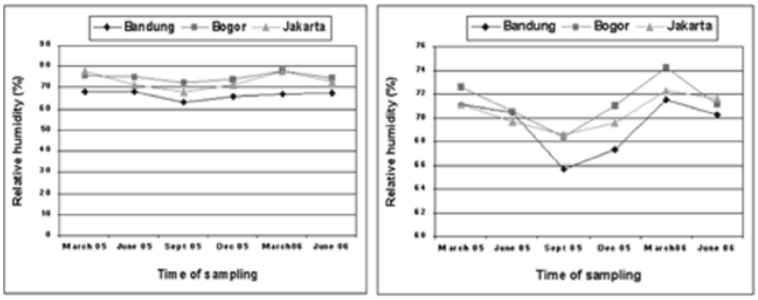

Figure 2 Relative humidity of storages at wholesale (a) and retail (b) levels in Bandung, Bogor and Jakarta in March 2005 – June 2006

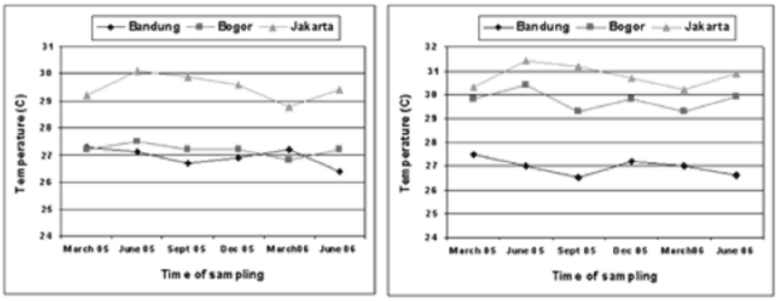

Figure 3 Temperatures of storages at wholesale (a) and retail (b) levels in Bandung, Bogor and Jakarta in March 2005 – June 2006

3.2. Aspergillus flavus infection

The percentage of kernels infected by *A. flavus* in peanuts collected from wholesaler had the same pattern as those collected from the retailers. The highest percentage of kernels infected by *A. flavus* either collected from wholesaler or retailer were in June 2005, while the lowest were in September 2005 (Fig. 4).

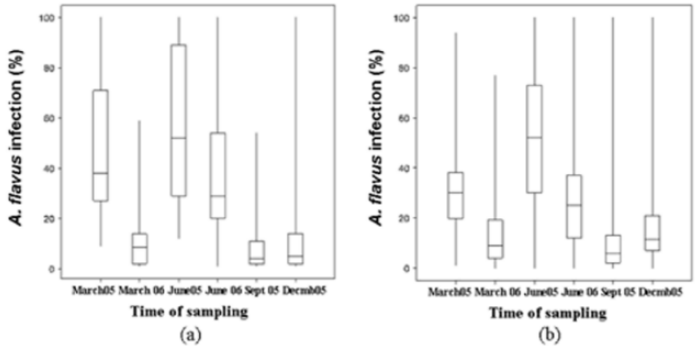

Figure 4 *Aspergillus flavus* infection in peanut samples collected from wholesaler (a) and retailer (b) in Bandung, Bogor and Jakarta in March 2005 – June 2006

The median and the highest range of percentage of kernels infected by *A. flavus* were in peanuts collected from Bandung, while the lowest were those collected from Bogor (Fig. 5). The median and the range of percentage of kernels infected by *A. flavus* collected from wholesaler were higher than those collected from retailers (Fig. 6).

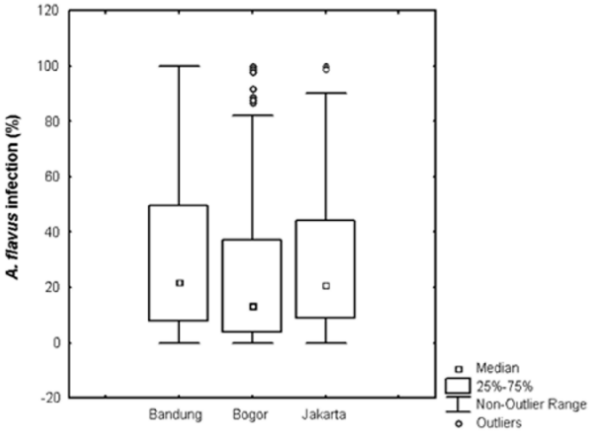

Figure 5 *Aspergillus flavus* infection in peanut samples collected from Bandung, Bogor and Jakarta in March 2005 – June 2006

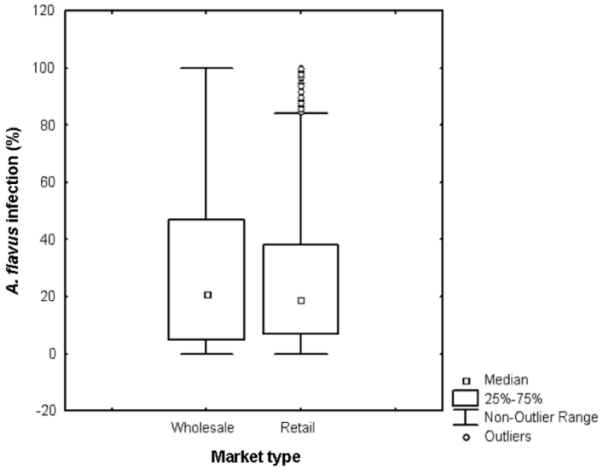

Figure 6 *Aspergillus flavus* infection in peanut samples collected from wholesaler and retailer in March 2005 – June 2006

3.3. Aflatoxin B_1 contamination

The median of aflatoxin B_1 content in peanut kernels either collected from wholesaler or retailer during sampling were relatively similar (< 20 ppb), nevertheless their broadest range occurred in June 2005 (Fig. 7). They were correlated with the percentage of kernels infected by *A. flavus* (Fig. 4).

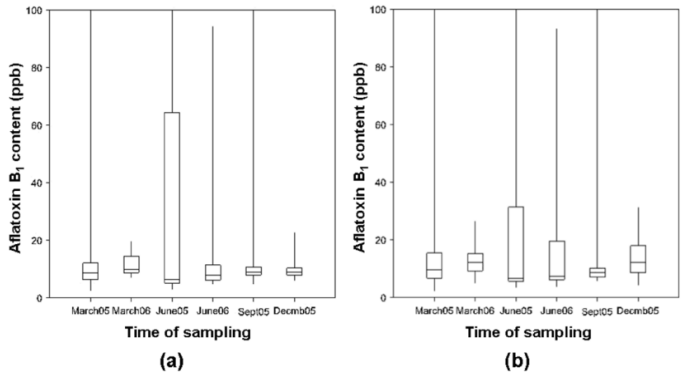

Figure 7 Aflatoxin B_1 contents in peanut samples collected from wholesaler (a) and retailer (b) in March 2005 – June 2006.

The highest median (± 20% of 204 samples) and the broadest range (10 – 60%) of frequency of samples contaminated with aflatoxin B_1 > 15 ppb were in samples collected from Bandung, followed by samples from Jakarta (the median ± 20% of 181 samples, the range 10 – 40%), and samples collected from Bogor (the median ± 15% of 180 samples, the range 10 – 40%) (Fig. 8).

The frequency of peanut samples contaminated with aflatoxin B_1 > 15 ppb collected from retailers was higher (the median 26% of 390 samples) than those collected from wholesaler (the median 18% of 175 samples) (Fig. 9), although the median and the range of percentage of kernels infected by *A. flavus* collected from wholesalers were higher than those collected from retailers (Fig. 6).

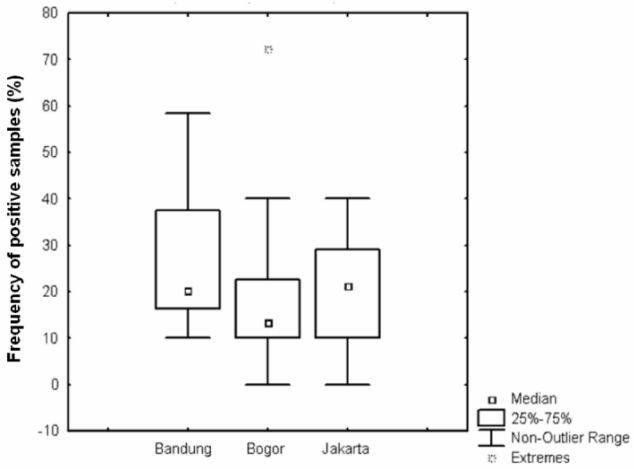

Figure 8 Frequency of aflatoxin B_1 content > 15 ppb of peanut samples collected from Bandung, Bogor and Jakarta in March 2005 – June 2006

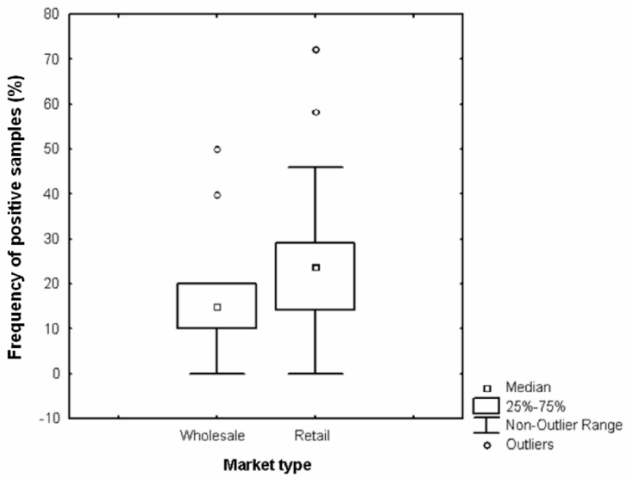

Figure 9 Frequency of aflatoxin B_1 content > 15 ppb of peanut samples collected from wholesaler and retailer in March 2005 – June 2006

4. Discussion

The moisture contentof peanut kernels collected at wholesaler or retailer in the cities (Bandung, Bogor and Jakarta) were at a safe level. According to SNI (1995) the safe moisture content for peanut kernels to be stored is around 6 – 8%. Christensen et al. (1992) stated that the moisture content of peanut kernels was in equilibrium with the relative humidity of the storage. Bala (1997) reported that the moisture content was also affected by the temperature of the storage.

The percentage of kernels infected by *A. flavus* is affected by the moisture content of the kernels, while the later is affected by the relative humidity of the storage. Relative humidity is related to the location of city from sea level as well as the month of the year. Post-harvest handling methods from farmer up to retailer levels also affect the degree of *A. flavus* infection. Antagonistic fungi can inhibit aflatoxigenic *A. flavus* growth, consequently aflatoxin production will also inhibited. Dharmaputra et al. (2001) reported that *in vitro A. niger* inhibited aflatoxigenic *A. flavus*, consequently aflatoxin production was also inhibited up to 80%. According to Pitt and Hocking (2009) aflatoxin was produced by certain strains of *A. flavus.* Dharmaputra et al. (2003b) reported that during the wet and dry seasons in 2003, 54% and 58% of 113 and 90 isolates of *A. flavus*, respectively, which were found in the soils of peanut farms in Wonogiri regency, produced aflatoxins.

5. Conclusions

- *A. flavus* infection and aflatoxin B_1 content in peanuts collected from wholesalers had the same pattern as those collected from retailers. They also correlated with the time of sampling.
- Aflatoxin B_1 content in peanut kernels either collected either from wholesalers or retailers during sampling were relatively similar (< 20 ppb), nevertheless their broadest range occurred during June 2005. Aflatoxin content was correlated with the percentage of kernels infected by *A. flavus*.
- The highest frequency of samples contaminated with aflatoxin B_1 > 15 ppb was collected from samples at Bandung, followed by samples from Jakarta and then Bogor. The frequency of peanut samples contaminated with aflatoxin B_1 > 15 ppb collected from retailers was higher than those collected from wholesalers.

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Multivariate analysis of the temporal changes of fungal communities in unsafe storage conditions of some common wheat varieties in relation to relative humidity level and rice weevil infestation

Fleurat-Lessard, F.*#[1], Fourar-Belaifa, R.[2], Bouznad, Z.[3]
[1] INRA – UR 1264 Mycology & Food Safety (MycSA) - 71 Av. Edouard Bourleaux - 33883 - Villenave d'Ornon Cedex, France. E-mail: francis.fleurat-lessard@bordeaux.inra.fr
[2] University of Blida - Department of Agronomical Science - 09000 Blida, Algeria -
[3] Institut National Agronomique - Laboratory of Plant Pathology & Molecular Biology – 12600 - El Harrach – Alger, Algeria –

* Corresponding author
# Presenting author

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Abstract

Fungal colonization of stored grain bulks is a major threat for mycotoxin contamination and reduction in viability of grain when stored under unsafe conditions, e.g. under high r.h. and insect presence. An investigation was carried out to identify the trends of the changes in the fungal species communities during storage of wheat grain under these unsafe storage conditions. The distribution change of fungi genera was monitored on small grain samples of three wheat varieties with different kernel size and hardness (soft, medium-hard and hard), during 160 d storage at constant temperature of 22-23°C, at two r.h. levels, and with or without an infestation by the rice weevil *S. oryzae*.

According to their behavioral differences related to grain water activity affinity, fungi genera were classified in three groups: i/ The hydrophilic group of field fungi (*Fusarium*, *Geniculifera*, *Sepedonium*, and *Chrysogenum*); ii/ The intermediate semi-xerophilic fungi (*Alternaria*, *Mucor*, *Ulocladium, Epicoccum,* and *Arthrobotrys*); iii/ The storage xerophilic fungi (*Penicillium* and *Aspergillus*). Temporal abundance of these three groups with grain storage time and condition was observed in weak relation with wheat variety and insect presence. The multivariate comparison of the different experimental situations revealed a difference in the susceptibility of varieties to fungal species colonization in close relationship with the final equilibrium level between ambient r.h. and grain moisture content which was observed variety-dependent. This difference was not related to grain hardness but rather to a different r.h. affinity. For one variety (*Apache*), the germination rate was declining more rapidly than for the two others with storage time. Any significant relation between sound and infested grain condition and the contamination rate by storage fungi could be found. The susceptibility of the three wheat varieties to critical storage conditions and fungal colonization may lead in one variety to a hot-spot formation.

Keywords: Common wheat, Variety, Fungal microflora, Insect pest, Fungi abundance change

1. Introduction

In France, freshly harvested wheat is never dried and is stored directly without any modification of its original physical-chemical condition at the harvest. The legal limit of moisture content recommended for safe storage of grain is fixed at 15.5% (wet basis) maximum. Nevertheless, part of the domestic production of wheat (common and durum wheat) is exported toward EU third countries located in Mediterranean or tropical climatic areas. In these warmer storage conditions, wheat has a much higher potential for quality loss by deteriorating agents, not only insect infestation but also by fungal microflora infection in relationship with the overcoming of the threshold of water activity allowing more or less xerotolerant fungi species development (Cahagnier at al., 2005). To face this quality retention storage issues that may occur in countries importing grain from developed countries, generally well equipped to prevent or reduce the grain quality deterioration process, grain stores managers are often without effective intervention means. When temperature and moisture content are high, grain respiration becomes active. The heat produced mainly by microorganisms living in the grain bulk increases the temperature of the grain that indirectly favored the fungal growth (Fleurat-Lessard, 2004). Among the various living organisms in the stored grain ecosystem, the storage fungi represent the major cause of deterioration of grain quality and of commercial value, in relationship with the potential of certain species for mycotoxin production, especially when grain is stored under a warm climate environment. This change in

temperature of stored grain can result in a spontaneous heating from the growth of fungi and in the colonization of the surface of grain bulks by thermophilic fungi and actinomycetes (Fleurat-Lessard, 2002; Magan and Aldred, 2007). In condition of medium wet or moist grain at harvest, the fungi species belonging to the group of the 'hydrophylic field flora' are predominant during the beginning of the storage period. During long-term storage, xerophilic fungi species (called the 'storage flora') progressively replace the field flora over a period of several months of storage (Pelhate, 1982; Frisvad, 1995; Fleurat-Lessard, 2002). In agreement with the concept of Wallace and Sinha (1981), the stored grain ecosystem must be considered by a holistic and ecological approach to enable a proper understanding of the processes occurring and to improve strategies of post-harvest protection against deteriorative forces (Tipples, 1995; Magan and Aldred, 2007). There is very little detailed information on the tolerance and susceptibility of wheat varieties actually cultivated in France to critical storage conditions. To improve the actual knowledge about wheat varieties sensitivity to poor storage conditions, the impact of critical conditions of storage on stored grain qualitative trait changes were investigated recently and results of this systemic study are presented in another session of the present Conference (Fourar et al., 2010). However, in the same trial, we examined more deeply and in an ecological manner the patterns of evolution of fungi species communities in comparing two opposite storage conditions, a safe environment and a critical condition of a_w and insect infestation.

Our main objective was to relate the dynamics of the changes in fungus species community to the deterioration process of wheat quality traits. The questions to be tackled were: i/ May grain hardness influence fungal species community change trends during long-term storage in critical physical-chemical conditions? ii/ The currently cultivated wheat varieties have they different susceptibilities to critical storage conditions and especially when the storage mycoflora may develop?

2. Materials and methods

2.1. Experimental design

A multidimensional laboratory trial was carried out to identify the key-factors of the overall quality traits changes, to understand their interactions in the process of deterioration, and finally to reveal underlying trends of critical storage conditions that may endanger grain quality retention. A large set of qualitative criteria was followed on grain batches from 3 wheat varieties with various qualities for cereal food processing, which were stored during 160 d at 22-23°C, under two different relative humidities (r.h.), and with or without an infestation by the rice weevil *S. oryzae*. The major factors involved in wheat grain quality trait changes in relation to the development of insect and fungi populations were periodically recorded each 40 d approximately. At each checking date, all the grain quality attributes were determined by standard methods or by laboratory proofed methods (Table 1).

Table 1 List of quality traits of wheat grain with the reference of each analytical method used for their quantification in the present study. *Grain quality traits are distributed in the different classes of quality attributes*.

Grain Quality trait analyse	acronym	Analytical method	Reference
1. Sanitary and soundness condition			
Adult insects counting	Insect_AD	Sieving – NF-V 03-742	Afnor, 1982
Insect hidden infestation counting	Insect_HI	Radiograph-ISO 6632-4	Afnor, 1982
2. Microbiological spoilage			
2.1 Qualitative analysis: Rate of fungi-contaminated kernel	Cont_Rate	Ulster's method	Cahagnier and Richard-Molard, 1997
2.2 Quantitative analysis: Isolation and identification of fungal colony-forming-unit (CFU) per g	Fungi_Q	NF V08-011	Afnor, 1996
3. Germination			
Germinative capacity	Germ_Cap	ISTA rules for seed testing	ISTA 1999
4. Physical-chemical condition			
Moisture content (wet basis)	MC	Oven-drying practical method NF V 03-707	Afnor, 1982

Grain Quality trait analyse	acronym	Analytical method	Reference
Kernel hardness	Hardness	Hardness point-meter	Hardness meter notice
Thousend grain mass	TGM	NF V 03-702	Afnor, 1982
5. Biochemical composition			
Lipid acidity (or fat acidity)	Lipid_Ac	NF V 03-712	Afnor, 1982
6. Statistical analyses			
Multivariate explanatory analyses		Multiple correlation - PCA	Addinsoft, 2005

The wheat varieties batches, that had been cultivated especially for this trial in center-northern France, were received just after 2007 harvest and were fumigated with phosphine before use. Then, a thorough mechanical and manual cleaning was achieved to sort all impurities and abnormal kernels before settling the experimental samples. The experimental design was hierarchical, composed with three levels of controlled factors: Variety, ambient r.h. and infestation with *Sitophilus oryzae* (L.) (*vs*. uninfested control). For each level of factor, four grain sample of 1.150 kg each were placed in aerated glass containers and put inside a controlled-r.h. storage enclosure. We applied the approach of the "fixed-effect-modelling" in which several qualitatively and logically distinct variables were checked on the same grain sample at different time intervals during a storage period of 160 d. This is a covariance analysis situation where several treatments (different grain varieties and storage conditions) were applied to the objects of the experiment (grain samples) to see if the response variable values changed along time. Among the observed variables, the fungi species distribution and occurrence were more particularly investigated.

2.2. Analysis of fungi community evolution during storage

During the 160-d storage period, the grain was sampled four times after 42, 75, 12, and 160 d. A part of the sample was used to determine the rate of contamination of kernels by the Ulster method (Cahagnier and Richard-Molard, 1997). Another part was used to achieve a global quantitative microbiological analysis on two replicates of each experimental unit (giving the global count of colony-forming-unit (CFU) per g of grain). The fungi species colonies observed on Petri dishes medium of the quantitative microbiological analysis were counted in separated classes according from their macroscopic external aspect. The formal identification of the genus of each separated "class" was performed after isolation on two different culture media (Potato dextrose agar (PDA), malt and yeast extract agar). The frequency of the distribution of each identified fungi genus was then calculated for each experimental condition (weighed mean percentage) and for each control date.

2.3. Statistical analysis

The multidimensional statistical analysis and chart plotting were achieved with Xlstat® (Addinsoft, Paris, France, 2007) software. In our experiment, multivariate data were expressed in a matrix form with *p* columns (measured variables including observed fungi species) and *n* rows (wheat varieties sample units distributed in various storage conditions of r.h. and insect infestation). The variables were ranged in two classes: i/ Explanatory variables: r.h. level (*r.h._Equi*), insect presence *vs*. absence (*Infested*), grain hardness (*Hardness*, related to varietal difference), and storage time (*Time*); ii/ Dependent variables: adult insect density per kg (*Insect_AD*), insect hidden infestation (*Insect_HI*), germination capacity (*Germ_Cap*), fungi CFU per g (*Fungi_Q*), grain contamination rate by fungi (*Cont_Rate*), fat acidity (*Fat_Acid*), and 11 different *Genera* of fungi recovered from wheat grain samples along the study.

The interactions between all variables were represented and analyzed in a multivariate global approach. A multivariate covariance analysis (ANCOVA) was performed in Xlstat® to assess the effect of storage conditions and of the prime characteristics of varieties (imbedded in *Hardness*) on all dependent variables, and especially on the fungi distribution variation trends. This data processing software allowed carrying out the calculation of simple and multiple correlation coefficients, as also the analysis of covariance enabling to model by multiple correlation the evolution of each dependant variable as a function of the explanatory variables. Next, data multivariate analysis results were processed using the principal component analysis (PCA). This procedure allowed extracting the Pearson's product moment correlation matrix of the binary correlations between all the dependant and explanatory variables. PCA

was performed to visualize the circular diagram of the strength of the correlations between all variables in eliminating the effects of redundancy between the variance of closely related variables (e.g. protein content and hardness). This diagram allowed to precisely appreciate their complex interactions from graphically represented independent variables taken in a whole set.

3. Results

Two main sets of results were intended: i/ descriptive results of the changes in the level of dependent variables during the 5 months storage period as also the comparison between variation trends related to safe or critical storage environment conditions; ii/ Explanatory analysis of the global interactions between storage conditions (in critical situation) and qualitative traits change with time allowing to understand the involvement of fungi species communities into the quality deteriorative process.

3.1. Analyse of variation trends in fungi distribution

A global overview of the changes in the rate of contaminated seeds of the three wheat varieties stored in the different conditions exposed above showed that all the kernels were harbouring at least a fungal germ, whatever the variety (Fig. 1). This 100% contamination level was declining during storage from 4 months storage time for *Caphorn* variety (hard wheat type), and from 3 months storage time for the two other varieties (*Apache* and *Crousty*, respectively medium-hard and soft). However, at the end of the storage period this global contamination rate was approximately the same for the three varieties (between 78 and100% fungi-contaminated kernels).

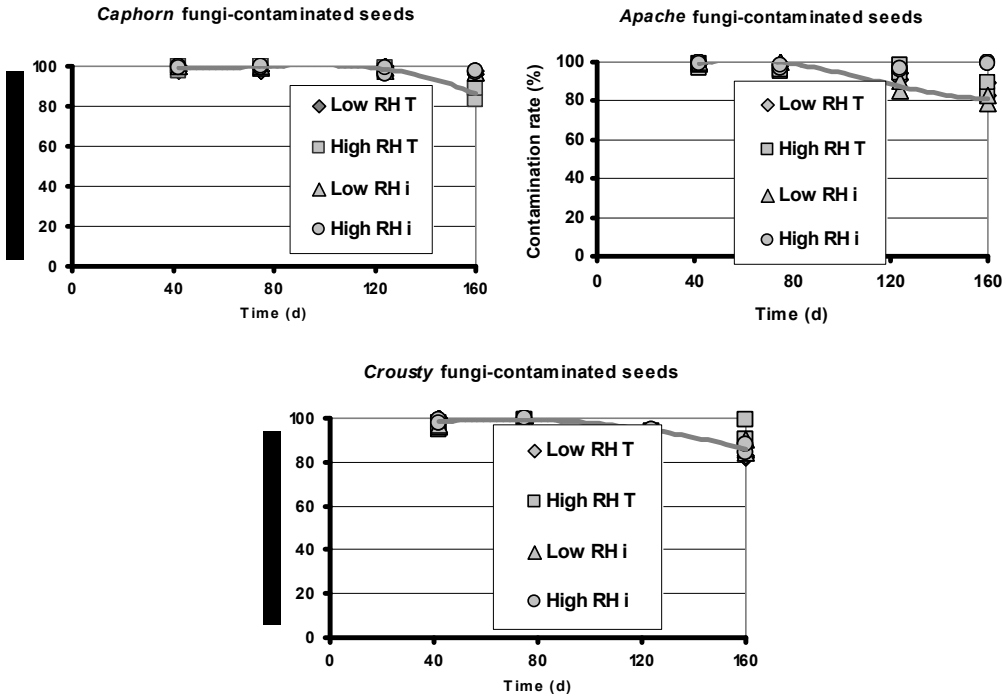

Figure 1 Changes in the rate of contaminated seeds observed on three wheat varieties stored under high or low r.h. and with and without an infestation by *S. oryzae* (T = uninfested control; i = infested).

The identification of fungi *Genera* enabled to establish a comparative distribution of fungi species in relation with storage conditions (Table 2).

Table 2 Comparative distribution of fungi genera isolated in samples of three wheat varieties during a 160-d storage period in two different r.h. levels and with and without an infestation by *S. oryzae.*

Wheat variety	**Caphorn**		**Apache**		**Crousty**	
Fungus Genus	**Control (sound)**	**Infested**	**Control (sound)**	**Infested**	**Control (sound)**	**Infested**
Fusarium	+++	+++	+	+	++	++
Epicoccum	+	+	+	+	+	+
Aspergillus	+	+	+	+	+	+
Penicillium	+	+	+	+	+	+
Alternaria	++	++	+	+	+	+
Chrysosporium	++	+	++	+	++	++
Geniculifera	+++	+++	+++	+++	+++	+++
Sepedonium	+	+	+	-	+	++
Ulocladium	++	+	++	+	++	++
Mucor	+	+	-	-	-	-
Arthrobotrys	++	++	+	++	++	+
Other *Taxa*	+	+	+	+	+	+

Among the 11 formally identified *Genera*, the most frequent fungi were: *Fusarium*, *Geniculifera*, *Chrysosporium*, *Alternaria*, *Ulocladium* and *Arthrobotris*. Most of these *Genera* corresponded to a primary contamination of grains at the harvest and they belong to "field mycoflora" or to "intermediate mycoflora" according to the classical grain fungi dynamics series concept (Sinha, 1979; Pelhate, 1982).

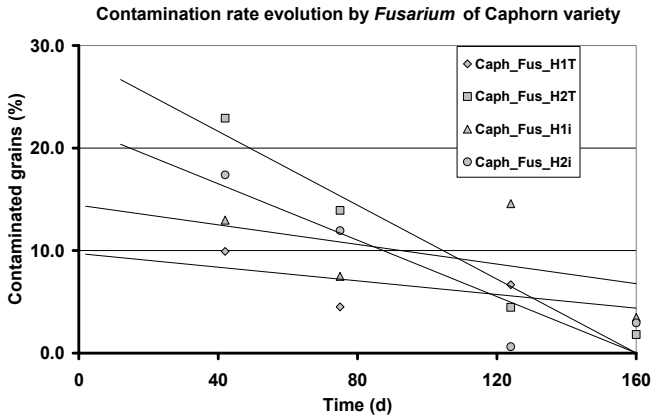

Figure 2 General trends of decrease of the contamination rate of *Caphorn* wheat variety by field fungi of *Fusarium* spp.

These two series of grain mycoflora had a different fate along the storage period. The contamination rate by "field fungi" *Genera* as *Fusarium* spp. was regularly declining during the 5-month storage period, down to complete disappearance (Fig. 3). The fate of the "intermediate mycoflora" showed a congruent figure with the concept of grain fungi dynamic series (Fig. 4). Again according to the theory, the appearance of "xerophilic storage fungi" occurred only after 4 months of storage (Fig. 5).

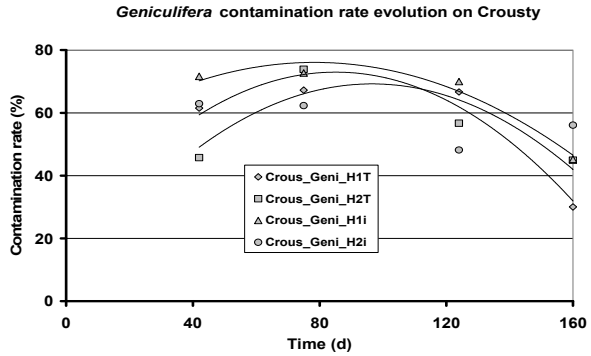

Figure 3 General trends of evolution of the contamination rate of *Crousty* wheat variety by semi-xerophilic fungi (intermediate mycoflora group) of *Geniculifera* spp.

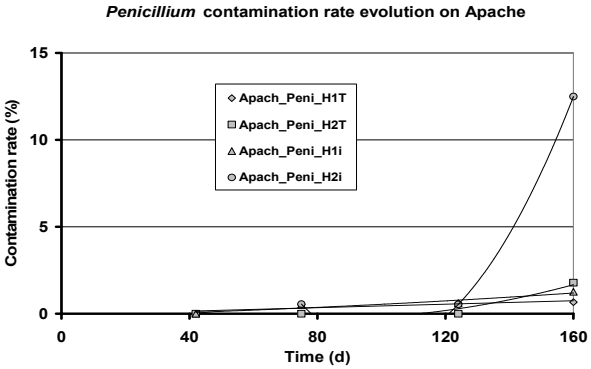

Figure 4 General trends of evolution of the contamination rate of *Apache* wheat variety by xerophilic fungi (storage mycoflora group) of *Penicillium* spp.

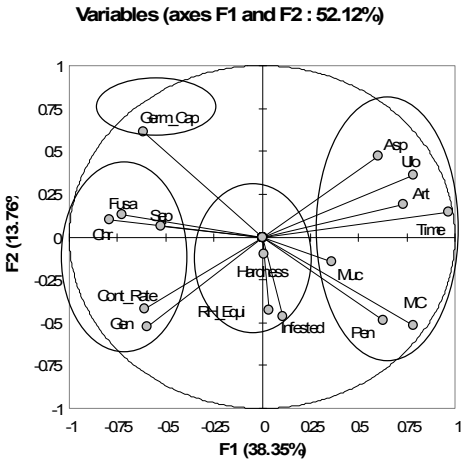

Figure 5 PCA: circular diagram visualising the correlations between all variables (dependent and explanatory) revealing the interactions in some qualitative traits and in fungal microorganism community structure during a 160-d storage period of 3 wheat varieties of different hardness at two different r.h. levels and with or without an infestation by *S. oryzae*.

3.2. Multivariate explanatory analysis of relationship between grain storage condition and fungi species distribution change

A linear multiple regression was computed for the predictive modelling of each dependent variable (including each fungi *Genus* frequency) as a polynomial function of the explanatory variables: kernel hardness (major discriminative attribute for each variety), r.h. level, presence or absence of insects, and variety specific effect. The analysis of this multiple linear regression showed that the dependent variables could be correlated with the set of explanatory variables, except total counts of fungal CFU (*Fungi_Q*) (Table 3).

Table 3 Descriptive parameters of multiple regression models predicting the dependent variables (see Table 1) as a polynomial function of explanatory variables: kernel hardness (imbedded in variety properties), r.h. level equilibrium, presence or absence of insect (*Infested* vs. control) and variety specific effect.

Explanatory variables	**# F-test value**	**Hardness**			**RH Equilibrium**			**Insect infestation**		
Dependent variables		**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**
Moisture content	19.405***	0			0			0		
Germ_Cap	11.606***	0			0			-0,332	-2,745	0.009**
Fungi_Qu	NS									
Cont_Rate	18.18***	0			0			0		
Fusarium spp.	27.628***	0			0			0		
Aspergillus spp.	13.029***	0			-16,16	-1,332	NS	0		
Penicillium spp.	9.155***	0			6,004	1,142	NS	0		
Chrysosporium sp.	31.166***	0			0			0		
Geniculifera sp.	18.920***	0			0			0		
Sepedonium sp.	10.517***	0			11,602	1,576	NS	0		
Ulocladium sp.	48.847***	0			-15,62	-0,887	NS	0		
Mucor spp.	8.296***	0			0			0		
Arthrobotrys sp.	30.464***	0			18,24	1,787	NS	0		

Continue:

Explanatory variables	**# F-test value**	**Storage time**			***Caphorn* variety**			***Apache* variety**		
Dependent variables		**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**
Moisture content	19.405***	0,02	6,784	< 0.001***	-0,331	-1,005	NS	0,788	2,393	0.021*
Germ_Cap	11.606***	-0,479	-3,959	< 0.001***	0					
Fungi_Qu	NS									
Cont_Rate	18.18***	-0,069	-6,11	< 0.001***	4,625			0,344	0,277	NS
Fusarium spp.	27.628***	-0,085	-7,515	< 0.001***	1,012	0,805	NS	-5,021	-3,992	< 0.001***
Aspergillus spp.	13.029***	0,066	4,928	< 0.001***	0			0		
Penicillium spp.	9.155***	0,024	4,124	< 0.001***	0			0		
Chrysosporium sp.	31.166***	-0,059	-9,014	< 0.001***	-0,844	-1,167	NS	-2,489	-3,441	0.001***
Geniculifera sp.	18.920***	-0,204	-6,553	< 0.001***	-2,69	-0,781	NS	9,504	2,758	0.008**
Sepedonium sp.	10.517***	-0,035	-4,307	< 0.001***	0			0		
Ulocladium sp.	48.847***	0,192	9,844	< 0.001***	0			0		
Mucor spp.	8.296***	0,012	3,144	0.003**	1,357	3,241	0.002**	-0,091	-0,216	NS
Arthrobotrys sp.	30.464***	0,086	7,6	< 0.001***	0			0		

The best fitted polynomial regressions were found with the following fungi *Genera*: *Ulocladium, Chrysosporium, Arthrobotrys, Fusarium, Geniculifera*, and *Aspergillus*. However, the explanatory variables did not explain the same amount of variance for each dependent variable. Thus, storage time (*Time*) was observed a very highly significant component of the predictive model of all dependent variables, except *Fungi_Q* (Table 3). Infested condition (*vs*. sound grain condition) was negatively and

highly significantly correlated with germination capacity and without relation with any fungi *Genus* variance. The contribution of the variety Apache to the variance of moisture content was significant. This positive correlation indicated that the higher increase in moisture content in *Apache* than in the two other varieties might be related to a higher r.h. affinity of this variety (Fourar-Belaifa et al., 2010). For the contribution of the varieties to the variance of fungi *Genus*, it was observed two negative correlations between *Apache* variety with *Fusarium* and *Chrysosporium Genera*, and a positive correlation with *Geniculifera Genus*. *Caphorn* variety had a significant positive correlation with *Mucor* Genus only.

The graphical representation of the magnitude of correlation between variables by PCA circular diagram allowed extracting the more relevant interactions between variables that are significantly involved in the deterioration process during storage in critical conditions (Fig. 5). With PCA graphical representation, it was observed that the first component axis contributed to more than 38% of total variance or overall correlations of the whole set of variables. The fungi *Genera* were distributed into two distinct groups at each end of this first component. The first group included *Genera* negatively correlated with storage time and moisture content variables. This meant that these fungi *Genera* regressed with storage time and low grain moisture content (Table 4). Thus, these Genera could be clearly classified as 'hygrophilic field fungi' type: *Fusarium*, *Sepedonium*, *Chrysosporium*, *Geniculifera*. At the opposite end of the first axis, the second group of fungi *Genera* was correlated to storage time in significant dependence with moisture content variation. This meant that the dynamics of these fungi *Genera* was dependent of long-term storage periods and that their occurrence was dependent of moisture content variance. Thus, these *Genera* might be classified as 'storage or intermediate mycoflora': *Aspergillus*, *Ulocladium*, *Penicillium*, *Arthrobotrys*, and *Mucor*. The proximity between explanatory variables "*Infested*" and "*r.h._Equi*" and dependent variables *Penicillium* and *Mucor* Genera indicated that the presence of insect and high r.h. might have induced an abundant proliferation of storage fungi, which was a situation observed with *Apache* variety at the end of the storage period (hot-spot induced by storage fungi of *Penicillium* Genus, Fig. 4). Neither hardness, nor r.h. level had a significant contribution to the overall correlation of the whole set of variables processed through PCA. The second axis of PCA contributed weakly to the overall covariance of the whole set of variables (less than 14%). Along this second axis, it was observed a negative influence of insect infestation on germination capacity. However, the influence of moisture content level on germination capacity was significantly correlated (correlation coefficient: -0.842; d.f.: 5, 42; $P \leq 0.05$).

Table 4 Direction of significant correlations between controlled factors and fungi species communities variance monitored on 3 wheat varieties stored during 160 d in safe or critical environmental conditions (r.h., insect infestation and kernel hardness according to selected wheat varieties)

	Time	**Infested**	**Var_Apache**	**Var_Caphorn**
germ_Cap	negative	negative		
Fungi_Qu	NS			
Cont_Rate	negative			positive
M.C.	positive		positive	
Fusarium	negative		negative	
Apergillus	positive			
Penicillium	positive			
Chrysosporium	negative		negative	
Geniculifera	negative		positive	
Sepedonium	negative			
Ulocladium	positive			
Mucor	positive			positive
Arthrobotrys	positive			

4. Discussion

Some correlations could be explained by the properties of the selected varieties. Thus, the fungi-contamination-rate of wheat kernels correlated with the hardness of kernels because the most contaminated variety at the harvest by field fungi was *Caphorn*, which was of the hard type (*vs*. medium-hard and soft for *Apache* and *Crousty*, respectively). Nevertheless, this situation could be also related to a

low susceptibility of *Apache* to the contamination by *Fusarium* spp., which was the most abundant fungi *Genus* found on *Caphorn* at the harvest. The least susceptibility to *Fusarium* contamination of Apache than the two other varieties was in agreement with the rating for intrinsic susceptibility of *Fusarium* spp. contamination published for all wheat varieties cultivated in France. An important result obtained in the present study was to see that the hardness type of wheat variety has no significant relationship with fungi *Genera* variations during post-harvest storage. Nevertheless, wheat variety intrinsic characteristics and properties had a positive or a negative dependence with several variables linked to the suitability for long-term storage without deterioration of quality traits (Table 4). From the results of this specific study investigating about the aptitude for safe storage time in a small set of three different wheat varieties currently cultivated in France, it could be deduced that the aptitude of high-yield productive wheat varieties to tolerate critical storage environmental conditions (high r.h., insect infestation and fungal spoilage risks) was not taken into account as an important quality attribute in variety creation.

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Effect of essential oils from *Eucalyptus* on the growth of aflatoxigenic species

Da Gloria, E.M.*#[1], Mengai, B.[1], Steffen Almeida, G.[1], Cuccovia Mazotti, N.C.[1], Tadeu dos Santos Dias, C.[1], Moreira e Moreira, R.[2], Gomes Vieira, I.[3], Da Silva, M.F.G.F.[4], Cruz Silva, S.[4], Calori Domingues, M.A.[1],
[1] Escola Superior de Agricultura "Luiz de Queiroz" ESALQ/USP, Av. Pádua Dias, 11, Piracicaba. SP, Brazil, CEP 13418-900. Email: emgloria@esalq.usp.br (E. M. da Gloria)
[2] Estação Experimental de Ciências Florestais de Itatinga, Estrada do Distrito do Lobo. s/ n^0, CEP: 18690-000.
[3] Instituto de Pesquisas e Estudos Florestais, Avenida Pádua Dias. 11, Piracicaba. SP. Brazil, CEP: 13400-970
[4] Departamento de Química, Universidade Federal de São Carlos. São Carlos – SP, Brazil, CEP 13565-905

* Corresponding author
# Presenting author

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Abstract

In Brazil, *Eucalyptus* species has been cultivated as source of energy and cellulose. They represent the most important cultivated forest in the country. In production areas, the leaves from the trees decay on the soil as green fertilizer. In this study were evaluated pure and blends of essential oils from different species of eucalyptus trees grown in Brazil for antifungal activity against aflatoxigenic species *Aspergillus flavus* and *A. parasiticus.* These fungal species can grow and contaminate grains during the storage period under high r.h. conditions, with an eventual production of aflatoxins. Antifungal activity was evaluated by the radial growth measurement of the fungi inoculated on maize meal extract agar basic medium. The eucalyptus oils were evaluated in a contact assay and a fumigant assay using pure and blended oils. Six concentrations of pure and blended oils were evaluated at the following doses: 0, 2, 4, 16, 32 and 84 µL per 20 mL of fungi culture medium. Fungal inocula from conidia suspensions containing 10^6 spores/mL was inoculated by a needle. Glass Petri dishes were incubated for 9 days at 28^0C (± 0.3°C) in the dark. Antifungal activity was observed in all pure and blended oils, in different concentrations of contact and fumigant assay, for both fungi. *Eucalyptus stageiriana* oil and *E. stageiriana* + the hybrid *E. grandis x E. urophylla* oils blend controlled the total fungal growth at the lowest dose (20 µL).

Keywords: Essential oil; *Eucalyptus* spp.; *Aspergillus flavus*; *Aspergillus parasiticus*; Antifungal activity.

1. Introduction

Combating pests and diseases that affect the pre and post-harvest crops of interest to man has been performed almost exclusively by the use of synthetic pesticide. However, due to all the risks for the environment and human health alternative methods of control have been researched and studies have been conducted with extracts of medicinal plants to control plant pathogens (Salvadori et al., 2003). Some studies have demonstrated the effect of essential oils from plants with several biological effects and the antifungal activity has been demonstrated (Bakkali et al., 2008). In Brazil, *Eucalyptus* species has been cultivated as source of energy and cellulose. They represent the most important cultivated forest in the country. In the production areas the leaves from the trees decay on the soil as green fertilizer. One possible destination for those leaves can be the essential oil extraction, but the *Eucalyptus* essential oil market is small, so new potential uses for this oil can help to increase the market. Essential oils of eucalyptus have several functions and have been considered as an insect repellent, inhibiting germination and growth of other plants, controlling microbial activity of some fungi and bacteria, among others (Boland et al., 1991).

In this study pure and blends of essential oils from different species of eucalyptus trees grown in Brazil were evaluated for antifungal activity against aflatoxigenic species *Aspergillus flavus* and *A. parasiticus.*

2. Materials and methods

2.1. Essential oil extraction

Essential oils used in this study were from *E. grandis, E. staigeiriana, E. citriodora*, and the hybrid *E. grandis x E. urophylla.* Volatile compounds were isolated from 200 g of fresh leaves by hydrodistillation in 2 L of water for 4 hours using Clevenger-type equipment. The oil was dried over anhydrous sodium sulfate and stored under refrigeration.

2.2. Essential oil composition

Four essential oils were characterized for their composition using a gas chromatograph (Shimadzu™ 17A) fitted with a capillary column (Atm 54 ms). Temperature was programmed initially at 50ºC for 3.5 min, and then increased at a rate of 7°C min up to 100^0C. Then the rate was increased at 10°C min up to 250°C, this latter temperature maintained for 3.5 min, and helium gas as carrier gas at a flow rate of 1 mL min. Injection was in split mode at 280°C. The chromatograph was coupled to a Shimadzu™ QP5000 mass selective detector - Electron impact mass spectrometry (EIMS) and the mass spectrum was recorded in the range of 70 eV and a mass / charge ratio (m / z) of 50 to 500.

The identification of the components was made by determination of their retention indices related to those of a homologous series of n-alkanes (Dool and Kratz, 1963), and fragmentation patterns in mass spectra with those stored on the spectrometer database and the bibliography (Adams, 2001).

2.3. Antifungal assays

To evaluate the effect of essential oils against aflatoxigenic species, *Aspergillus flavus* and *A. parasiticus,* the oils were evaluated in a contact assay and a headspace volatile exposure assay for fumigant activity determination (Villela et al., 2009) with three replicates. Six different doses (0, 2, 4, 16, 32 and 84 µL per 20 mL basic medium) of the pure and blend of two essential oils from *E. grandis, E. staigeiriana, E. citriodora*, and the hybrid *E. grandis x E. urophylla* were tested. All doses of oils and blend were dissolved with 200 µL of acetone, and acetone alone was used in the control.

The basic medium was the maize meal extract agar (MMEA) as used by Marin et al. (1995). Twenty mL of this medium was placed in 9 cm diameter glass Petri dishes. The fungal species inoculum was obtained from colonies of *A. flavus* and *A. parasiticus* maintained at constant temperature of 28°C for 7 days. The inocula in water suspension were adjusted to 10^6 spores mL in water with 1.0% DMSO.

In contact assay, the doses of essential oil were added to the autoclaved and cooled but still liquid basal medium. The inoculation was performed after basal medium solidification by a needle that was immersed in the inoculum stock solution and applied in one point of the basal medium. In the headspace volatile assay the oil was applied over a round filter paper dish of approximately 2 cm diameter placed in the inner of plate lids. The inoculation was performed similarly as in the contact assay and Petri dish was incubated with the lid upside down. For both assays, the Petri dishes were sealed and incubate in the dark at constant temperature of 28°C (± 0.3°C) and the mycelial growth was evaluated after 9 d by colony diameter measurements.

Mycelium growth means of each treatment were compared by Tukey's test with probability level < 0.05 using Statistical Analysis System software (SAS, 2004).

3. Results and discussion

Several peaks were detected in the oil chromatograms but only for *E. staigeiriana* oil was possible to identify most of them (Table 1). The great number of observed compounds in this oil probably was due to the hydrodestilation time of 4 h that allowed the extraction of monoterpenes and sesquiterpenes from leaves (Viturro et al., 2003; Franco et al., 2005).

Table 1 Number of peaks detected and identified in oils extracted from Eucalyptus leaves.

Species	Peaks detected	Peaks identified (%)
Hybrid *E. grandis* x *E. urophylla*	47	21 (44.6)
Eucalyptus grandis	37	20 (54.0)
Eucalyptus staigeiriana	39	38 (97.4)
Eucalyptus citriodora	40	21(52.5)

Citronellal, α-pinene and limonene were the major components observed in the oils from *E. citriodora*, *E. grandis* and *E. staigeiriana*, respectively. In a previous study, Boland (1991) observed the same major compounds in oils of *Eucalyptus* spp. leaves but with different percentages for major components. The hybrid *E. urograndis* showed α-cimene as a major compound (Table 2).

Table 2 Volatile compounds identified and their percentage in the oils of *E. urograndis, E. grandis, E. staigeiriana* and *E. citriodora.*

Compounds	*E. urograndis*	*E. grandis*	*E. staigeiriana*	*E. citirodora*
isopropyl butanoate	0.24	-	-	-
alpha-pinene	0.66	26.97	2.7	0.04
p-cimene	41.32	0.84	1	-
limonene	2.27	-	17.66	-
1.8-cineole	4.78	10.92	3.89	0.33
gamma-terpinene	3.56	0.32	-	-
exo-fenchol	0.47	-	-	-
trans-limonene oxide	0.23	-	-	-
trans-pinocarveol	0.94	-	-	-
borneol	1.16	-	-	-
terpinen-4-ol	2.6	-	-	-
alfa-terpineol	2.82	3.58	-	-
piperitona	0.34	-	-	-
thymol	0.65	-	-	-
carvacrol	0.39	-	-	-
drima-7.9-(11)-diene	0.14	-	-	-
flavesona	4	3.8	-	-
globulol	1.59	-	-	-
sesquithuriferol	0.99	-	-	-
leptospermone	9.96	5.14	-	-
beta-eudesmol	0.83	-	-	-
campen	-	0.74	-	-
beta-pinene	-	0.06	5.56	0.05
α-cimene	-	5.91	-	-
trans-verbenol	-	0.75	-	-
camphene hydrate	-	0.4	-	-
pinocarvona	-	0.53	-	-
bornyl acetate	-	0.17	-	-
trans-beta-guaiane	-	0.1	-	-
beta-atlantol	-	0.3	-	-
khusimore	-	0.88	-	-
mirane	-	-	0.59	-
alpha-phellandrene	-	-	4.37	-
alfa-terpinene	-	-	0.18	-
beta-e-ocimene	-	-	0.21	-
gama-terpinene	-	-	1.53	-
trans-oxide linanol	-	-	0.08	-
p-mentha-2nd. 4 (8)-diene	-	-	6.68	-
linalool	-	-	1.72	-

Compounds	*E. urograndis*	*E. grandis*	*E. staigeiriana*	*E. citirodora*
1-terpineol	-	-	0.13	-
trans-p-mentha-2-en-1-ol	-	-	0.17	0.02
neoiso-3-tujanol	-	-	0.18	-
iso-isopulegol	-	-	0.23	-
neoiso-isopulegol	-	-	0.11	-
p-mentha-1 .5-dien-8-ol	-	-	1.02	-
terpinen-4-ol	-	-	1.65	-
meta-cymene-8-ol	-	-	0.28	-
alfa-terpineol	-	-	5.39	0.26
trans-piperitol	-	-	0.17	-
citronellol	-	-	2.36	14.81
neral	-	-	9.34	-
geraniol	-	-	4.31	-
geranial	-	-	10.84	-
lavandulil acetate	-	-	0.4	-
methyl geranato	-	-	5.62	-
acetate citronellyl	-	-	1.02	0.02
neril acetate	-	-	2.81	-
geranyl acetate	-	-	5.05	-
beta-z-farnesene	-	-	0.14	2.99
germacrene a	-	-	0.07	-
alfa-e.e-farnesene	-	-	0.12	-
globulol	-	-	0.17	-
espatulenol	-	-	0.43	-
z-sesquilavandulol	-	-	0.4	-
gamma-pinene	-	-	-	0.09
p-mentha-3.8-diene	-	-	-	0.04
terpinolene	-	-	-	0.07
trans-rose oxide	-	-	-	0.03
citronella	-	-	-	19.45
3-tujanol	-	-	-	6.25
format citronellyl	-	-	-	0.02
dehidro-anomadendrano	-	-	-	0.2
alfa-muuroleno	-	-	-	0.17
trans-beta-guaiane	-	-	-	0.16
alfa-cardineno	-	-	-	0.29
7-epi-alfa-eudesmol	-	-	-	0.51

Results observed in headspace volatile assay could be considered statistically the same observed for contact assay for all oils and blends (Table 3). Exceptions to this similarity were observed with *E. grandis* oil for both fungi and with *E. urograndis* oil for *A. parasiticus*. For *E. grandis* oil, the headspace volatile assay offered antifungal activity at lower doses than contact assay and for *E. urograndis* oil, contact assay showed lower efficient doses than in volatile assay.

All pure oils and blends showed some antifungal activity against *A. flavus* and *A. parasiticus* in both types of assays with different doses. The total fungal growth control for both fungi was achieved for all oils and blends with exception of *E. grandis* oil and the blend of E*. grandis* and the hybrid *E. grandis x E. urophylla* oil for *A. flavus* in the contact assay. The oil of *E. staigeiriana,* the blend of oils from *E. staigeiriana* and the hybrid *E. grandis x E. urophylla* showed an antifungal activity at the lowest dose (20 µL) (Tables 4-7).

Table 3 Effect of antifungal activity for contact or volatile activity of *Eucalyptus* spp leaves extracts against the fungi *A. flavus* and *A. parasiticus*.

Oils	*A. flavus* - P Value[1]	*A. parasiticus* - P Value
G[2]	0.0049	0.0138
U	0.3455	0.0500
S	0.1783	0.3506
C	0.1828	0.7065
G+U	0.4116	0.3506
S+G	0.8449	0.2932
C+G	0.9008	0.3910
S+U	0.3632	0.3632
S+C	0.3434	0.8449
C+U	0.2534	0.2048

[1]Level of significance 5% to compare contact versus votalite.[2] = G = *E. grandis*; C = *E. citriodora*; U= the hybrid *E. grandis x E. urophylla*; S=*E. staigeiriana*

Table 4 Mycelial growth in antifungal test for contact activity of *Eucalyptus* spp leaves extracts against *A. flavus.*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
G	37. Aa	37 Aa	31 Aa	30 Aa	10 Bb	6 Ca	25.2 a
C	37 Aa	34 Aa	33 Aa	28 Aa	24 Ba	2 Cb	26.6 a
C+G	37 Aa	31 Aa	21 Bb	19 Bb	13 Bb	0 Cb	20.3 b
G+U	37 Aa	23 Bb	19 Bb	14 Cb	13 Cb	5 Da	18.4 b
U	37 Aa	26 Bb	20 Bb	17 Cb	4 Dc	2 Db	18.4 b
C+U	37 Aa	30 Ba	25 Bb	21 Bb	0 Cc	0 Cb	18.7 b
S+G	37 Aa	40 Aa	33 Aa	4 Bc	0 Bc	0 Bb	17.8 b
S+C	37 Aa	36 Aa	33 Aa	0 Bc	0 Bc	0 Bb	19.0 b
S	37 Aa	19 Bc	17 Bc	0 Cc	0 Cc	0 Cb	12.1 c
S+U	37.Aa	12 Bc	4.Cd	0 Cc	0 Cc	0 Cb	8.8 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 5 Turkey test for treatments (oils) x dose (µl) in antifungal test for contact activity of *Eucalyptus* spp leaves extracts against *A. parasiticus*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
C	36 Aa	34 Aa	31 Aa	25.Aa	23 Aa	5 Ba	25.6 a
G	36 Aa	36 Aa	29 Aa	25 Aa	10 Bb	6 Ba	23.6 a
G+U	36 Aa	22 Bb	16 Bb	12 Bb	12 Bb	5 Ca	17.1 bc
U	36 Aa	24 Bb	17 Bb	14 Bb	4 Cc	3 Ca	14.9 c
C+G	36 Aa	22 Bb	12 Cb	12 Cb	7 CDc	0 Da	18.5 b
C+U	36 Aa	26 Bb	25 Ba	21 Ba	0 Cc	0 Ca	17.7 b
S+G	36 Aa	39 Aa	35 Aa	0 Bc	0 Bc	0 Ba	17.9 b
S+C	36 Aa	39 Aa	32 Aa	0 Bc	0 Bc	0 Ba	16.5 bc
S	36 Aa	17 Bb	16 Bb	4 Cc	0 Cc	0 Ca	12.2 d
S+U	36 Aa	14 Bb	5 Cc	5 Cc	0 Cc	0 Ca	10.0 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 6 Turkey test for treatments (oils) x dose (µl) in antifungal test for volatile activity of *Eucalyptus* spp leaves extracts against *A. flavus*

	Colony diameter (mm)[2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
C+G	34 Aa	32 Aa	29 Aa	21 Aab	4 Bab	2 Ba	20.3 bc
S+U	34 Aa	34. Aa	36 Aa	34 Aa	2 Bb	0 Ba	23.4 a
G+U	34 Aa	41 Aa	35 Aa	26 Aa	11 Ba	0 Ca	24.5 a
C+U	34 Aa	32 Aa	31 Aa	18 Bb	4 Cab	0 Ca	19.5 cd
S+G	34 Aa	31 Aa	30 Aa	21 Bab	3 Cab	0 Ca	19.8 bcd
S+C	34 Aa	38 Aa	38 Aa	11 Bb	0 Cb	0 Ca	20.0 bcd
C	34 Aa	46 Aa	38 Aa	17 Bb	0 Cb	0 Ca	22.6 ab
G	34 Aa	30 Aa	29 Aa	16 Bb	1 Cb	0 Ca	18.5 cd
U	34 Aa	30 Aa	24 Aa	10 Bb	8 BCa	0 Ca	17.6 cd
S	34 Aa	39 Aa	30 Aa	0 Bc	0 Bb	0 Ba	172 d

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

Table 7 Turkey test for treatments (oils) x dose (µl) in antifungal test for volatile activity of *Eucalyptus* spp leaves extracts against *A. parasiticus.*

	Colony diameter (mm) [2] Dose (µL)						
Oils[1]	**0**	**10**	**20**	**80**	**160**	**420**	**Mean**
G+U	31 Aa	40 Aa	33 Aa	18 Ba	8 Cab	0 Ca	21.6 a
C+G	31 Aa	20 Bab	19 Bab	20 Ba	15 Ba	0 Ca	17.4 cde
S+U	31 Aa	36 Aa	25 Aa	28 Aa	5 Bb	0 Ba	20.9ab
U	31 Aa	28 Aa	15 Bab	7 Cb	7 Cab	0 Ca	14.8 def
C+U	31 Aa	23 ABab	18 Bab	18 Ba	2 Cb	0 Ca	15.5 cdef
G	31 Aa	29 Aa	25 Aa	19 Aa	3 Bb	0 Ba	17.8 cd
S+G	31 Aa	17 Bab	16 Bab	20 Ba	1 Cb	0 Ca	18.6 bc
C	31 Aa	30 Aa	30 Aa	9 Bb	0 Bb	0 Ba	16.6 cde
S+C	31 Aa	41 Aa	40 Aa	0 Bb	0 Bb	0 Ba	14.3 f
S	31 Aa	34 Aa	3 Bb	0 Bb	0 Bb	0 Ba	14.4 f

[1]See Table 3 for description of oils. [2]Means followed by same capital letter in the row or the same letter in column do not differ, by Tukeys multiple range test at 5% significance.

The blends of oil from different *Eucalyptus* species or hybrid showed different antifungal activity in comparison to pure oils. In some blends the antifungal activity was much lower than the one of pure oils for one of the tested fungus and better than the one of pure oils for the other. In some blends the antifungal activity was the same as observed for pure oils. Only *E. citriodora* + *E. grandis* blend in contact assay showed better antifungal activity than the both pure oils.

Even if all *Eucalyptus* oils and blends have showed antifungal activity in laboratory bioassay there is a need for further studies in the future. The oil extracted from *E. staigeiriana* was the one that showed the best potential. The major components of this oil were limonene and geranial that have confirmed the antifungal activity already observed (Adegoke et al., 2000; Lee et al., 2008). However, antifungal activity could be due to other minor compounds, individually or with a synergistic effect of several of them, as already observed by Vilela et al. (2009) and Burt (2004).

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Aflatoxin control and prevention strategies in maize for Sub-Saharan Africa

Hell, K.*#[1], Mutegi, C.[2], Fandohan, P.[3]
[1] CIP (International Potato Center), 08 BP 0932 Tri Postal, Cotonou, Republic of Benin. Email: K.Hell@cgiar.org
[2] Kenya Agricultural Research Institute, P.O Box 57811-00200, Nairobi, Kenya [3] Programme on Agricultural and Food Technology, National Institute of Agricultural Research of Benin, P. O. Box 128, Porto-Novo, Benin

* Corresponding author
# Presenting author

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Abstract

Mycotoxins are secondary fungal metabolites that contaminate agricultural commodities and can cause sickness or death in humans and animals. Risk of mycotoxin contamination of food and feed in Africa is increased due to environmental, agronomic and socio-economic factors. Environmental conditions especially high humidity and temperature favour fungal proliferation. Farming practices in Africa sustain fungal and toxin contamination of food and feed. The socio-economic and food security status of the majority of inhabitants of sub-Saharan Africa leaves them little option in choosing good quality products.

Several technologies have been tested in Africa to reduce mycotoxin risk. Field management practices that increase yields may also prevent aflatoxin. They include use of resistant varieties, timely planting, fertilizer application, weed control, insect control and avoiding drought and nutritional stress. Other options to control the toxin causing fungi *A. flavus* contamination in the field are use of atoxigenic fungi to competitively displace toxigenic fungi, and timely harvest. Post-harvest interventions that reduce mycotoxins are rapid and proper drying, sorting, cleaning, drying, smoking, post harvest insect control, and the use of botanicals or synthetic pesticides as storage protectant. Another approach is to reduce the frequent consumption of 'high risk' foods (especially maize and groundnut) by consuming a more varied diet, and diversifying into less risky staples like sorghum and millet. Chemo-preventive measures that can reduce mycotoxin effect include daily consumption of chlorophyllin or oltipraz and by incorporating hydrated sodium calcium alumino-silicates into the diet. Detoxification of aflatoxins is often achieved physically (sorting, physical segregation, flotation etc.), chemically (with calcium hydroxide, ammonia) and microbiologically by incorporating pro-biotics or lactic acid bacteria into the diet. There is need for efficient monitoring and surveillance with cost-effective sampling and analytical methods. Sustaining public education and awareness can help to reduce aflatoxin contamination.

Keywords: Aflatoxin, Sub-Saharan Africa, Control measures

1. Introduction

Aflatoxins are secondary metabolites primarily produced by the fungi *Aspergillus flavus* Link, *A. parasiticus* Speare and to a lesser extent *A. nomius* Kurtzman et al. (CAST, 2003). Optimal conditions for fungal development are 36 to 38ºC, with a high humidity of above 85% (Diener et al., 1987). Suitable conditions for the growth of the fungi and toxin production occur in most areas of Africa and aflatoxin contamination of food is a widespread problem across the continent, which has been reviewed by several authors (Sibanda et al., 1997; Shephard, 2003; Bankole and Adebanjo, 2003; Bankole et al., 2006; Wagacha and Muthomi, 2008). African communities and populations are exposed to aflatoxins before birth and throughout their lives with serious impact on their health (Williams et al., 2003).

Aflatoxins are the most potent natural carcinogenic substance and they have been linked with a higher prevalence of hepatocellular cancer in Africa (Strosnider et al., 2006). There is a very high risk of Hepatitis B and Hepatitis C carriers to develop liver cancer when they are exposed to aflatoxin (Williams et al., 2003). There have been recent outbreaks of acute aflatoxicosis in Kenya (Probst et al., 2007), but chronic exposure to aflatoxins has much wider health effects than these rare acute poisonings (Williams et al., 2004). Aflatoxins have been linked to immune suppression by Turner et al. (2005) and Jiang et al. (2005). Children in areas of high aflatoxin exposure have been found to have stunted growth (Gong et al., 2004). Aflatoxin contamination has been linked to micronutrient deficiencies in animals (Williams et al., 2004), but Gong et al. (2004) reported that there was no relationship between aflatoxin-albumin, the biomarker of aflatoxin exposure, and micronutrients.

Aflatoxin contamination in several foodstuffs in Africa has been a recurrent problem (Shephard, 2003). In many parts, maize has become the preferred cereal for food, feed and industrial use, displacing traditional cereals such as sorghum and millets. However, it was significantly more heavily colonized by aflatoxin-producing *Aspergillus* spp. than either sorghum or millet (Bandyopadhyay et al., 2007). This review paper outlines some of the potential solutions to controlling toxins in Africa that are being developed by researchers either within or from outside Africa. Some of the potential solutions to controlling mycotoxins in Africa that are being developed by researchers are presented. These strategies can be broadly divided into: stopping the infection process (host plant resistance, biocontrol); control of environmental factors (temperature, rainfall, relative humidity, evapotranspiration, soil type) including efforts to build predictive models; crop management strategies (good agricultural practices (GAP), pre- and post-harvest management); post-harvest strategies (harvesting, drying, storage, use of plant extracts and preservatives) and decontamination (sorting, processing).

2. First strategy: stopping the infection process

2.1. Breeding for resistance

Several screening tools have been developed and used to facilitate corn breeding for developing germplasm resistant to fungal growth and/or aflatoxin contamination (Brown et al., 2003). Sources of resistance to *Aspergillus* infection and aflatoxin contamination in corn have been identified, but commercial hybrids have not been developed. This is largely due to the difficulty in finding elite lines that maintain high yields and maintain resistance within multiple environments (Clements and White, 2004). Brown et al. (2001) tested aflatoxin resistance in thirty-six maize inbred lines selected in West and Central Africa for moderate to high resistance to maize ear rot for their resistance to aflatoxins, more than half the inbred accumulated aflatoxins at levels as low as or lower than the resistant U.S. lines. In 2008, six tropical maize germplasm lines with resistance to aflatoxin were registered by the same research group (Menkir et al., 2008) and their distribution to national programs will start soon for the development of locally adapted hybrids.

Many new strategies that enhance host plant resistance against aflatoxin involving biotechnologies are being explored and are reviewed by Brown et al. (2003). These approaches involve the design and production of maize plants that reduce the incidence of fungal infection, restrict the growth of toxigenic fungi or prevent toxin accumulation. In the long term the identification of compounds that block aflatoxin biosynthesis would significantly enhance mycotoxin control.

2.2. Biological control

Another potential means for toxin control is the biocontrol of fungal growth in the field. Numerous organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, and nontoxigenic (atoxigenic) strains of the causal organisms (Yan et al., 2008) of which only atoxigenic strains have reached the commercial stage. Biological control of aflatoxin production in crops in the US has been approved by Environmental Protection Agency and a commercial product based on atoxigenic *Aspergillus flavus* strains is being marketed (Afla-Guard®). In Africa, two isolates of *A. flavus* have been identified as atoxigenic strains to competitively exclude toxigenic fungi in the maize fields. These strains have been shown to reduce aflatoxin concentrations in both laboratory and field trials, reducing toxin contamination by 70 to 99% (Atehnkeng et al., 2008b). A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin has gained provisional registration (AflaSafe) to determine efficacy in on-farm tests and candidate strains have been selected for Kenya and Senegal.

3. Second strategy: control of environmental factors

To design strategies for the prevention or reduction of aflatoxins, a thorough understanding of the factors that influence the infection of the plant with the aflatoxin causing fungi and the conditions that induce their formation is required. Environmental factors that favor *A. flavus* infection in the field include high soil and/or air temperature, high relative humidity, high rates of evapotranspiration, reduced water availability, drought stress, nitrogen stress, crowding of plants and conditions that aid the dispersal of conidia during silking (CAST, 2003). Some of these factors have been included in a model to predict toxin contamination in peanut systems in Mali. Weather and satellite based variables that could be used to indicate aflatoxin presence in peanut were identified (Boken et al., 2008).

Significant correlations exist between Agroecozones (AEZ) and aflatoxin levels, with wet and humid climates and drier regions after longer storage periods increasing aflatoxin risk (Hell et al., 2000). Agroecozones are geographic areas that share similar biophysical characteristics for crop production, such as soil, landscape, and climate. Kaaya et al. (2006) observed that aflatoxin levels in Ugandan maize samples were higher in more humid areas compared to the drier areas and similar results were obtained in maize samples from Nigeria (Atehnkeng et al., 2008a); these trends could used to elaborate predictive models. Modelling of interactions between host plant and environment during the season can enable quantification of preharvest aflatoxin risk and its potential management (Boken et al., 2008; Chauhan et al. 2008). Predictive growth models for fungal and mycotoxin developments are available and have been reviewed by Garcia et al. (2009).

Factors that influence the incidence of fungal infection and subsequent toxin development include invertebrate vectors, grain damage, oxygen and carbon dioxide levels, inoculum load, substrate composition, fungal infection levels, prevalence of toxigenic strains and microbiological interactions. Insects vector fungi and cause damage that allows the fungi to gain access, increasing the chances of aflatoxin contamination, especially when loose-husked maize hybrids are used (Dowd, 2003). High incidence of the insect borer *Mussidia nigrivenella* Ragonot, was positively correlated with aflatoxin contamination of maize in Benin (Setamou et al., 1998). Storage pests, in particular *Cathartus quadricollis* Guerin and *Sitophilus zeamais* Motschulsky, play an important role in the contamination of foods with fungi, especially those that produce toxins (Hell et al., 2003; Lamboni and Hell, 2009).

4. Third strategy: crop management strategies

Controlling or reducing infection and regulating the factors that increase the risk of contamination in the field for maize will go a long way in controlling aflatoxins. Management practices that reduce the incidence of mycotoxin contamination in the field include timely planting, optimal plant densities, proper plant nutrition, avoiding drought stress, controlling other plant pathogens, weeds and insect pests and proper harvesting (Bruns, 2003). In Africa, crops are cultivated under rain fed condition, with low levels of fertilizer and practically no pesticide application. These management practices promote *A. flavus* infection in fertility stressed plant. Any action taken to interrupt the probability of silk and kernel infection will reduce aflatoxin contamination (Diener et al., 1987).

Pre-harvest measures that are efficient in reducing aflatoxin levels are the same as those that will enhance yields. Crop rotation and management of crop residues also are important in controlling *A. flavus* infection in the field.

Tillage practices, crop rotation, fertilizer application, weed control, late season rainfall, irrigation, wind and pest vectors all can affect the source and level of fungal inoculum, maintaining the disease cycle in maize (Diener et al., 1987).

4.1. Timely harvesting

Extended field drying of maize could result in serious grain losses during storage (Borgemeister et al., 1998; Kaaya et al., 2006), and as such harvesting immediately after physiological maturity is recommended to combat aflatoxin problems. Kaaya et al. (2006) observed that aflatoxin levels increased by about 4 times by the third week and more than 7 times when maize harvest was delayed for 4 wk. However, after early harvesting products have to be dried to safe levels to stop fungal growth. Leaving the harvested crop in the field prior to storage promotes fungal infection and insect infestation, this is common practice in Africa often due to labour constraints, and the need to let the crop dry completely prior to harvest (Udoh et al., 2000).

4.2. Rapid drying

Moisture and temperature influence the growth of toxigenic fungi in stored commodities. Aflatoxin contamination can increase 10 fold in a 3-d period, when field harvested maize is stored with high moisture content (Hell et al., 2008). The general recommendation is that harvested commodities should be dried as quickly as possible to safe moisture levels of 10 – 13 % for cereals. Achieving this through simple sun-drying under the high humidity conditions of many parts of Africa is difficult. Even, when drying is done in the dry season, it is not completed before loading grains into stores like observed by Mestre et al. (2004) and products can be easily contaminated with aflatoxins. There are several

technologies to increase the efficacy of grain drying and reduce the risk of toxin contamination even under low-input conditions; these are the use of drying platforms, drying outside the field, drying on mats (Hell et al., 2008). Farmers should be able to determine the actual moisture content of their products. A simple moisture meter has been developed by IRRI using standard electronic components and can be produced locally:

(http://www.irri.org/irrc/streams/farmer friendly moisture meter.asp).

There are technological solutions that could aid in reducing grain moisture rapidly, which are reviewed by Lutfy et al. (2008). However, these dryers are not used by African farmers because large capital investments are needed to acquire them. However, Gummert et al. (2009) described the very positive effect dryers had on maintaining rice quality and reducing mycotoxin risk in Southeast Asia.

5. Fourth strategy: post harvest crop management practices

Aflatoxin contamination of foods increases with storage period (Kaaya and Kyamuhangire, 2006). It is compounded in Africa through excessive heat, high humidity, lack of aeration in the stores, and insect and rodent damage resulting in the proliferation and spread of fungal spores. Thus strategies to minimize quantitative and qualitative post harvest losses have been developed (Hell et al., 2008). These improved postharvest technologies have been used successfully to reduce the blood aflatoxin-adducts level in populations in Guinea, where exposure was more than halved 5 mo after harvest in individuals from the intervention villages (Turner et al., 2005).

Traditional storage methods in Africa can be divided into two types, namely temporary storage that is mainly used to dry the crop and permanent storage that takes place in the field or on the farm. The latter includes containers made from plant materials (woods, bamboo, thatch) or mud placed on raised platforms and covered with thatch or metal roofing sheet. The stores are constructed to prevent insect and rodent infestation and to prevent moisture from getting into the grains. It is difficult to promote new storage technologies, such as the use of metal or cement bins, to small-scale farmers due to their high cost. Many farmers nowadays store their grains in bags, especially polypropylene which are not airtight, but there is evidence that this method facilitates fungal contamination and aflatoxin development (Hell et al., 2000; Udoh et al., 2000). Presently there are efforts to market improved hermetic storage bags in Africa, based on triple bagging developed for cowpea (Murdock et al., 1997) which has been or is being tested for other commodities (Ben et al., 2009).

5.1. Disinfestation methods

Smoking is an efficient method of reducing moisture content and protecting maize against infestation by fungi. The efficacy of smoking in protecting against insect infestation was found to be high. About 4 to 12% of farmers in the various ecological zones in Nigeria used smoke to preserve their grains, and this practice was found to be correlated with lower aflatoxin levels in farmers' stores (Udoh et al., 2000). Other compounds used for seed fumigation like ethylene oxide and methyl bromide were found to significantly reduce the incidence of fungi including toxigenic species on stored groundnuts and melon seeds (Bankole et al., 1996). Among the chemical compounds tested in feeds, propionic acid, sodium propionate, benzoic acid and ammonia were the best anti-fungal compounds, followed by urea and citric acid (Gowda et al., 2004). Farmers use local plant products for controlling insect infestation, past studies have looked at the use of these substances for the control of fungi mostly proving their efficacy in-vitro (Hsieh et al., 2001), but these products have not proven there efficiency in farmers stores. There is need to review the efficacy of the multiple products used by farmers and tested by researcher to get a complete picture about their potential in reducing toxin contamination. Use of pesticides to control mycotoxins and their efficacy, have been reviewed by D'Mello et al. (1998), but their use by farmers in Africa is not always well practiced and deaths due to pesticide use have been reported. Extension workers should educate farmers on the importance of using recommended chemicals for specific crops at appropriate concentrations and within a safe delay before consumption.

5.2. Physical separation and hygiene

Aflatoxin is unevenly distributed in a seed lot and may be concentrated in a very small percentage of the product (Whitaker, 2003). Sorting out of physically damaged and infected grains (known from colorations, odd shapes and size) from the intact commodity can result in 40-80% reduction in aflatoxins

levels (Park, 2002; Fandohan et al., 2005; Afolabi et al., 2006). The advantage of this method is that it reduces toxin concentrations to safe levels without the production of toxin degradation products or any reduction in the nutritional value of the food. This could be done manually or by using electronic sorters. Clearing the remains of previous harvests and destroying infested crop residues are basic sanitary measures that are also effective against storage deterioration. Cleaning of stores before loading in the new harvests was correlated to reduced aflatoxin levels (Hell et al., 2008). Separating heavily damaged ears i.e. those having greater than 10% ear damage also reduces aflatoxin levels in maize (Setamou et al., 1998). Wild hosts, which constitute a major source of infestation for storage pests, should be removed from the vicinity of stores (Hell et al., 2008).

5.3. Reduction through food processing procedures

Sorting can remove a major part of aflatoxin contaminated units, but levels of mycotoxins in contaminated commodities may also be reduced through food processing procedures that may involve processes such as sorting, washing, wet and dry milling, grain cleaning, dehulling, roasting, baking, frying, nixtamalization and extrusion cooking. These methods and their impact on mycotoxin reduction have been reviewed by Fandohan et al. (2008). The effect of extrusion cooking on mycotoxins in cereals was reviewed by Castells et al. (2005). Dehulling maize grain can reduce aflatoxin contamination by 92% (Siwela et al., 2005). The effect of nixtamalization in reducing aflatoxin contamination (Park, 2002) has lately been questioned with Méndez-Albores et al. (2004) reporting that nixtamilization is reversible.

Fermentation can increase the safety of some food products contaminated with mycotoxins. However, the available reports are contradictory, with some showing very efficient reductions in mycotoxins associated with fermentation, whereas others find lesser or no effects. Fandohan et al. (2005) found that processing maize into makume (a solid state fermented maize based product) resulted in 93% reduction of aflatoxin, while reduction levels were 40% for 'owo' which is a non-fermented dry milled maize porridge. The authors identified sorting, winnowing, washing, crushing combined with dehulling of maize grains as the critical mycotoxin reducing steps in the production chain, while fermentation and cooking appeared to have insignificant effect. There are diverse processing methods for the highly predisposed commodities (maize and groundnuts) in different parts of Africa and investigations of the effect of these processing techniques will identify those methods that expose consumers to less aflatoxins.

5.4. Other strategies

The other strategies to reduce the risk of aflatoxin ingestion in Africa are dietary change, chemoprevention, detoxification and vaccination against hepatitis B would significantly reduce liver cancer risk (Strosnider et al., 2006).

6. Conclusions - perspectives of aflatoxin research in Africa

It is clear that aflatoxin contamination in agricultural crops is widespread in Africa, but food insecurity together with drought are a major obstacle to improvements in food safety. Increased pressure on limited food resources and undernutrition exacerbates the mycotoxin problem by increasing the likelihood of human consumption of contaminated foods and by rendering the population more susceptible to the consequent adverse health effects. Even though considerable research efforts have been made to control toxin contamination, there are several factors that lead to high aflatoxin risk in Africa:

1) Lack of political commitment to mycotoxin research,
2) Shortage of trained personnel especially for mycotoxin monitoring,
3) Limited awareness on risks at all levels and insufficient knowledge on options to reduce aflatoxin contamination in production to consumption chain.

The perspectives of aflatoxin research in Africa can be foreseen as follows:

i. Getting policy makers in the sub-region to recognise that the stimulation of the postharvest sector is an important avenue to increase food production and ensure food safety for the protection of the health of their citizens.
ii. Educating stakeholders on the danger of commercializing and consuming mouldy foods.
iii. Training personnel at all levels (scientists, technicians, extension agents) in sampling protocol and modern methods of mycotoxin analysis.
iv. Conducting food baskets surveys for aflatoxin contamination using uniform sampling protocols and modern analytical methods to obtain sound and reliable data on aflatoxin incidence in different food crops, which could then be used to define control strategies.
v. There should be a co-ordinated and collaborative effort on aflatoxin research in Africa to minimize repetitions so that resources can be focused on identified priority areas, including documenting the impact of aflatoxin on health and economies in Africa.
vi. Investigation should focus on the effect of different pre- and post-harvest crop management systems on aflatoxin contamination in different agro-ecologies in Africa and the effect of different traditional food processing methods on aflatoxin production so that technologies that result in a significant reduction in aflatoxin levels could be promoted.

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The use of essential oils to protect rice from storage fungi

Magro, A.*#[1], Matos, O.[2], Bastos, M.[3], Carolino, M.[4], Lima, A.[5], Mexia, A.[5]
[1] Instituto de Investigação Científica Tropical - IP. Trav. Conde da Ribeira, 9. 1300-142 Lisboa. Portugal. Email: ana.magro@netcabo.pt
[2] Instituto Nacional Recursos Biológicos – IP, Estação Agronómica Nacional. Av. da República, Quinta do Marquês, Nova Oeiras, 2784-505 Oeiras. Portugal
[3] Universidade do Porto, Faculdade de Engenharia (LEPAE). Rua Dr. Roberto Frias. 4200-465 Porto. Portugal.
[4] Universidade de Lisboa, Faculdade de Ciências, Centro de Biologia Ambiental. Edifício C2, Campo Grande. 1149-016 Lisboa. Portugal
[5] Universidade Técnica de Lisboa, Instituto Superior de Agronomia. Tapada da Ajuda.1349-017 Lisboa. Portugal

* Corresponding author
# Presenting author

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Abstract

Rice (*Oryza sativa*) is the main food of half of the population of our planet. The growth of fungi closely associated to the eventual occurrence of mycotoxins can be responsible for serious economic losses and public health risks. Knowledge about the origin of the growth of toxigenic fungi is a prerequisite to the establishment of mycotoxin control programs. Socio-economical and environmental factors led to an extreme reduction of rice availability, while the estimated rice production losses increased in all continents what increases the importance to develop new harmless strategies for the control of fungi affecting stored rice. Natural products from plant origin were screened for the control of main pernicious fungi.

In this work we have collected rice samples from different origins (national and imported) and these samples were analysed for fungal infection. Several fungi taxa were isolated: *Absidia, Alternaria, Aspergillus, Bipolaris, Botrytis, Chaetomium, Curvularia, Cunninghamela, Epicoccum, Fusarium, Geotrichum, Helicoma, Nigrospora, Penicillium, Pyricularia, Rhizopus, Scytalidium, Stemphylium, Sordaria, Trichoconiella, Trichoderma, Trichothecium* and *Ulocladium*. Some of the fungi isolated are potentially mycotoxigenic. We also studied a way to control the growth of some of these fungi using plant extracts and essential oils from *Syzyginum aromaticum* and *Laurus nobilis*. Promising results were obtained.

Keywords: Rice, Cereals, Fungi, Bio-pesticides, Plant extracts.

1. Introduction

Rice (*Oryza sativa* L.) is a staple food for over half of the world population and is grown on approximately 146 million ha, i.e. more than 10% of the total available land for agriculture. In the tropics, rice is the primary source of human nutrition, and is one of the cheapest sources of food energy and protein (Cantrell, 2001; Mexia, 2003).

Portugal is the biggest consumer of milled rice in Europe (15 kg per person and per year), with an annual paddy rice production of about 129 000 tonnes of *japonica* variety (short-grain), 26 000 tonnes of *indica* variety (long-grain) distributed mainly by Sado, Tejo and Mondego Valley companies. Besides national production, 98 000 tonnes of rice are imported to satisfy reach consumers needs (Brites et al., 2006; INE, 2007).

Paddy rice is a seasonal crop in Portugal, so storage of paddy and milled rice is of major importance for year-round availability. In storage, the development of fungi, especially *Aspergillus* spp. and *Penicillium* spp., is an unsolved problem. These fungi are responsible for rice quantitative and qualitative losses and are mycotoxins potential producers. Mycotoxins are hazardous to animal and human health and constitute a factor for economic losses in food products worldwide (Omidbeygi et al., 2007; Pitt and Hocking, 2009).

Safety, residual toxicity and resistance to known chemical preservatives led to an increased search on novel strategies of food preservatives. Naturally occurring antimicrobial compounds for food

preservation receive increasing attention due to consumer awareness about natural food products and a growing concern of microbial resistance towards conventional preservatives (Skandamis et al., 2001; Schuenzel and Harrison, 2002). The objectives of this work were: (1) Isolation, identification and evaluation of fungi abundance in rice samples; (2) *in vitro* fungicidal activities of the essential oils of clove (*Syzygium aromaticum* (L.) Merr. & Perry.) and laurel (*Laurus nobilis* L.) on the fungi isolated from the rice samples.

2. Materials and methods

2.1 Mycoflora analysis

Rice samples, namely paddy rice, brown rice and milled rice were collected regularly during the experimental period in a rice mill at different places of the milling process. Five samples were collected in sterilized containers and taken into the laboratory. In the laboratory, the rice samples were sub-divided in samples with 110 grains. These sub-samples were disinfected at surface with 1% sodium hypochlorite, during two minutes, as describe by Pitt and Hocking (1997) and Magro et al. (2008). Ten disinfected grains were placed on Petri dishes with 20 mL of Potato Dextrose Agar (PDA) medium with chloramphenicol (1%) (Fig. 1). For each sample, ten replicates were made.

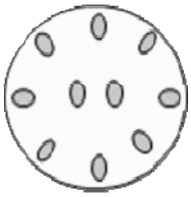

Figure 1 Schematic representation of the grain distribution on a Petri dish with PDA medium supplemented with chloramphenicol.

The grains in Petri dishes were incubated at 28ºC for 8 d and then examined under a stereomicroscope for fungal growth. Isolation of the colonies was made to obtain pure cultures. Slides of fungal growth were prepared and observed under a high magnification microscope for fungal morphology study. The identification was carried out using identification keys (Carmichael et al., 1980; Domsch et al., 1980; Onions et al., 1981; International Mycological Institute, 1991; Hanlin, 1997; Malloch, 1997; Pitt and Hocking, 1997; Barnett and Hunter, 1998; Samson et al., 2004).

2.2 Extraction of essential oils

The essential oil of laurel used in this study was obtained by hydro-distillation of air-dried leaves in a modified Clevenger-type apparatus for 3 h. The extracted oil was dried over anhydrous sodium sulfate and stored in a sterilized amber bottle at 4ºC until used. The essential oil of clove was supplied by the Portuguese company Segredo da Planta, reference No. 127005582.

2.3 Fungi selection for growth inhibition test

The fungi used for the bioactivity tests were *Aspergillus candidus*, *A. niger*, *Fusarium culmorum* and *Penicillium islandicum*. These fungi were obtained from samples of rice grains collected in a Portuguese rice processing factory.

2.4. Determination of effect of essential oils in solid media (Potato Dextrose Agar -PDA)

For the determination of effect of essential oils on the growth of the fungi tested, different amounts of essential oils were deposited on the surface of PDA, namely, 10, 25, 50, 100, 250, 500 and 750 µL. The Petri dishes were inoculated with a 5 mm diameter disk of fungi grown on potato dextrose agar (PDA) medium for 8 days at 28ºC. This disk was placed on the agar surface and incubated at 28ºC. Inhibition of fungal growth according to the effects of essential oils were determined by a periodic measurement of the fungal colony diameter change with time carried out during 25 weeks. In the control, equal amounts of sterilized water were placed on the surface of PDA. The mean radial mycelia growth of the fungi was determined by measuring the diameter of the colony in two directions when the plate surface of the control Petri was covered by fungus, 7 days after inoculation. For each concentration, four replicate dishes were used.

3. Results and discussion

3.1. Mycoflora analysis

Field and storage fungi were detected and identified in all samples (Table 1). The field genera isolated from rice grain samples were: *Absidia, Alternaria, Bipolaris, Botrytis, Chaetomium, Cunninghamella, Curvularia, Epicoccum, Geotrichum, Helicoma, Nigrospora, Pyricularia, Rhizopus, Scytalidium, Sordaria, Stemphylium, Trichoconiella, Trichoderma, Trichothecium* and *Ulocladium.*

Table 1 Fungi *taxa* identified on samples of paddy, brown and long grain rice from different origins.

	Step of Processing		
	Paddy	**Brown**	**Long grain**
National rice	*Absidia corymbifera*	*Alternaria* sp.	*Aspergillus* spp.
	Alternaria sp.	*Aspergillus* spp.	*A. fumigatus*
	Aspergillus spp.	*A. flavus*	*A. penicillioides*
	A. candidus	*A. fumigatus*	*Penicillium* sp.
	A. flavus	*A. niger*	*Trichoconiella padwickii*
	A. fumigatus	*A. terreus*	
	A. niger	*Bipolaris* sp.	
	A. terreus	*Chaetomium* sp	
	Bipolaris sp.	*Epicoccum* sp.	
	Botrytis sp.	*Fusarium* spp.	
	Chaetomium sp.	*Geotrichum* sp.	
	Curvularia sp.	*Nigrospora oryzae*	
	Cunninghamella sp.	*Penicillium* spp.	
	Fusarium culmorum	*Pyricularia* sp.	
	Geotrichum sp.	*Scytalidium* sp.	
	Nigrospora oryzae	*Stemphylium botryosum*	
	Penicillium spp.	*Trichoconiella padwickii*	
	P. islandicum	*Trichoderma harzianum*	
	Rhizopus oryzae	*Trichothecium roseum*	
	Scytalidium sp.	*Ulocladium atrum*	
	Sordaria fimicola		
	Trichoconiella padwickii		
	Trichoderma harzianum		
	Trichothecium roseum		
Imported rice		*Aspergillus* spp.	*Alternaria* sp.
		A. flavus	*Aspergillus* spp.
		A. fumigatus	*A. flavus*
		A. niger	*A. fumigatus*
		Bipolaris sp.	*Stemphylium botryosum*
		Curvularia sp.	*Trichoconiella padwickii*
		Fusarium spp.	
		Helicoma sp.	
		Nigrospora oryzae	
		Penicillium sp.	
		Trichoconiella padwickii	
		Rhizopus oryzae	

The storage species (or field fungi species remaining alive during storage) isolated were: *Aspergillus* spp., *A. candidus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Fusarium* spp., *F. culmorum*, *Penicillium islandicum* and *Penicillium* spp. The samples of paddy rice presented a higher number of *taxa* (Table 1), that may be the result of not having been processed and of storage conditions. On the other hand, the long grain rice, presented a lower number of *taxa* in relation to the other samples studied, probably due to the processing of the rice from paddy to long grain rice. This reduction of the number of taxa observed on polished rice was probably related to the effect of the abrasive process (Lima et al., 2000). In the brown rice samples 20 and 12 *taxa* (Table 1) were detected and identified in domestic production and imported rice, respectively. These results are in accordance with the results obtained by Mourato (1984)

and Manabe and Tsuruta (1991). According to these authors, the field fungi are gradually replaced by storage fungi as the storage period increases. However, with imported rice the storage period is not always well defined.

Field fungi colonize rice grains only when the water activity (a_w), temperature and relative humidity are high. However, as a result of an adaptation to low a_w, the fungi belonging to *Aspergillus* spp., and *Penicillium* spp., also designated as storage fungi, are able to invade the rice grains stored at levels of a_w considered as safe. They are frequently responsible for causing serious losses, even before they were visually detected. They affect negatively the product's appearance, flavor, odour and nutritional content. They also may produce mycotoxins with high impact in public health (Magro, 2001). It is important to emphasize that *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp., resist to the processing of rice. Since these fungi are potential mycotoxins producers, it is fundamental to improve and control the rice storage conditions as well as the cleaning process before processing. We can concluded that the processing of the rice grains is responsible for the reduction of the number of fungi *taxa*, and that storage duration should be reduced to less than one year, when possible.

3.2. Fungicide activity of essential oils in PDA

The ability of the essential oils of clove and laurel to inhibit the growth of rice fungi was evaluated (Tables 2, 3). Since the storage durability is an important factor in the storage of food products, in this work it was stipulated that the assays would have the durability of 40 weeks for the clove essential oil and 25 weeks for the laurel essential oil.

Table 2 Determination of the effect of clove essential oil on the growth of storage fungi in solid media (*in vitro* bioassay).

Fungi	Volume µL)	Incubation time (weeks)								
		1	5	10	15	20	25	30	35	40
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Fusarium culmorum	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Penicillium islandicum	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Aspergillus candidus	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○
	250	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○
Aspergillus niger	50	○	○	○	○	○	○	○	○	○
	25	○	○	○	○	○	○	○	○	○
	10	○	○	○	○	○	○	○	●	●
	Control	●	●	●	●	●	●	●	●	●

Note: (○) – without growth (●) – with growth.

Table 3 Determination of the effect of laurel essential oil on the growth of storage fungi in solid media.

Fungi	Volume µL)	Incubation time (weeks) 1	2	3	4	5	6	7	8	9	10	11	12	13
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Fusarium culmorum	250	○	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Penicillium islandicum	250	○	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	●	●	●
Aspergillus candidus	250	○	○	○	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●
	750	○	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○	○
Aspergillus niger	250	○	○	○	○	○	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●	●

Continue

Fungi	Volume µL)	Incubation time (weeks) 14	15	16	17	18	19	20	21	22	23	24	25
	750	○	○	○	○	○	○	○	○	○	○	○	○
	500	○	○	○	○	○	○	○	○	○	○	○	○
Fusarium culmorum	250	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○
	Control	○	○	○	○	○	○	○	○	○	○	○	○
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	○	○
Penicillium islandicum	250	○	○	○	○	○	○	○	○	○	○	○	○
	100	○	○	○	○	○	○	○	○	○	○	○	○
	Control	●	●	●	●	●	●	●	●	●	●	●	●
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	●	●
Aspergillus candidus	250	●	●	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●
	750	●	●	●	●	●	●	●	●	●	●	●	●
	500	○	○	○	○	○	○	○	○	○	○	○	○
Aspergillus niger	250	●	●	●	●	●	●	●	●	●	●	●	●
	100	●	●	●	●	●	●	●	●	●	●	●	●
	Control	●	●	●	●	●	●	●	●	●	●	●	●

Note: (○) – without growth (●) – with growth

The clove essential oil showed a strong antifungal potential along the 40 weeks, being active at the lowest dose of the extract against *F. culmorum*, *P. islandicum* and *A. niger* and at a dose of 25 µL against to *A. candidus*. For the essential oil of laurel, the total control of *F. culmorum* was reached with the dose of 100 µL, of *P. islandicum* with the dose of 250 µL, while *A. niger* and *A. candidus* were controlled only when the dose of 750 µL was applied and only for 13 and 10 weeks, respectively. The results obtained in this study are similar to these obtained by Nielsen and Rios (2000), Guynot et al. (2003), Lopez et al. (2005), and Matan et al. (2006). But our results are relevant, because we used different species of fungi and we get no fungi growth for several months, which is extremely important when we deal with stored grains. The essential oils of clove and laurel showed inhibitory effects on the four tested fungi, *Aspergillus candidus*, *A. niger*, *Fusarium culmorum* and *Penicillium islandicum*, at all concentrations but the clove essential oil was more efficient in the control of fungi that laurel essential oil. These findings

clearly indicate that the essential oils used should be more studied in order to better characterize their potential for future use as a substitute for chemical fungicides.

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Ozone treatment effects on microbial count on maize

McClurkin, J.D.#[1], Maier, D.E.*[2],
[1] Department of Agricultural and Biological Engineering, Purdue University, 225 S University Street, West Lafayette, IN 47907, USA.
[2] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA.

* Corresponding author
# Presenting author

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Abstract

The ultimate goal of this research was to develop a semi-continuous flow grain treatment system and predictive model that will reduce microorganisms on grain kernel surfaces with ozone. The focus of this research was to determine the concentration-time product (CTP) of ozone required to eliminate various levels of microbial growth on grain kernels. To examine the effect of ozone on surface microbes, samples of freshly-harvested and stored maize were treated with ozone for 1 and 3 hours at average ozone concentrations of 1752 ppm, 915 ppm and 37 ppm. Microorganisms were significantly decreased by 28 to 57% after maize samples were ozonated for 1 h at 37 to 1752 ppm and 45 to 80% for 3 h at 37 to 1752 ppm. Linear regression analysis of the CTP data indicated that percent mold reduction increased at a rate of 0.0088 times the CTP. The modified Gompertz equation applied to the microbial inactivation data indicated that a 0.5 to ~1 log mold reduction on maize kernels was attained for ozone concentrations between 37 and 1752 ppm. When compared to preliminary field data from a semi-continuous flow grain treatment system, the laboratory data and the model-predicted values were reasonably close with respect to the microbial load reduction observed on maize samples taken from the system.

Keywords: Ozone, Microorganisms, Treatment, Sterilization, Ozone concentration.

1. Introduction

There is great potential for the use of ozone to replace insecticides and fungicides used to treat stored grain and grain-based food and feed products. Stored grain products, such as maize, can harbor multiple fungi such as *Aspergillus* species that produce mycotoxins harmful to both human and animal health (Sharma, 2005). Ozone is an environmentally friendly gas that is produced by the excitation of electrons in air. Ozone has been shown to effectively sterilize grain and is thus a viable option for deterring grain loss. However, in order to effectively remove mold from grain, it is necessary to determine the best treatment approach. These approaches involve adjusting the ozone concentration and treatment times to yield an optimum concentration-time product (CTP). CTP is a measurement that relates gas concentration with treatment time. Once various CTPs are evaluated the optimum concentration and treatment time to reduce the microbial load on grain can be determined. Analyzing the microbial load on grain before and after ozone treatment will yield the percent in mold reduction for various CTPs. As a result, ozone treatment effects on grain can be quantified and modeled.

In order to control the efficacy of ozone on mold growth on maize in a scaled up treatment system, a model needs to be developed. The model equation needs to describe the inactivation of mold as a function of reduction in colony forming units (CFU) on maize at varying ozone concentrations. A modified Gompertz model equation and a linear model equation will be considered to express fungal inactivation of maize due to ozone treatment. The objective of this research was to quantify the treatment effects on grain exposed to high ozone concentrations with respect to microbial load reduction and to develop a model to quantify microbial load reduction as a function of ozone concentration and time.

In a study by Fan et al. (2007) ozone treatment with a concentration of 100 nanoliters per liter (nL L^{-1}) for 2 hours reduced the microbial population by 2-3 log CFU per milliliter (mL). The average time to obtain a 2 log CFU mL^{-1} reduction was 1.3 hours at 20°C and 2.5 hours at 5°C. This was determined by fitting the Gompertz model to an ANOVA statistical program. The model was used to determine the time required for a 2 log CFU mL^{-1} reduction. White et al. (2010) treated high moisture maize with ozone to determine its ability to control deterioration of high moisture maize. White et al. (2010) was able to decrease dry matter loss with ozone treatments of 2.4 and 4.8 mg min^{-1} for 24 hr.

According to Strait et al. (1998) grain not previously treated with ozone has inherent sites on its surface that react with ozone during an initial treatment (i.e., Phase 1). After the sites have reacted with the ozone (Phase 2), the rate of ozone degradation decreases (Kells et al., 2001). The reactions in Phase 1 will continue to occur until all possible ozone-surface reactions have completed. Therefore, when treating with ozone, it may be necessary to sterilize the inside of a container with ozone for a period of time before running experiments.

2. Materials and methods

2.1. System set-up

An ozone treatment and intermittent grain sampling system was designed and fabricated for this project. Five identical chambers were connected to make the complete system and allow grain to be placed in the individual chambers. Each chamber was equipped with a drawer that allowed removal of the bottom layer of grain. The bottom of each drawer consisted of a perforated screen to hold grain and allow ozonated air to pass through. The design held approximately 1 kg of grain kernels per chamber, which was about 130 g of maize per drawer pull.

2.2. Ozone generator and ozone analyzer

The ozone generator was manufactured by O_3Co Inc. (Idaho Falls, Idaho) and utilized the corona discharge to produce ozone. The unit contains four electrodes where the oxygen-to-ozone transformation takes place. The generator produces about 2.75 gh^{-1} of ozone at 115 V with dry air as the feed gas. The input voltage was varied from about 50 V to produce 0.5 g/h to 140 V to produce 3.5 $g.h^{-1}$.

The ozone analyzer used to monitor the ozone concentration from the generator was an IN2000LC unit from IN USA, Incorporated (Norwood, Massachusetts). According to the analyzer's manual, the unit has a measuring range from 0 to 2000 ppm, and is calibrated according to US NIST traceable standards (+/- 1%). The ozonated air mixture is pumped into the analyzer at 1.0 $l.min^{-1}$. When multiple chambers were filled with grain and ozonated concurrently, an automatically operated valving system was used to switch ozone readings from chamber to chamber.

2.3. Microbial load analysis

The percentage of mold growth is a variable that needed to be determined in each sample by microbial load count which was attained through dilution plating followed by counting colony forming units (CFU). To measure the effect of ozone on fungal populations samples were serially diluted onto malt salt agar medium. After 3 days of incubation, colonies were counted giving CFU values. Maize kernels were also plated and number of CFUs were counted prior to and after ozone treatment. There was an ozone concentration and treatment time (CTP) combination associated with each percent difference value. The CTP versus the percent difference was graphed to compare the percent reduction in surface mold to the CTP.

2.4. Models for mold growth analysis

The modified Gompertz equation was used to fit CFU counts at various ozone concentrations. By comparing the CTP and percent mold reduction values, an inactivation curve was developed. The graph of the curve is a log (N) versus time graph. The N value is the number of microorganisms at time t and N_0 is the number of microorganisms at time t = 0. The log of N is dependent upon the lower N value, the inactivation rate of the microbial load and the phase of disappearance. This model was useful in determining the reduction in microbial load over time.

A linear correlation of CTP and percent mold reduction was also explored. This correlation was used to directly compare treatment CTP to the percent mold reduction. This will be useful for treatment system scale up in order to determine the expected percent mold reduction for the CTP used to perform sterilization. After the optimal CTP is determined, it can be used in the modified Gompertz curve to model the inactivation of the surface microbes over time.

Fan et al. (2007) used a modified Gompertz model developed based on the statistical analysis performed in their research. The experimental factors of concentration, exposure time, half-life time as a function of airflow, temperature and relative humidity made up the factorial combinations. These combinations were

used for the experimental series. In this current research, the modified Gompertz model was used to compare the inactivation response data to the ozone treatment parameters over time.

3. Results

3.1. Microbial load reduction

During the treatment, 30 g of maize was removed after one hour and the remaining 100 g were treated for two additional hours. The controls for these tests were the results from the samples which did not receive treatment. Three replications were performed. Samples from replications two and three were taken from a different bag of grain. The results show that there was a reduction in CFUs after ozone treatment for one hour, and a further reduction after three hours. These results showed reduction in CFU by up to 100% after 3 hours of treatment. Previous literature (Fan et al., 2007) also showed reduction in microbial load after high ozone treatment.

In Figure 1 the average percentages of mold reduction during the 1752 ppm, 915 ppm and 37 ppm treatments for 1 and 3 hours are graphically illustrated. The standard error bars represent a 95% confidence interval. The 3-hr results indicate increased mold reduction as ozone concentration increased from 37 to 1752 ppm as would be expected. Unfortunately, the 1-hr results for the 1752 ppm concentration did not display the same trend. These results were not consistent, considering the fact that this was the highest ozone concentration tested.

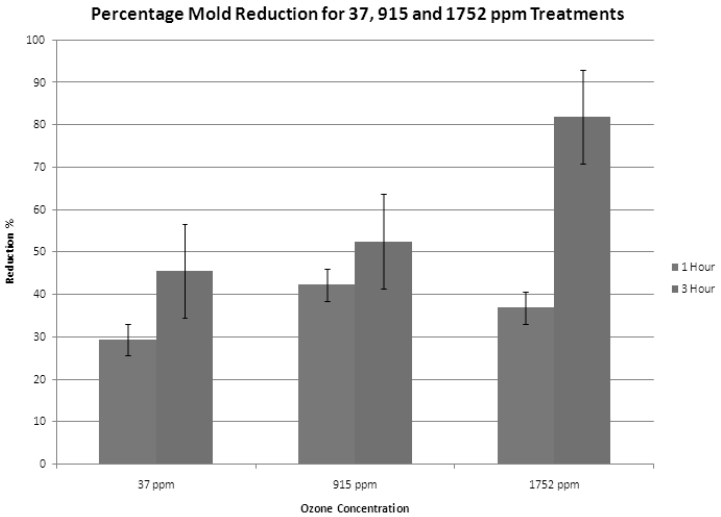

Figure 1 Average of three replications of percent mold reduction for ozone treatments of maize at 1752 ppm, 915 ppm and 37 ppm and treatment periods of 1 and 3 hours.

Because of the variation in mold reduction for the 1-hr results, a trend in percent reduction could not be identified. The 37 ppm results show that the percent reduction went from 31% for 1 hour to 50% reduction after 3 hours. At an ozone concentration of 915 ppm the percent reduction went from 47% for 1 hour to 57% for 3 hours. The percent reduction at 1752 ppm after 1 hour was 38% and went to 80% for 3 hours. An additional replication was run and the four percentage reduction values for 1752 ppm after 1 hour were 24.3%, 45.3%, 43% and 8.8%, which caused the average percent reduction to remain lower than for the 915 ppm treatment effect. Based on the results from replications 2 and 3 for the 1-hr treatment at 1752 ppm, the average percent mold reduction was 44.2%, which would be similar to the effect achieved at 915 ppm. That would be a more reasonable result than the observed reduction. Therefore, the 3-h treatment effect appears to be more predictable and reliable with respect to the percent mold reduction than a 1-hr treatment as the ozone concentration increased. During one replication (i.e., 1752 ppm and 3 h) a complete (100%) sterilization of surface molds was observed.

3.2. Linear model

Figure 2 depicts the x-y relationship for mold reduction and CTP for the ozone treatments. The linear correlation for the CTPs initially showed a low R^2 value of 0.38 indicating that the fit was not very strong. There were several outliers in the data which caused the linear fit line not to be as accurate. These outliers were identified and removed from the data set analyzed statistically. The data analysis was then performed again and a higher R^2 value (0.65) was obtained, indicating a better fit. The final equation obtained through Microsoft Excel (2007) by fitting a linear regression to the plot of scattered points (Eqn. 1) was:

$$y = 0.0088 * x + 44.097 \quad (1)$$

where x represents the CTP and y gives the percent mold reduction. From Equation 1 a CTP of 392 ppm-hr predicts a 47.5% reduction and for 340 ppm-hr a 47% reduction. This comparison of data from grain bins shows that the values are reasonably close, and thus the linear equation appears useful in predicting the percent mold reduction. Additional tests will need to be performed to determine how effective Equation 1 is in predicting field data.

For field applications it would be expected that the lower range of Figure 2 would be most commonly used, as ozone generators currently used on grain bins do not have sufficient capacity to produce sufficient ozone concentrations for the higher range.

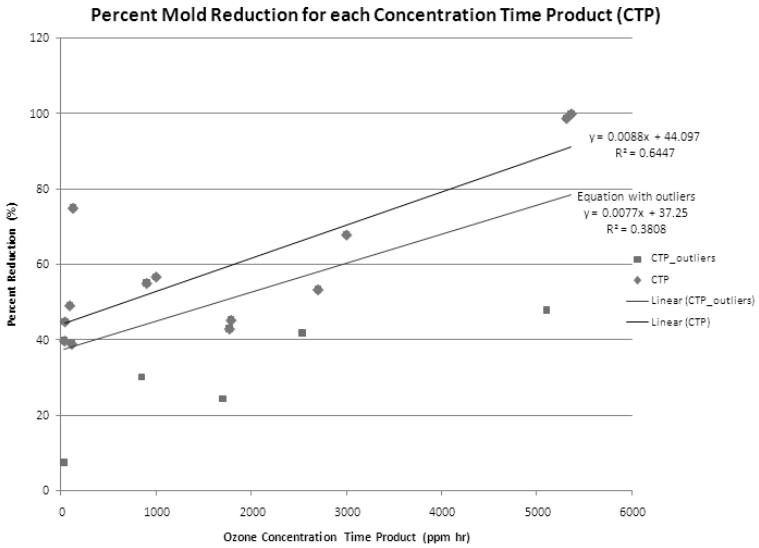

Figure 2 Linear graph of percent reduction versus ozone concentration time product for tested values with and without outliers.

3.3. Modified gompertz model

The modified Gompertz model was applied to each ozone concentration and the microbial reduction previously determined. Values for N and N_0 were obtained by taking the number of CFU present on the grain at time t and time t = 0. These values were then used to determine A, (log (N/N_0)), μ (min^{-1}), and λ (min). The model application gave the inactivation curve in Figure 3 over time and is comparable to the Gompertz inactivation curve (Erkman, 2001). The most identifiable value is the inactivation rate (μ), which is the slope of the line (see Figure 3). The slope for the line which represents 1752 ppm concentration of ozone was μ = -0.008, for 915 ppm the slope was μ = -0.004, and for 37 ppm the slope was μ = -0.0035. The lag phase for each concentration began around 120 minutes. This is the period when microbial reduction has been completed and no further reduction is expected. There is a large difference between the 915 ppm and 1752 ppm curves. As the concentration increases, the inactivation

rate increases substantially. A log reduction near 1 log is seen at the 1752 ppm concentration but only a log reduction of < 0.5 log is observed for the 37 ppm and 915 ppm concentrations. This is not comparable to Fan et al. (2007) who showed a log reduction of 2 in microbial load. However, with a larger initial mold concentration, a log reduction of 2 could be attained.

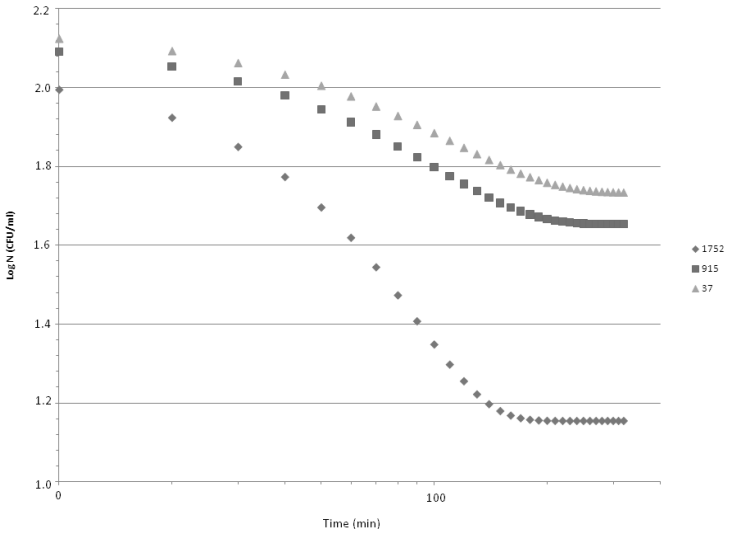

Figure 3 Graph of Log_{10} CFU ml^{-1} for three ozone concentrations representing the inactivation curves.

4. Discussion

As a result of the experiments conducted in the laboratory ozone treatment and grain sampling system, the following conclusions can be drawn in terms of quantifying the treatment effects on grain exposed to high ozone concentrations with respect to microbial load reduction and developing an equation to quantify microbial load reduction as a function of ozone concentration and time. First, the experimental results showed a reduction in mold CFUs on maize of 47.4% and 56.6% at 915 ppm ozone treatment after one and three hours, respectively, versus 37.5% and 80.2% at 1752 ppm after one and three hours, respectively. The reduction in mold CFUs after one and three hour treatments at ozone concentrations of 37 ppm was only 28% and 45%, respectively. The treatment effect after 1 hour was lower for 1700 ppm than for 800 ppm and no reasonable explanation for that behavior could be found. Secondly, linear regression analysis of the CTP data indicated that percent mold reduction increased linearly at a rate of 0.0088 times the CTP. This showed that higher ozone concentrations and/or longer treatment periods can decrease the amount of CFUs found on maize samples. Lastly, the modified Gompertz equation was successfully applied to the microbial inactivation data. From the results it was concluded that a 0.5 to ~1 log mold reduction on maize kernels was attained for ozone concentrations between 37 and 1752 ppm.

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Bt maize: a tool for improving food safety of grains at harvest

Regnault-Roger, C.*#[1], Folcher, L.[1], Delos, M.[2], Jarry, M.[3,6], Weissenberger, A.[4], Eychenne, N.[5]
[1] Université de Pau et des Pays de l'Adour - UMR CNRS 5254 IPREM - EEM - IBEAS BP 1155 - 64013 Pau. Email: catherine.regnault-roger@univ-pau.fr
[2] Ministère de l'Agriculture et de la Pêche - Service Régional de la Protection des Végétaux - "Midi-Pyrénées" - Bât. E - Bd Armand Duportal - F 31074 Toulouse
[3] Université de Pau et des Pays de l'Adour - UMR 1224 ECOBIOP, IBEAS BP 1155 - F-64013 Pau
[4] Chambre d'Agriculture du Bas-Rhin - Filières végétales - 2 route de Rôme - BP 30022 Schiltigheim - 67013 Strasbourg cedex
[5] Fédération Régionale de Défense contre les Organismes Nuisibles- FREDEC Midi-Pyrénées, Bt 43, 2 route de Narbonne, B.P.12267- F 31322 Castanet Tolosan
[6] INRA, UMR 1224 ECOBIOP, F-64310 Saint-Pée sur Nivelle

* Corresponding author
# Presenting author

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Abstract

A new EU (European Union) regulation came into force in 2007 with Regulation (EC) No. 1126/2007 which established maximum levels for fumonisins B_1 and B_2 (4000 ppb), deoxynivalenol (1750 ppb) and zearalenone (350 ppb) in maize and maize products. In order to evaluate French maize food safety, studies were carried out by the national Biological Risk Monitoring (BRM) Network. In this study, field trials involving 84 plots were conducted with Bt maize (MON 810) and its isogenic non-Bt counterpart in 2005 and 2006 in South-western France. Mycotoxin levels were determined in grain at harvest. Fumonisins B_1 and B_2, deoxynivalenol, and zearalenone were analyzed by LC-MS-MS and the results treated statistically using non parametric tests for mycotoxins and analysis of variance test for weather variables. As the climate was homogenous inside the experimental area, the transgenic event introduced into the maize was the only key parameter which differed between Bt and non-Bt maize plots.

Our results showed that all mycotoxin families were not impacted in the same way. The efficacy of *Bt* maize reduced mycotoxins more than 90% for fumonisins and more than 50% for zearalenone although deoxynivalenol was lightly increased. Therefore a competition between the different *Fusarium* spp. which produced fumonisins or trichothecenes is hypothesized. According to Regulation (EC) No. 1126/2007, 93% of the maize of Bt maize plots were able to be commercialized compared to only 45% for non-Bt maize plots. The results of this work showed that Bt maize improved food safety and constituted an useful tool to reduce significantly mycotoxin levels in harvested and stored grains.

Keywords: *Bt* (MON810) maize, Fumonisins B_1 and B_2, Deoxynivalenol (DON), Zearalenone, EC regulation 1126/2007 thresholds

1. Introduction

Maize (*Zea mays* L.) faces up to infestations by toxigenic fungi including *Fusarium* spp. These fungi cause diseases - e.g., ear rot - that affect plant growth, yield and crop quality. Toxins produced by *Fusarium* spp. were identified within the grain and kept up in grain-derived products, i.e. in human food and animal feeds, These mycotoxins cause several severe diseases in animals and humans. A recent review described the main diseases which were observed in the past provoked by the chronic contamination of small grains and maize and updated the challenges to face these contaminations (Miller, 2008). The main mycotoxins contaminating maize in France are: (i) fumonisins B_1 and B_2 [FB1-B2] produced by *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F.moniliforme* J. Sheld.) and *F. proliferatum* (T. Matsushima) Nirenberg, (ii) several trichothecenes, for the most part deoxynivalenol [DON] and (iii) zearalenone [ZEA] produced by *F. graminearum* Schwabe, and *F. culmorum* (Wm. G. Sm.) Sacc. *(*syn. *F. roseum* Link. Because DON, fumonisins and ZEA do not impact human and animal health in the same way and with the same intensity, it is important to determine when a fungal contamination occurs in a crop, which kind of mycotoxins still remain at harvest time and then in stored grains. The European Union (EU) decided to establish maximum levels of mycotoxins in cereals for human consumption and animal feed. The new EC regulation No. 1126/2007 (modifying EC regulation

No. 1881/2006) establishes the regulatory thresholds for fumonisins B_1 and B_2, deoxynivalenol (DON) and zearalenone. Since October 2007, the thresholds (maximum trace tolerable limit, MTL) were set at 4000 ppb for FB1-B2, 1750 ppb for DON and 350 ppb for ZEA.

Within this framework, and in order to maintain maize production in France below the EC mycotoxin threshold values, studies were carried out by the national Biological Risks Monitoring (BRM) Network supervised by the French Ministry of Agriculture. Field trials were conducted in France to compare the incidence of several plant protection approaches including prophylactic plant protection methods, agrochemicals and *Bt* technology.

In a previous study, we observed that field trials conducted with agrochemicals showed that the insecticide deltamethrine (20 $g.ha^{-1}$), which controlled the two main maize borers - *Ostrinia nubilalis* Huebner [Lepidoptera: Crambidae], and *Sesamia nonagrioides* Lefebvre [Lepidoptera: Noctuidae] was more efficient than the fungicide tebuconazole (250 $g.ha^{-1}$) to reduce mycotoxins levels in maize grains (Folcher et al., 2009a). According to Sobek and Munkvold (1999), insect borer damages help ear rot disease development by spore transport and kernel epidermis alteration. Beside temperature and relative humidity, genetic parameters impact the susceptibility of maize varieties for borer infestation and *Fusarium* spp. *Bt* maize, which was genetically modified (GM) for controlling Lepidoptera, reduced significantly *Fusarium* ear rot infection in kernels (Munkvold et al., 1997).

To complete these observations, field trials with *Bt* and *non-Bt* corn were conducted in southwestern France by the national BRM Network. The biocontrol of *O. nubilalis* and *S. nonagrioides* by several *Bt* maize events (Bt 176 and MON 810) were evaluated. These two *Bt* events encode for the same protein Cry1Ab but their promoter genes differ, and the control levels of the insects were different. The results showed that MON 810 controlled efficiently in the field the two corn borer pests both in ears and in stems (Folcher et al., 2006). Then, a reduction of mycoflora *Fusarium* spp. was observed with *Bt* (MON 810) maize (Folcher et al, 2009b).

In this study, the experimental work focused on a comparative experimentation involving *Bt* maize (MON 810) *vs*. its isogenic counterpart for evaluating the mycotoxin levels at the harvest. The following points were taken into account: (i) it was carried out under non differential conditions of cultivations for *Bt* and *non-Bt* maize to avoid any interference other than the one produced by the transgenic event; (ii) chemical analysis was conducted to quantify separately DON, ZEA and FB1-B2 levels. We aimed to determine if all mycotoxins were reduced within Bt maize vs its non Bt counterpart and, if not, which mycotoxin families were more reduced. One of the expected results was also to compare them to the new UE thresholds.

2. Materials and methods

Our experiments involved two varieties of maize (*Zea mays* L.): a *non-Bt* PR33P67 and its GM counterpart PR33P66 transformed by MON 810 event. The choice of these cultivars was guided by the duration of crop development (forwardness factor) in the Southwestern France (the so-called Région Midi-Pyrénées). Experiments were located in 21 sites in 5 departments: Haute-Garonne (9 sites), Tarn (4), Tarn et Garonne (5), Gers (2), and Ariège (1).

The bioassays were conducted under natural conditions twice in summers (2005 and 2006). The 21 fields (>1 ha), i.e. 84 twin plots (42 *non-Bt vs*. 42 GM *Bt* maize plots) for the two years, were seeded over a period of 20 days beginning April 15, 2005 and 2006. No insecticide neither fungicide treatments were done during cropping. The meteorological data (temperature, relative humidity (r.h.) and rainfalls) were recorded to verify if conditions were ensured for *Fusarium* spp. growing.

These trials were located in an area where by maize borers *O. nubilalis* and *S. nonagrioides* regularly infested maize. In this area, both pests are multivoltine. During cultivation and at the harvest, the infestation by caterpillars of the two borers was checked by dissection of 20 stalks and ears from each plot. Two 1kg samples of kernels were taken from 20 mixed ears collected randomly in each plot (i.e. 4 kg per field) and analyzed for mycotoxins. One hundred and sixty-eight samples were gathered. Mycotoxins were analysed by LC-MS-MS. DON, Fumonisin B1, Fumonisin B2 and ZEA were checked.

The following variables were subjected to statistical analyses (84 data per variable): FB1-B2, DON, ZEA and Mycotox (total mycotoxins). The weather variables involved temperature, rainfall and relative

humidity (r.h.). The Shapiro normality tests (Shapiro and Wilk, 1965) indicated that the weather variables (rainfall, temperature and r.h.) followed a normal distribution but the mycotoxin variables did not ($P < 0.05$). Consequently we used non-parametric bilateral Wilcoxon signed-rank tests (noted *Tw* test) and Mann-Whiney (Noted *U* test) tests (Siegel and Castellan**,** 1998) to analyse these variables. ANOVA test was used for normally distributed variables. Differences are considered significant if $P < 0.05$. Analysis was conducted using the software StatBox (Version 6.1).

The efficacy (E) parameter was calculated on the average of all plots as follows:

$$E\ (\%) = [(Control - Treatment)/Control] \times 100$$

in which 'control' and 'treatment' were the mycotoxin levels in respectively *non-Bt* and *Bt* plots. Efficacy may be negative if value in average for Treatment is upper than in Control.

3. Results

The comparison of *Bt vs*. *non-Bt* samples revealed distinct patterns for each mycotoxin family. The amounts of fumonisins B1 and B2 was significantly lower in *Bt* than in *non-Bt* maize for both year 2005 ($Tw = 231$; N = 21; $P < 10^{-4}$) and year 2006 ($Tw = 3$; N = 21; $P < 10^{-4}$) (Table 1). The efficacy E was respectively 95.66% for year 2005 and 92.44% for year 2006. DON levels were significantly lower for *non-Bt* maize than *Bt* cultivars for both year 2005 ($Tw = 65$; N = 21; $P = 0.04$) and year 2006 ($Tw = 51$; N = 21; $P = 0.01$). Parameter E was negative in this case with -31.04% for year 2005 and -75.52% for year 2006. A reduction of ZEA level was observed with *Bt* maize, but this reduction was smaller than the one observed for FB1-B2. It was not statistically significant for either of the two years (year 2005: $Tw = 64$; N = 21; $P = 0.24$, and year 2006: $Tw = 99$; N = 21; $P = 0.27$). Efficacy E was respectively 50% and 54%. Considering total mycotoxin levels, for both 2005 and 2006, the reduction was highly significant for GM *Bt* maize: respectively ($Tw = 231$; N = 21; $P < 10^{-4}$) and ($Tw = 212$; N = 21; $P = 3.98.10^{-4}$). Efficacy E was 92.6% for year 2005 and 76.3% for year 2006.

Table 1 Comparison of mycotoxin levels (mean ± SE in ppb) of GM *Bt* maize *vs*. its isogenic *non-Bt* counterpart.

Mycotoxin	Cultivars	2005	2006	*U* test[2]
Fumonisins B_1/B_2	GM	265.621 ± 114.062	425.076 ± 249.144	$U = 265$, $P = 0.120$
	Isogenic	6114.931 ± 1292.660	5620.036 ± 1453.458	$U = 259$, $P = 0170$
	Tw test[1]	$Tw = 231$, $P < 10^{-4}$	$Tw = 3$, $P < 10^{-4}$	
Deoxynivalenol	GM	185.691 ± 46.763	975.605 ± 471.796	$U = 146$, $P = 0.030$
	Isogenic	113.576 ± 57.199	238.805 ± 56.096	$U = 93.5$, $P < 10^{-4}$
	Tw test	$Tw = 65$, $P = 0.040$	$Tw = 51$, $P = 0.010$	
Zearalenone	GM	9.373 ± 3.030	1.567 ± 1.422	$U = 163$, $P = 0.070$
	Isogenic	18.954 ± 8.857	3.471 ± 2.313	$U = 121$, $P = 0.005$
	Tw test	$Tw = 64$, $P = 0.240$	$Tw = 99$, $P = 0.270$	
Total Mycotoxins	GM	460.685 ± 116.457	1402.248 ± 573.679	$U = 193$, $P = 0.240$
	Isogenic	6247.461 ± 1282.183	5862.312 ± 1466.050	$U = 259$, $P = 0.170$
	Tw test	$Tw = 231$, $P < 10^{-4}$	$Tw = 212$, $P = 3.980 \times 10^{-4}$	

Legend: [1] *Tw* test*:* Results of Wilcoxon signed rank test ($P < 0.05$); [2] *U* test*:* Results of Mann-Whitney test ($P < 0.05$).

The inter-annual variability of mycotoxin levels in 2005 and 2006 is detailed in Table 1. The Mann-Whitney test showed no significant difference between the levels of fumonisins B1 and B2 for non-Bt maize ($U = 259$; N = 21; $P = 0.17$) neither for Bt-maize ($U = 265$; N = 21; $P = 0.12$). Consequently, FB1-B2 levels of the two cultivars could be considered as homogenous for the two years. Conversely the DON levels were significantly higher in 2006 than in 2005 for both *non-Bt* ($U = 94$; N = 21; $P < 10^{-4}$) and Bt maize ($U = 146$; N = 21; $P = 0.03$). It was also to be underlined that DON biosynthesis was more important in 2006 than in 2005. Regarding ZEA, the level was significantly higher for *non-Bt* cultivar in 2005 compared to the year 2006 (U = 121; N = 21; $P = 0.005$), but not for Bt maize (U = 163; N = 21; *P*

= 0.07). In both cases, *Bt* maize gave a reduction of the ZEA levels. The level of the mycotoxins FuB1B2, DON and ZEA taken all together was not statistically different for the two years 2005 and 2006 for *non-Bt* maize (U = 259; N = 21; *P* = 0.17) neither for *Bt* maize (U = 193; N = 21; *P* = 0.24).

These mycotoxins are biosynthesized by *Fusarium* spp. So it was important to consider if meteorological data set up environmental conditions to develop *Fusarium* spp. Several rainfalls higher than 5 mm are required to release the spores of fungi from the ascospores and a daily RH up to 90% is required to induce spore germination (Marin et al., 1995; Brennan et al., 2005). Consequently, the number of days with an average moisture higher than 90% was considered over the 4 months covering the trials (beginning of June until the end of October) as well as the number of days with rainfalls higher than 5 mm (spore release). The percentage of days suitable for spore release and germination was calculated for each trial location. Table 2 shows that mean temperatures varied from 18.9°C and 20.6°C. Recorded rainfalls during the bioassays varied from 259 mm to 345.5 mm with a number of rainy days (with rainfall > 5 mm) between 12 to 21. In all locations, the percentage of favorable days for spore release varied from 6.52 to 11.41% during the summers although the percentage of favorable days for spore germination fluctuated between 1.09 to 3.80%. In all cases, conditions required for fungi development were met (Table 2).

Table 2 Meteorological data within Region Midi-Pyrénées area during the summers (May 1st to October 31th 2005 and 2006).

Sites location (Department)	Year	Temp. mean (°C)	Rain fall (mm)	Raining day number (>5mm)	Favourable days for spore release (%)	Hygrometry days number (>90%)	Favourable days for spore germination (%)
Haute-Garonne	2005	19.75	285.5	18	9.78	7	3.80
Haute-Garonne	2006	20.58	259	12	6.52	4	2.17
Tarn	2005	19.22	345.5	16	8.70	2	1.09
Tarn	2006	20.00	309.5	15	8.15	2	1.09
Tarn et Garonne	2005	19.21	318.4	17	9.24	7	3.80
Tarn et Garonne	2006	19.96	335.2	21	11.41	2	1.09
Gers	2005	18.86	341	19	10.33	5	2.72
Gers	2006	19.69	266	16	8.70	3	1.63

The climatic conditions in the bioassays, temperature, r.h. and rainfall, were favorable to the development of *F. verticillioides* and *F. proliferatum* as well as *F. graminearum* and *F. culmorum*. The climate changes with rainfall and drought events interfered with DON and fumonisin production (Abbas et al., 2007). Regarding the levels of mycotoxins and because field trials were conducted in strictly identical conditions (except the transgenic event introduced into the maize variety), we verified that climate was homogenous inside the experimental area (Region Midi-Pyrénées). Table 3 gave the conclusion that no statistical difference should be noted. Moreover, in each field, *Bt* maize and *non-Bt* counterparts growing in twin plots were rigorously submitted to similar weather conditions. Consequently, the MON 810 event introduced into the maize was the key parameter which differed between the two twin plot series. As the climate in the experimental area was similar for the two years of the study, the difference of mycotoxins levels observed within the *non-Bt* maize and its *Bt* counterpart can be considered as a consequence of the transgenic event.

Table 3 Climatic conditions in Region Midi-Pyrénées during the summers (May 1st to October 31th) 2005 and 2006 according to sites and years.

Variables	Site			Year			Site / Year		
	F^1	df	P^2	F^1	df	P^2	F^1	df	P^2
Temperature	0.190	3,40	0.9025	0.887	1,40	0.3519	0.001	3,40	1.0000
Rainfall	0.237	3,40	0.8703	0.318	1,40	0.5758	0.124	3,40	0.9453
Relative Humidity	0.522	3,40	0.6696	1.506	1,40	0.2269	0.261	3,40	0.8530

Legend: [1] *F* value; [2] Results of Two-Way ANOVA ($P < 0.05$)

4. Discussion

The level of total mycotoxins was significantly reduced with MON 810, but difference was observed according to mycotoxin families: FuB1B2 were strongly reduced, ZEA also but not significantly, DON was even increased compared to non-Bt maize.

Some field trials, conducted in USA in 2000-2002, demonstrated that fumonisins levels were frequently lower in grains of *Bt* hybrids than in *non-Bt* varieties (Hammond et al., 2004). Other experiments carried out in central Europe concluded that Bt maize hybrids slightly reduced the fusariotoxin level of maize (Magg et al., 2002). Field studies conducted in Ontario (Canada) showed that DON concentrations were reduced in Bt maize but mainly dependent of *O. nubilalis* density in the field (Schaafsma et al., 2002). A mycotoxin characterization campaign on maize conducted in the whole of France in 2004 by the national BRM network underlined a geographical distribution of *Fusarium* spp. The two main species producing fumonisins, *F. verticillioides* and *F. proliferatum* need warmer temperatures to develop than *F. graminearum* and *F. culmorum*. They predominate in Southwestern France while *F. graminearum* and *F. culmorum* are prevalent in Northern and Eastern. The Northern was characterized by predominance of DON or ZEA. Southern France with a more limited contamination by trichothecenes suffered high fumonisin levels (Delos et al., 2007). The higher levels of FB1-B2 in our bioassays corroborated this conclusion.

The complexity of the relations between insects, fungi and mycotoxins is well known. It was established that the level of infestation of maize ears by both *F. verticillioides* and *Aspergillus flavus* Fresen was affected by a competition correlated with insect activity that damaged the plant (Cardwell, 2000). Reid et al. (1999) observed from an evaluation of ergosterol (a metabolite biosynthesized by fungi and considered to be a biomarker of fungal activity), that *F. graminearum* developed a higher activity than *F. verticillioides*. Contamination of the grains by different species of fungi gave an idea of the competition between species colonizing the ears of maize (Velluti et al., 2000).

In this present study, it can be hypothesized a competition occurring between *F. verticillioides* and *F. graminearum* within the maize grains. The control of lepidopteran larvae by MON 810 event decreased the fumonisins levels, but increased the DON level in Bt maize, higher than non-Bt variety. Following this observation, we hypothesized that the control of insects limited the invasion of *F. verticillioides*, an opportunist fungi, and, as a consequence, favored the development and the activity of *F. graminearum* which infested the plant. This phenomenon was observed on wheat spike and has been named “flora inversion” (Ioos et al., 2004). However, because the level of DON compared to fumonisin was not so important, the development or the activity of *F. graminearum* might be lower than *F. verticillioides*. To verify/falsify this hypothesis, further works taking into account a qualitative and quantitative evaluation of occurring *Fusarium* spp. ought to be carried out. The ecological dimension of *Fusarium* spp., their geographical distribution as well as the gene control would also be taken into consideration (Yates and Sparks, 2008).

Regarding the thresholds requirements of EC regulation No. 1126/2007 for food safety, it was observed that for FB1-B2, 9 plots were below the 4000 ppb threshold and 12 above for year 2005 and also 10 plots below the threshold and 11 above for year 2006. But for GM *Bt* maize, all the plots were below the threshold for year 2005 and one plot was above the threshold for year 2006. All the plots with non-Bt maize were below the 1750 ppb threshold for both 2005 and 2006 for DON, although all the GM maize plots were below the threshold for 2005, but 3 of 21 plots were above the threshold for year 2006. Considering ZEA, all the twin plots had levels below the EC threshold (Figure 1).

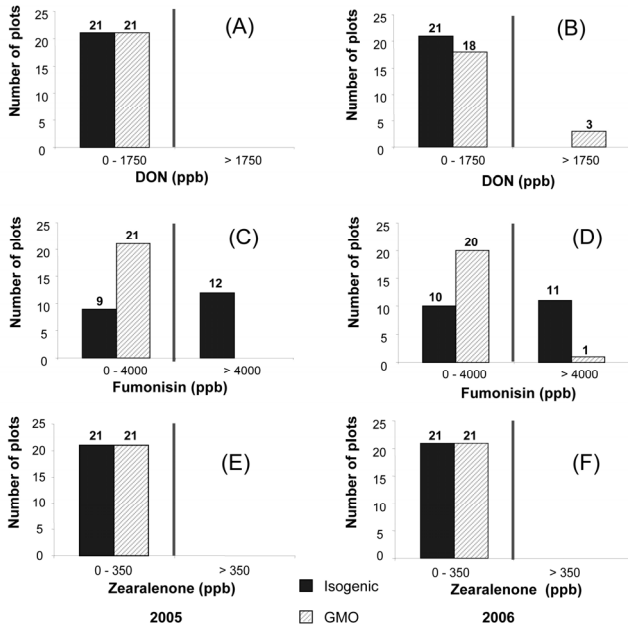

Figure 1 Grain deoxynivalenol (A & B), fumonisins B1 and B2 (C & D) and zearalenone (E & F) levels at harvest in Bt and non-Bt (isogenic) maize plots for 2005 and 2006 years. Red bars represent the present EC thresholds according the EC Regulation 1126/2007 of 2007-09-28.

Consequently, if this experimental crop would be carried on the market, the balance for the two years would be 7% of uncommercialized maize for *Bt* maïze compared to 55% i.e. more than half of the production that could not be commercialized for conventional *non-Bt* maize without any treatment. The loss for *Bt* maize was undoubtedly lower than for *non-Bt* maize with all economic consequences that could be induced by this situation.

From these results, it can be concluded that Bt maize improves the food safety of maize harvested grains and limits the risk of mycotoxins. It is also an useful tool to reduce the economic impact of such contamination for harvested and stored grains.

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Effect of oxygen reducing atmospheres on the quality and safety of stored shelled Brazil nut packs

Scussel, V.M.*#, Tanello, A.C., Giordano, B.N., Manfio, D., Galvão, S., Rodrigues, M.N.F.
Laboratory of Mycotoxicology and Food Contaminants, Department of Food Science and Technology, Center of Agricultural Sciences, PO. Box 476, Federal University of Santa Catarina, Florianopolis, SC, Brazil.
Email: vildes@cca.ufsc.br

* Corresponding author
# Presenting author

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Abstract

High moisture content, relative humidity, temperature and environment rich in oxygen (O_2) are the main factors for tree nuts to get infected by fungi and so aflatoxins (AFLs) contaminated. During storage and commercialization dry Brazil nuts packs need to maintain their safety and quality. Modified atmospheres in storage (macro-environment) and packaging (micro-environment) have been used to prolong food shelf life by reducing O_2 concentration with inhibitory gases or, more recently, by adding O_2 absorber pads. This work reports the application of O_2 atmosphere reducing methods on stored shelled Brazil nut packs aiming fungi and AFL degradation as well as hygienic conditions improvements. The methods applied were: (a) ozone - O_3, (b) carbon dioxide - CO_2 and (c) O_2 absorber pads with and without vacuum. Nuts were submitted to microbiological tests (fungi, aflatoxigenic strains, yeast and bacteria), moisture content and AFLs analysis. From all O_2 reducing atmosphere evaluated, the best performance was obtained with O_3. A reduction on fungi growth (1.8×10^4 cfu.g^{-1} to 2.6×10 cfu.g^{-1}) and yeast destruction after the first month of storage were registered. Also O_3 was the only nut treatment that was able to degrade AFLs. None of the spiked (AFLs: 15 ppb) nut samples O_3 treated had AFLs detected up to the LOQ of the method (0.36 μg.kg^{-1} for AFB_1+AFB_2+AFG_1+AFG_2) i.e., much lower than the allowed by the European Union regulation (MRL: 4 and 2 ppb for total and AFB_1, respectively), thus producing safer nuts. All other treatments stabilized and/or inhibited microorganisms growth. Add CO_2 and O_2 pads played an important role on nut quality. Further study will be carried out in order to adjust O_3 concentration and application conditions for longer period of storage.

1. Introduction

In the natural environment, Brazil nuts (*Bertholletia excelsa* Humb. and Bonpl) that grow in the Amazon forest may get contaminated by fungi and aflatoxins (AFLs) (Steiner et al., 1992, Pacheco and Scussel, 2009), as do other tree nuts. The aflatoxigenic *Aspergillus* species that have been isolated from Brazil nuts are *A. flavus, A. parasiticus* and *A. nomius* (Cartaxo et al., 2003; Castrillon et al., 2003; Arrus et al., 2005; Scussel, 2004; Olsen et al., 2008). Their growth is directly related to the climate conditions of that region and to the conditions during their storage, transport and commercialization, if there is no control of moisture content (m.c.) and temperature. That can also occur if nuts are packaged in a microclimate rich in oxygen (O_2) and m.c. enough to allow microorganisms to grow (McKenzie et al., 1998; Pacheco and Scussel, 2006).

Studies have reported the use of modified atmospheres in food storage, extensive to packaging, to reduce O_2 concentration by adding gases such as nitrogen, carbon dioxide (CO_2) and ozone (O_3) which lead to microorganisms (fungi, yeast and bacteria) inhibition, maintenance of lipid stability and reduction of grains/nuts/vegetable respiration (Zhao and Cranston, 1995; Kim and Yousef, 2000; Achen and Yousef, 2001; Sharma et al., 2002; Yelsincemin and Murat, 2006; Olmez, 2009). Vacuum also is an alternative for O_2 reduction and in recent years the addition of O_2 absorber pads have been the newest alternative in packaged food (Mexis et al., 2010; Freshpax, 2009; Ageless, 2009). Studies has been reported the effect O_3 and CO_2 on controlling microorganism growth in agricultural commodities (Mason et al., 1997; Maskan et al., 1999; Mazza et al., 2001; Yelsincemin and Murat, 2006). CO_2 is a promising and efficient inactivating microorganisms' gas for application on non-thermal sterilization process (Kaliyan et al., 2007; Van der Steen et al., 2009). Maeba et al. (1988) reported the destruction and disinfection of AFB_1 e AFG_1 in agricultural products treated with 1.1 ppm of O_3 during 5 min. An advantage of O_3, apart from being a powerful disinfectant, oxidant and AFLs degrader, is that it decomposes quite fast into O_2 and

does not have toxic effects (Samarajeeva et al., 1990; Mckenzie et al., 1998). This work reports the application of O_2 atmosphere reducing methods (vacuum, CO_2, O_3, and O_2 absorber) and their influence on fungal growth and AFL degradation on stored packaged shelled Brazil nuts.

2. Materials and methods

2.1. Samples

Shelled dry (processed) Brazil nuts (15 kg) were provided by Renmero Factory, Cameta city, in the State of Para, northern Brazil. The nut type and condition were as follows: medium size, 40-50 mm of length according to standard nut size by De Melo and Scussel (2007); initial m.c. and total fungi load of 6.5% and 1.83 log cfu.g^{-1}. No AFLs contamination was detected up to the method LOQ applied, respectively.

2.2. Chemicals, reagents and culture media

For analytical purposes, the following reagents and chemicals were used: potassium iodine, sulphuric acid, sodium thiosulphate (J.T. Baker); Solvents: methanol, ethanol, acetonitrile, benzene and toluene (Carlo Erba); Starch indicator (Synth). Ultrapure water (MilliQ system, Millipore); AFL standards: AFB_1, AFB_2, AFG_1, AFG_2 (Sigma); malt extract agar-MEA (Himedia), *A. flavus* and *parasiticus* agar-AFPA (Fluka), peptone agar (Himedia) and Tween 80 (CRQ); Violet red bile agar, Baird Parker agar tellurite potassium, serenity cysteine broth, tetrathionate broth, brilliant-green and phenol-red lactose sucrose agar (Merck).

2.3. Equipment and apparatus

The materials that were used: homogenizer (IKA T 25-Ultra Turrax); water bath (Quimis-Dubnoff Q226D); autoclave (Phoenix); microscope (100-400x PZO); incubator set at 20-25°C (ZET); microscope stereoscope (Carl Zeiss); colonies counter (Phoenix); microbiological oven (OLM); analytical (Mettler) and semi-analytical (CAB) scales; thermometer and hygrometer (CE); Altima C_{18} column (150 x 3.2 mm, 5 µm) (Alltech) at 30ºC; liquid chromatograph (LC) system (Waters Alliance 2695 separation module) with a 20 µl injection loop (Waters Corp.) coupled to a Quatro Ultima triple quadrupole mass spectrometer (Micromass) equipped with APCI as ionization source.

2.4. Application of O_2 reducing atmospheres

Shelled Brazil nuts were divided into two groups. (a) Group I - as Controls: nuts packed (a.1) loose - only air inside and (a.2) under vacuum. (b) Group II - AFL 15 ppb spiked nuts with O_2 reducing atmosphere: nuts were divided into the following sub-groups: packed (b.1) loose - only air inside; (b.2) vacuum; (b.3) O_3 treated* (packed with and without vacuum); (b.4) CO_2 gas added into packs; and (b.5) O_2 absorber pads (packed with and without vacuum). The series *O_3 (11.14 mg.L^{-1} - 90 min) was applied on the spiked nuts separately and then aseptically packaged. O_3 concentration checking was performed by the iodine metric test (APHA, 1980).

2.5. Packaging and storage conditions

Packs dimensions for length and width were of 20x25 cm, respectively, made with polypropylene film (with O_2 and UV barrier). For storage, the packs (260 g nut portions each) were heat sealed and stored in an incubator at 27ºC during two months.

2.6. Sample collection for analysis

Individual packs of shelled Brazil nuts were collected at Day one and every 30 days. Samples collected for analysis were in duplicate (n = *2*).

2.7. Shelled Brazil nut analysis

The analyses carried out were microbiological, m.c., temperature and AFLs. The methods applied for total fungi count was of Pit and Hocking (1997). The aflatoxigenicity of fungal strains was checked utilizing the AFPA by Pitt et al. (1983) and the identification of fungi in genus and species was carried out according to the keys of Samsom et al. (2004). *Salmonella* spp.*, Staphilococcus* spp. and coliforms (45ºC) were checked by APHA (1997). Moisture content was determined by gravimetry (AOAC, 2005) and AFLs content by LC tandem mass spectrometry (Xavier and Scussel, 2008) (limit of quantification - LOQ: 0.358 $\mu g.kg^{-1}$ for AFB_1+AFB_2+AFG_1+AFG_2, respectively).

2.8. Statistical analysis

The results were expressed as the mean values and standard errors. Statistical analysis was performed by analysis of variance (ANOVA) and included the Tukey´s test to evaluate significant differences among the means ($p < 0.05$). Figure 1 shows the flowchart on the whole study.

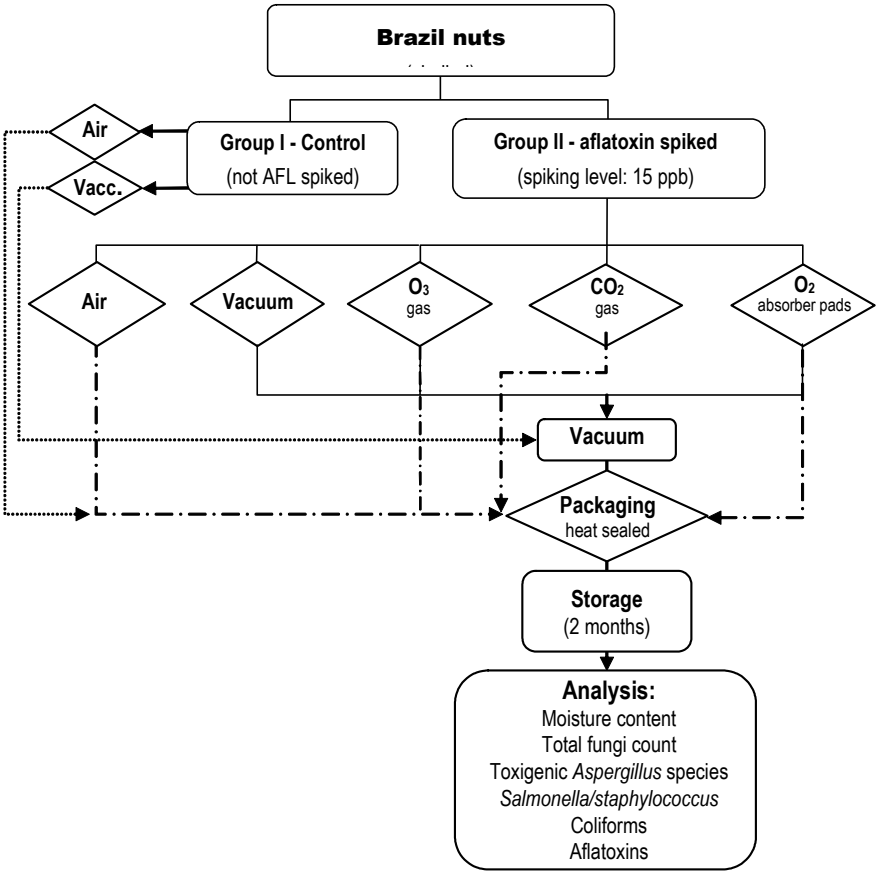

Figure 1 Flow chart of the oxygen reducing atmospheres application on shelled Brazil nuts stored in packages.

3. Results and discussion

All modified atmosphere treatments applied presented better nut quality after the period of study, when compared to the Control Group of nut packed loose i.e., with air inside (high total fungi and yeast count) (Table 1).

Table 1 Effect of O_2 reducing atmosphere on packs of shelled Brazil nuts microorganisms and aflatoxins

Storage	**Microorganisms (log cfu/g)**				**m.c. (%)**	**Aflatoxin total (ppb)**
Atmosphere Day	**Fungi/toxigenic strain**	**Coliform**	***Salmonella***	***Staphylococcus***		
Group I – Control[a]						
Air						
Initial	1.83/*A.f.; A.p.*[c]	ND	ND	ND	6.5	15.00
Final	2.69/*A.f.;A.p.*	ND	ND	ND	7.1	15.00
Vacuum						
Initial	1.83/*A.f.;A.p.*	ND	ND	ND	4.2	15.00
Final	0.70/*A.f.;A.p.*	ND	ND	ND	4.2	15.00
Group II[b]						
Air						
1	1.83/*A.f.;A.p.*	ND	ND	ND	6.5	15.00
30	2.96/*A.f.;A.p.*	ND	ND	ND	7.1	15.00
60	6.30/*A.f.;A.p.*	ND	ND	ND	7.1	14:89
Vaccum						
1	1.83/*A.f.;A.p.*	ND	ND	ND	4.2	15.00
30	0.56	ND	ND	ND	4.2	15.00
60	0.10	ND	ND	ND	4.2	14:99
Ozone						
1	1.83/*A.f.;A.p.*	ND	ND	ND	5.0	ND
30	NG	ND	ND	ND	4.9	ND
60	NG	ND	ND	ND	4.7	ND
Ozone + vacuum						
1	1.83/*A.f.;A.p.*	ND	ND	ND	3.1	ND
30	NG	ND	ND	ND	33.1	ND
60	NG	ND	ND	ND	3.0	ND
Carbon dioxide						
1	1.83/*A.f.;A.p.*	ND	ND	ND	6.5	15.00
30	NG	ND	ND	ND	7.0	15.00
60	NG	ND	ND	ND	7.0	14:99
Oxygene absorber pad						
1	1.83/*A.f.;A.p.*	ND	ND	ND	6.5	15.00
30	NG	ND	ND	ND	6.5	14.90
60	NG	ND	ND	ND	6.5	15:00
Oxygene absorber pad + vacuum						
1	1.83/*A.f.;A.p.*	ND	ND	ND	4.0	15.00
30	NG	ND	ND	ND	3.9	15.01
60	NG	ND	ND	ND	4.0	1498

ND: not detected [a] not aflatoxin spiked (AFL total < lower than the method LOQ: 0.350 µg/kg), [b] AFLs-15 ppb spiked *A. parasiticus A. flavus NG no grow*

3.1. Total fungi and aflatoxigenic strains

A substantial fungi reduction was observed after the study both with O_2 absorber and O_3 packaged under vacuum as well as nuts O_3 loose packed. CO_2 also plays an important role on the microorganism reduction in the current experiment ranging from 1.8 x 10^4 cfu.g^{-1} to 2.6 x 10 cfu.g^{-1}. Applying vacuum improved quality further. As far as mycoflora and contamination the main genera and species isolated from the untreated shelled nuts received from the factory were *Acremonium* sp., *A. ochraceus*, *Cladosporium* sp., *P. corylophium and Rhizopus* sp. followed by *A. niger; A. parasiticus, A. versicolor and P. crustosum*. However, infection was reduced when atmospheres were applied.

3.2. Hygienic bacterial indicators

Regarding to what was observed for fungi and yeast O_3, all gases and O_2 absorbers as well as vacuum did not allow neither *Salmonella*, *Staphylococcus* or coliform to grow showing the safe power of the treatments for microbial population control.

3.3. Moisture content and AFLs

Nuts presented m.c. reduction during the after vacuum application specially the O_3 treated throughout the whole storage period which kept nuts cruncher. That was probably due to the fact that during O_3 application occurs an exposure of nuts to 90 minutes with O_3 stream that can take moist from nut surface. The lower total fungi count was detected in the packs that were submitted to O_3 (reduction of 5.01 %) suggesting that apart from the fungi destruction by the O_3/vaccuum application, the reduction of m.c. powered fungi reduction.

3.4. Aflatoxins

It was possible to observe in the AFLs spiked samples, that O_3 was able to degrade them because none when analysed had AFLs detected up to the method LOQ used when compared to the Control Groups. That was different of the other O2 reducing atmospheres. They were able, only to stabilize/reduce the microorganisms growth keeping nuts safe but AFLs. In that sense the pack with O_3 and vacuum applied brings an alternative for AFL degradation and also m.c. reduction, a factor that is directly related to fungi proliferation and development of possible aflatoxigenic strains. Nuts treated with O_3 in the study showed to be good for consumption, as no AFLs were detected in them.

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Reduction of in-shell Brazil nut (*Bertholletia excelsa* H.B.K.) aflatoxin contamination by ozone gas application during storage

Giordano, B.N.E.[1], Simao, V.[1], Manfio, D.[1], Galvao, S.[1], Scussel, J.N.[2],Scussel, V.M.*#[1]
[1] Food Science & Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, P.O.Box 476, Florianopolis, SC, CEP 88034-001, Brazil. Email: vildescussel_2000@yahoo.co.uk
[2] Mechnical Engeneering Dept Technological Center, Federal University of Santa Catarina, PO.Box 476, Florianopolis, SC, Brazil.

* Corresponding author
# Presenting author

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Abstract

The susceptibility of the in-shell Brazil nut mycoflora and aflatoxins (AFLs) contamination to ozone (O_3) gas during storage is reported. In-shell Brazil nuts obtained from retail market were submitted to O_3 gas atmosphere at different concentrations immediately before to be stored. Samples were collected just after the gas exposure and every 30 days during the storage period to carry on mycological tests and AFLs analysis. A sensorial evaluation by descriptive quality analysis was carried out to check treated nuts sensory attributes according to consumer acceptance after gas exposure. The O_3 treatment applied within 5 h at 31 mg/L was able to successfully inhibit the viability of fungi of the nut-contaminating microflora and so the toxigenic *Aspergillus species* from the day of application. AFLs were totally degraded in all samples whatever O_3 concentration applied. No significant changes on sensory attributes were observed that could affect nut acceptability after the O_3 treatments and storage conditions applied in the present experiment. This procedure is tentatively applied at an Amazon State nut factory for checking its potential in mycotoxin risk contamination of in-shell Brazil nuts safeguarding under the Amazon region environment.

Keywords: In-shell Brazil nut, Ozone, Mycoflora, Aflatoxin, Storage, Sensory evaluation.

1. Introduction

Prevention of aflatoxin (AFL) contamination in tree nuts by controlling the environment conditions for toxigenic fungi growth has not always been effective. The risk of AFL contamination in Brazil nuts (*Bertholletia excelsa* H.B.K.) can occur, either in the forest or during storage and it can vary with the year of harvest (Pacheco and Scussel, 2006; 2009). Nut contamination by fungi, especially the aflatoxigenic species of *Aspergillus*, are directly related to the climatic conditions (high humidity and warm conditions) in the Amazon forest during the period of harvesting (at wet season: December to May) (Arrus et al., 2005; Pacheco and Scussel, 2006; 2007; 2009; Olsen et al, 2008). The presence of AFLs is a concern for exporters of Brazil nuts according to European Community (EC) reduction the maximum tolerance limit of aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) and AFB_1 to 4 and 2 µg/kg, respectively (ECC, 2006) either in-shell or shelled. Thus, there is a need for the development of safe technologies at the forest environment level enabling to control fungi proliferation and reduce toxin contamination.

Fungi can growth both, on the shell and inside the shell on the nuts when shells are cracked or through its opercule. Thus, AFLs have been detected on the surface of shelled nuts and/or inside of cracked, or brown spotted, in-shell nuts (De Mello and Scussel, 2007; 2009). Recently, it was found that the main site of the in-shell nut contaminated by AFLs is located between the shell and the the nut peel (Conforcast, 2008). Several environmental factors are known to influence fungi growth and AFL production, being temperature and relative humidity (r.h.) the most critical (Mangan and Aldred, 2007). Ozonation, an oxidation method has been studied for detoxification of AFLs in foods (Shamarajeewa et al., 1990). The oxidation decreases AFL concentration over time (McKenzie et al., 1997). O_3 modified atmospheres have been developed for dried figs, barley, pistachio among other foods (Oztekin et al., 2006). The attractive aspect of O_3 is that it decomposes rapidly (half-life of 20–50 min) to molecular oxygen after application without leaving a residue (Mason et al., 1997; Frazier and Westhoff, 1988; Maeba et al., 1988; Perez et al., 1999; Kells et al., 2001; Yesilcimen and Murat, 2006; Inan et al., 2007; Olmez et al., 2009). It can be applied to food as a gas or dissolved in water.

One of the most important applications of O_3 in agriculture is in the post-harvest treatment of crops and the main purposes of gas application are: inactivation of bacterial growth, prevention of fungal decay, destruction of pesticides and chemical residues, and control of storage pests. Storage period can be doubled when some fruits and vegetables are held in an environment with O_3 (Frazier and Westhoff, 1988; Inan et al., 2007; Olmez et al., 2009). Regarding *fungi* and AFLS in different food commodities, Oztekin et al. (2006) reported a significant reduction of microorganism counts on dried figs at 5 mg/L of O_3, decreasing the total yeast/mould counts of 72% and Perez, et al. (1999) observed a fungal decay of strawberry after 4 days of storage under ozonation. Five mg/L of O_3 inhibited the surface growth, sporulation and mycotoxin production of cultures of *Aspergillus flavus* and *Fusarium moniliforme* (Mason et al., 1997). Yesilcimen and Murat (2006) studied pistachio exposure to O_3: 5.0, 7.0 and 9.0 mg/L and found AFB_1 and total AFLs reduction by 23 and 24% at 9 mg/L. Maeba et al. (1988) observed the destruction and detoxification of AFB_1 and AFG_1 with O_3 in model experiments.

We report here the application of O_3 gas at different times and concentrations during the storage of in-shell Brazil nut to improve nuts safety regarding fungi and AFLs contamination.

2. Materials and methods

2.1. Sample

Dry (processed) in-shell Brazil nuts (14 kg), export Medium Size Type 40-50 mm length (De Melo and Scussel, 2007). A naturally contaminated batch was chosen for the study with initial AFLs (AFB_1 / AFB_2 / AFG_1 / AFG_2) of 11.58 µg/kg obtained in the retail market. That level is allowed by regulations from USA, Canada, Brasil and Mecosur (15, 15, 30, 20 µg kg, respectively). Total fungi count: 4.83 log cfu/g and 9.43% and m.c.

2.2. Storage

*S*even vertical silos, build with vinyl polychloride (PVC) with the following dimensions 80 x 15 x 0.2 cm for height, diameter and width, respectively; containing an upper lid and two apertures (top and lower part of the silos) for sample collection and O_3 application, respectively; connected to an ozone generator (Megazon™) (Fig. 1).

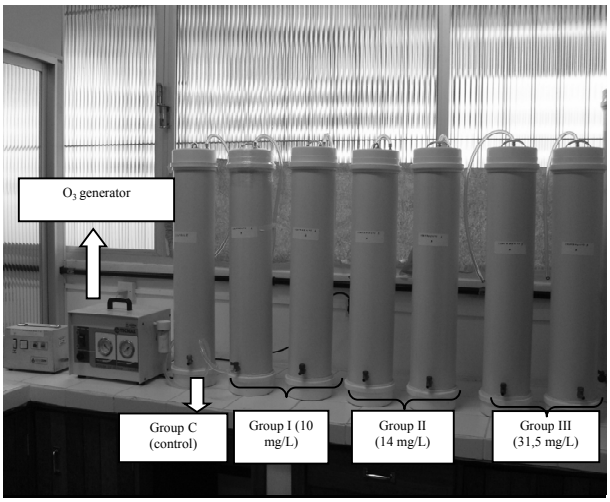

Figure 1 Brazil nut ozone treatment and storage system: silos (n=7) and O_3 generator

2.3. Chemicals, reagents and culture media

The reagents, potassium iodine, sulphuric acid, sodium thiosulfate, 2-thiobarbituric acid (TBA), trichloroacetic and butylated hydroxytoluene were of Analytical grade (Vetec) and utilized for the TBA test. The solvents were methanol, ethanol, acetonitrile, benzene and toluene from Carlo Erba, starch indicator from Synth and the ultrapure water from MilliQ system, Millipore. The standards of AFL

(AFB_1, AFB_2, AFG_1 and AFG_2) were obtained from Sigma. Malt extract agar-MEA was purchased from Himedia™; *A. flavus* and *parasiticus* agar-AFPA from Fluka™, peptone agar from Himedia™ and Tween 80 from CRQ™.

2.4. Equipment and apparatus

The equipment and apparatus utilized for sample preparation, microbiological and AFL analysis were homogenizer from IKA T 25-Ultra Turrax; water bath from Quimis-Dubnoff™ Q226D; autoclave from Phoenix™; microscope from 100-400x PZO; incubator set at 20-25°C from Dist™; microscope stereoscope from Carl Zeiss Iena™ (Germany); colonies counter from Phoenix™ and microbiological oven from Fanen™. Analytical cales from Mettler™ and the semi-analytical one from CAB™. Industrial Brazil nut cracker was provided by CIEX™ (Manaus, AM, Brazil). The spectrophotometer model E005 was from Hitachi™. The liquid chromatograph comprised an isocratic pump model 305 and fluorescence detector model 121 from Gilson™. The column was of C_{18} with 15 cm length, 4.6 mm diameter, and particle size of 5 µm from Phenomenex™. Thermometer and hygrometer utilized for environment temperature and moist readings were from CE™.

2.5. Sample preparation for O_3 application

In-shell Brazil nuts previously analysed for fungi load, m.c. and AFLs contamination were aseptically weighted into portions of 2 kg to be added into each silo for the O_3 treatment.

2.6. Preparation of the storage Silos, ozone application and iodinemetry

Silos were filled with nuts and tightly closed. They were divided into 4 groups for O_3 application: Group C (Control = no O_3 application), Group I (O_3 conc. = 10 mg/L), Group II (O_3 conc. = 14 mg/L) and Group III (O_3 conc. = 31.5 mg/L) (Fig. 1). Each treatment was carried out in duplicate (n = 2) except for Group C. After closing the upper part of the silos, O_3 gas was applied through the lower lateral aperture to get the following concentration in each silo: 10, 14 e 31.5 mg/L, with 1, 3 and 5 h exposure time, (n = 2) for silo Groups 2, 3 and 4, respectively. Those concentrations were checked by iodinemetrical analysis (APHA, 1980). The storage time was expanded on a 180-d period (May to October).

2.7. Sample collection for analysis

Individual 200 g samples of Brazil nuts were aseptically collected from each silo at day-one and every 30 d. This sampling was carried out from the top silo aperture. Samples collected for analysis were made in duplicate (n = *2*).

2.8. Mycology, m.c., r.h., temperature, AFLs and sensory analysis

For total fungi count the method used was from Pit and Hocking (1997). For fungi toxigenicity (for AFLs) the method was from Machida and Saito (1999). The identification of fungi in genus and species was carried out according to the keys of Samsom et al. (2004). The strains aflatoxigenicity was checked utilizing the AFPA method developed by Pitt et al. (1983). The m.c. was carried out by a gravimetric method (AOAC, 2005). Relative humidity and temperature were monitored daily utilizing hygrometer and thermometer, respectively. In parallel, data on r.h. and temperature was obtained from the National climatic recordings database EPAGRI/SC (May to October). AFLs analysis was performed by liquid chromatography with fluorescence-detection, with a limit of quantification (LOQ) at 0.50, 0.17, 0.50, and 0.17 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2, respectively (Sobolev et al., 2007).

2.9. Sensory evaluation

The descriptive quantitative analysis by Stone and Sidel (1993) was conducted using a team of 18 trained (specifically for Brazil nut taste) panelists and four sessions (n = 4). The hedonic scale was separated into 5 degrees as follows: 1: dislike very much, 2: dislike, 3: neither like nor dislike, 4: like, and 5: like very much. Six sensory attributes of the Brazil nuts were recorded: shell appearance, nut appearance, strange odor, roasted flavor, rancidity and firmness.

2.10. Statistical analysis

The results were expressed as the mean values and standard errors. Statistical analysis was performed by analysis of variance (ANOVA) and included the Turkey´s test to evaluate significant differences among the means ($p < 0.05$).

3 Results and discussion

3.1. Effect of O_3 on the in-shell Brazil nut mycoflora

The total fungi load, aflatoxigenic *Aspergillus* species and levels of AFLs variation during the storage of in-shell Brazil nuts under O_3 atmosphere are shown in Table 1. There was a clear reduction on the total fungi count, AFLs and m.c. when compared to the Control Group. However this reduction trend was dependent upon the O_3 concentration used and time of storage.

Table 1 Fungi, aflatoxigenic *Aspergillus* species and aflatoxins levels in Brazil nut stored under ozone at room temperature

Storage			Brazil nut										
	O_3 treatment		Fungi				m.c./loss (%)		Aflatoxins (in-shell / after shelling) (µg/kg)[a]				
days			total count (log cfu/g)		*Aspergillus* aflatoxigenic species		in-shell	after shelling					
	group	conc. (mg/L)	in-shell	after shelling	in-shell	after shelling			∑AFLs	AFB_1	AFG_1	AFB_2	AFG_2
1	C	0[b]	4.83	2.54	*A. flavus*, *A. parasiticus*	ng ng	9.43 (NA)	5.14 (NA)	11.58/6.61	3.48/1.16	3.57/1.89	2.21/2.02	2.32/1.74
	I	10	3.5	ng	*A. flavus*, *A. parasiticus*	Ng ng	7.72 (6.61)	3.97 (6.25)	3.01/ND	3.01	ND	ND	ND
	II	14	3.3	ng	*A. flavus*, *A. parasiticus*	ng ng	7.71 (7.58)	3.96 (6.89)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.68 (8.56)	3.95 (7.52)	ND	ND	ND	ND	ND
30	C	0	4.84	2.57	*A. flavus*, *A. parasiticus*	ng ng	9.57 (NA)	5.28 (NA)	12.06/8.01	3.69/1.37	3.78/2.73	2.23/2.05	2.36/1.86
	I	10	ng	ng	ng	ng	7.67 (10.22)	3.95 (8.04)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.66 (10.60)	3.94 (8.67)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.64 (11.74)	3.93 (9.29)	ND	ND	ND	ND	ND
60	C	0	4.86	2.60	*A. flavus*, *A. parasiticus*	ng ng	9.63 (NA)	5.32 (NA)	12.24/7.95	3.83/1.16	3.82/2.83	2.22/2.08	2.37/1.88
	I	10	ng	ng	ng	ng	7.63 (11.65)	3.93 (9.03)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.61 (12.59)	3.90 (9.65)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.58 (13.34)	3.88 (10.28)	ND	ND	ND	ND	ND
90	C	0	4.88	2.62	*A. flavus*, *A. parasiticus*	ng ng	9.84 (NA)	5.46 (NA)	12.34/8.03	3.86/1.14	3.86/2.86	2.25/2.10	2.37/1.93
	I	10	ng	ng	ng	ng	7.56 (14.65)	3.87 (11.38)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.54 (15.75)	3.87 (12.29)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.51 (16.11)	3.60 (12.50)	ND	ND	ND	ND	ND
120	C	0	4.89	2.65	*A. flavus*, *A. parasiticus*	ng ng	9.89 (NA)	5.51 (NA)	12.49/8.08	3.94/1.14	3.88/2.88	2.27/2.11	2.40/1.95
	I	10	ng	ng	ng	ng	7.47 (16.15)	3.85 (12.23)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.43 (17.78)	3.84 (13.14)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.41 (17.96)	3.82 (13.34)	ND	ND	ND	ND	ND
180	C	0	4.91	2.69	*A. flavus*, *A. parasiticus*	ng ng	9.93 (NA)	5.63 (NA)	12.55/8.17	3.95/1.13	3.90/2.91	2.28/2.17	2.42/1.96
	I	10	ng	ng	ng	ng	7.37 (18.82)	3.80 (12.99)	ND	ND	ND	ND	ND
	II	14	ng	ng	ng	ng	7.35 (19.89)	3.78 (13.79)	ND	ND	ND	ND	ND
	III	31.5	ng	ng	ng	ng	7.32 (19.89)	3.76 (14.19)	ND	ND	ND	ND	ND

[a] nuts were evaluated for fungi whole (in-shell + edible part) and after shelling (only edible part); nuts Groups: [b] C = control (no O_3 treatment); I (O_3 conc. = 10 mg/L), II (O_3 conc. = 14 mg/L) and III (O_3 conc. = 31.5 mg/L); NT = not treatment ; NA = no applicable; ng = no growth; mc = moisture content; total fungi load initial = $6.9x10^4$; initial mc= 9.43%/ ND = not detected; [c] = concentration AFLs µg kg in duplicate; LOD 0.25; 0.08; 0.25 and 0.08 µg/kg e LOQ 0.50; 0.17; 0.50 and 0.17 µg/kg, to AFB_1, AFB_2, AFG_1 and AFG_2 respectively; [d] $AFB_1 + AFG_1 + AFB_2 + AFG_2$. Temperature: 20°C (16.6 – 20.6°C); r.h.: 80% (75.8 – 85)

3.1.1 Total fungi load

As expected, the in-shell Brazil nuts ozonation showed to be effective on the fungi/spores destruction during the period of storage. A reduction of total fungi count was registered as soon as the first day after O_3 application in all treated nut Groups. However, the complete destruction of fungi (no growth: 'ng' label) was reached at different stages of storage depending upon the gas concentration applied. Total fungi destruction started at the first day when the highest O_3 concentration was applied (31.5 mg/L) and after 30 days of storage, when O_3 exposure was achieved at a lower concentration i.e., at 14.0 and 10 mg/L. Thus, no fungi growth was registered in all O_3 treated nut Groups after a month of storage up to the end of the six month. From the original (untreated nuts) total fungi load of 4.83 log cfu (Control), it went down at day one of O_3 treatment to 3.5 and 3.3 log cfu/g for groups I (O_3: 10.0 mg/L) and II (O_3: 14.0 mg/L), respectively; and no fungi growth was detected in the nuts treated with the highest O_3 concentration (Group III – O_3: 31.5 mg/L). These figures were very different of those observed with the

nuts of control groups, where the fungi load remained somewhat stable with an insignificant increase during the whole period of storage i.e., from -zero/-one (4.83 log cfu/g) to180 d (4.91 log cfu/g). Similar situation happened to those nuts when analyzed after being shelled, however in a lower extend from D-one (2.54 log cfu/g) to 180 d (2.69 log cfu/g) – control group) which was expected, as the edible part was protected by the shell. Some works have reported the effect of ozonation in food and nuts and they have similar findings for fungi load reduction as obtained in the present experiments, however applying lower O_3 concentrations. We utilized higher O_3 concentrations due to the fact that our aim was more than just fungi disinfection. We wanted to obtain a significant AFLs degradation too (to be discussed in the next Session) and for that purpose the O_3 concentration should be higher. Zhao and Cranston (1995) studied the effect of O_3 on black pepper at a lower gas concentration (6.7 mg/L) than in our study. The authors reported fungi load reduction from 7 to 4 log cfu/g. In another study carried out in dried figs (Oztekin et al., 2006), O_3 reduced the total fungi load from initial 1.46 to 1.00, 0.57, and 0.40 log cfu/g using O_3 at 1, 5, and 10 mg/L, respectively. In a work carried out on Brazil nut, however treating the nuts with aqueous O_3 solutions (0, 1, 10 and 20 mg/L) the authors showed that these treatments were effective to fungi control reaching an inactivation rate of 100% for *A. parasiticus* and 96% for *A. flavus*. However O_3 application in water can lead to an increase of moisture, reducing crunchy and firmness (Freitas and Venancio, 2008).

3.1.2. Aflatoxigenic species of Aspergillus

Regarding the isolation of aflatoxigenic species of *Aspergillus* (*A. flavus* and *A. parasiticus*) in the Brazil nuts just after the O_3 treatments (Group I, II, III), only the highest O_3 concentration treated nuts (Group III - 31.5 mg/L) did not allow them to growth since day one after gas application. The same occurred to the Groups of lower O_3 concentrations, but only after 30 d of storage. On the other hand, the Control Group was contaminated permanently by these species throughout the whole storage period. Similar situation happened when Mason et al. (1997) applied O_3 (5 mg/L) in cultures of *A. flavus* and *Fusarium verticillioides*. Ozone treatment in these conditions inhibited the growth, sporulation and mycotoxin production. Kells et al. (2001) studied the efficacy of O_3 as a fumigant to disinfest stored maize from insects. At a concentration of 50 mg/L, these authors observed a reduction of 63% of the kernel contamination level by *A. parasiticus*. It seems important to emphasize that the species *A. nomius* was not detected in the present experiments either due to the fact that after nut dehydration, fungi were destroyed by the heating temperature or fungi competition as nuts were purchased in the market in the South of Brazil, or also because the AFPA media does not give a clear response, *i.e.* not enhancing the characteristics of that *Aspergillus* species.

3.1.3. Environmental conditions after O_3 treatment: r.h. level

During the period of the in-shell Brazil nut storage in Southern Brazil were rather mild with average temperature of 20°C (min 16.6 and max 20.6°C) and r.h. of 80% (min 75.8 and max 85.3%).

3.1.4. Effect of O_3 on AFL contamination

In contrary to the nuts Control Group, a reduction on the AFL levels was detected throughout the whole storage period of the in-shell Brazil nuts O_3 treated (Table 1). Just after the O_3 treatments, either at gas concentration of 14 or 31.5 mg/L, the Brazil nuts did not present contamination by AFLs – up to the limit of quantification of the method used (0.50, 0.17, 0.50, and 0.17 µg/kg, for AFB_1, AFB_2, AFG_1 and AFG_2, respectively). Although the three concentrations applied were able to degrade the toxins, some AFLs were still detected: 3.01 µg/kg (74% reduction) at the lower O_3 concentration (10 mg/L) after the first month of storage, the toxins was able to be totally degraded after 30 d. As far as the storage period and the AFL degradation are concerned, all the groups did not present any AFLs contamination after one month of storage onwards. As expected with the Control Group, the nuts AFL level remained stable or slightly increased from the beginning to the end of storage (11.58 to 12.55 µg/kg, respectively). That could be explained by the controlled experiment environment and storage conditions applied, and additionally, by the mild/low temperatures in Southern Brazil. The fact that other AFLs were detected, i.e., AFB_2 and AFG_2 in the Control Group, was probably the consequence of the origin of the nuts which were purchased in the retail market of Southern Brazil and not in the Amazon region, thus exposed to different fungi species contamination through manipulation and different environments, from tropical to temperate climate down in the south, respectively.

3.1.5. O_3 nuts sensory attributes for quality acceptance

No significant changes ($P < 0.05$) were found between shell and nut appearances, strange odor, roasted flavor, rancidity and firmness scores of the ozonated Brazil nuts samples stored and they did not differ greatly among the concentrations used too. They were between 3 (indifferent) and 4 (like), different of the Control Group that received score 2 for most of the attributes except for nut firmness (score 3), showing that the treatment with O_3 and the period of storage of the in-shell Brazil nuts, did not affect their sensory quality attributes for all O_3 treated Groups. Also the shell received score 4, except for roasted flavor (score 3). Therefore, regarding sensory evaluation, the gas treatment kept nuts sensory attributes thus still palatable apart from being safer.

Prevention is a better strategy than detoxification which is much more complicated and so the implications to human and animal health. Despite of the findings, there is a need of more studies, especially on application in pilot plants utilizing larger amounts of nuts under the Amazon forest environment (first and second storages) prior factory processing, in order to establish the optimal applicable O_3 gas concentration and time of exposure for maximum effectiveness utilizing the present findings. This work is being applied in a pilot plant at a Brazil nut factory in the Amazon State for checking its effectiveness under the Amazon region environment. Important to emphasize that gaseous O_3 decomposes to form O_2 and it does not affect the environment, nor leave residues in the nuts.

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Use of technology radiation as a method of reducing the microorganism and conservation postharvest of caja during storage

Silva, J.M.*#[1], Correia, L.C.S.A.[2], De Moura, N.P.[2], Maciel, M.I.S.[2], Villar, H.P.[1]
[1] Centro Regional de Ciências Nucleares-NE, CRCN/CNEN. Av. Profo. Luiz Freire, 200, Cidade Universitária, 50.740-540, Recife, PE/Brazil. Email: jmnilda@yahoo.com.br; hpvillar@cnen.gov.br
[2] Depto. de Ciência dos Alimentos, Univ. Federal R. de Pernambuco, Recife, PE, 52171-900, Brazil, aidil_alencar@yahoo.com.br; nairapaesmoura@yahoo.com.br; marines@ufrpe.br

* Corresponding author
# Presenting author

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Abstract

The caja fruit (*Spondias* spp.) are among the species considered exotic presents excellent view of economic exploitation to the Northeast of Brazil. However, contamination by microorganisms is a major factor in postharvest losses of fruits and other cultivated in Brazilian territory. Use of ionizing radiation aiming to reduce the use of pesticide products in pest control and microorganisms phytopathological and maintaining the quality of agricultural products has been studied by several researchers of postharvest fruit. This technology meets the requirements of food safety imposed by the various organs of public health. In order to evaluate the action of ionizing radiation on agents phytopathological and the effects on the quality of postharvest of caja fruits when stored under temperature of shelf, the experiment was conducted with application of doses 1.0 and 2.0 kGy, dose zero as fruit control, and storage under temperature of 23°C for twelve days. Control fruit had higher contamination by microorganisms phytopathological throughout the storage period. Analysis of total soluble solids, total titratable acidity, hydrogen potential, soluble solids/titratable acidity ratio, ascorbic acid and color of the flesh have suffered minor variations between the different doses studied.

Keywords: *C*onservation; caja fruit; gamma radiation; postharvest storage; microorganism spoilage.

1. Introduction

The Brazilian Northeast has a wide variety of species fruit considered exotic, with good acceptance by local consumers. Many species still have a inadequate harvest that affect their quality and cause heavy losses during the marketing because extensive contamination in fruit by microorganisms (Schöttler and Hamatschek, 1994; Mattietto et al., 2003; Mata et al., 2005).

The caja *(Spondias* spp.), is among the species that presents excellent prospects for economic exploitation for the Northeast Region of Brazil. Its fruit has excellent qualities, plus high vitamin C content and medicinal properties to fight infection and increases immune efficiency (Barroso et al., 1999, Lee et al., 2002). However, the consumption of fruits of *Spondias* species occurs mostly in the form of juice and ice cream industrialized, especially where exploitation occurs in the form of extraction and non-commercial planting (Gomes, 1990; Lima et al., 2002; Gouveia et al., 2003). The trade of raw fruits is almost nonexistent on supermarket shelves, situation which can be attributed to the lack of application of techniques for postharvest retention of fruits arrived at the consumer's table. The technical knowledge of the fate of qualitative traits of these species after harvest will increase raw caja consumption, with positive effect on population health, meanwhile to contribute to the economy of the northeast region as an additional source of income for small and medium producers in the region. Application of new technologies to reduce postharvest losses and maintain the quality of the fruit until the consumer has been the subject of research already for several decades. Use of ionizing radiation with the order of reducing the attack of pathogenic microorganisms and maintain the quality of agricultural products has been studied by several researchers in the field of postharvest. The results of these studies has been the basis to meet the requirements of food quality and safety required by various agencies related to public health, in view of the large amount of chemicals that has harmed the health of the population. Phytosanitary measures imposed for fresh fruit has favored the use of ionizing radiation, which has been recognized as effective method for food safety insurance and economy of many developed countries (Moraes, 2000; Marin-Huachaca et al., 2002). The objective of this research was to study the effects of

ionizing radiation on pathogenic microorganisms and the characteristics of postharvest quality of the caja (*Spondias* spp.), stored at a temperature of 23ºC during twelve days.

2. Material and methods

Caja fruit (*Spondias* spp.), taken with a complete development solution, average size of about 1.5 cm in diameter and free of injury, were placed in trays Izopor and covered with PVC film of 12 μm thickness, then irradiated at a dose of 1.0 and 2.0 kGy with a source of Cobalt-60 delivering 8.993 kGy/h (vs. zero-dose for control fruits). The fruits were stored for 12 days at 23ºC. Symptoms of leaf rust disease were observed every four days and the results expressed in percentage of fruit affected with rot. Chemical and sensory analysis were carried out at the end of storage.

2.1. Sensorial analyses

Acceptance test of acceptability with the participation of 38 panellists was conducted to evaluate the external appearance. We used a hedonic scale divided in seven levels (7 = like extremely; 6 = liked very much; 5 = like slightly; 4 = neither liked nor disliked; 3 = dislike slightly; 2 = dislike very much; 1 = disliked extremely). The samples were numbered with three-digit numeral and each evaluator received a sample treated by each dose (including control) in separate cabins and with appropriate lighting.

2.2. Physical-chemical and biochemical analyzes

Chemical analyzes were carried out in the pulp with two replicates per sample and the results expressed in the units described in the literature for each analysis.

2.2.1. Total soluble solids

They were determined by the method of refraction through the refractometer of brand Atago, model Master-T. The results were expressed in Brix' degree (°Brix), as recommended by the Institute Adolfo Lutz (IAL, 1985).

2.2.2. Total titratable acidity

It was determined by the procedure electrometric titration with NaOH 0.1 N until pH reach 8.1 (referring to the pH color change indicator phenol phtaleín), as recommended by the Institute Adolfo Lutz (IAL, 1985). The results were expressed as percentage of citric acid equivalent in the pulp.

2.2.3. Soluble solids / titrable acidity ratio

This ratio was determined by dividing the values found in soluble solids and total acidity.

2.2.4. Hydrogenic potential (pH)

PH was determined with a digital pH-meter with a glass electrode immersed in the solution containing the pulp.

2.2.5. Color of pulp

The colour index was determined with the Minolta colorimeter CR-300 model, operating system illuminant D65 and 2° standard observer.The results were expressed by the color parameters L*, a* and b* according with McGuirre (1992).

2.2.6. Amount of C vitamin

The vitamin C content was determined in the pulp after addition of 0.8% oxalic acid solution as a stabilizer. We used the methodology specified by Carvalho et al. (1990), which uses as main reagent a solution of 2.6-dichlorophenolindophenol. The results were expressed in mg of ascorbic acid per 100 g of pulp.

2.3. Delineation statistics

We used the fully case-to-case delineation, represented by a cultivar, three doses of ionizing radiation and four replicates with twenty fruits. The results were submitted to analysis of variance by F test and mean differences by Duncan test at 5% probability according to Gomes (2002).

3. Results and discussion

After four days storage, the fruits control showed that 15% of control fruits already were contaminated byrot, while the fruits irradiated at doses of 1.0 and 2.0 kGy had no symptom of decay (Figure 1A). This indicated a very positive effect on the action of ionizing radiation on control of these phytopathogenic mould that cause rot in caja, affecting its external appearance during marketing, and preventing its raw consumption. Wen et al. (2006), studying the effect of radiation on various fungi and Lycium fruit on their sensory characteristics, concluded that a dose of 14 kGy was ideal for the decontamination of fruit and for the retention of sensory quality and the extension of the shelf life.

After eight days of storage, with the fruits receiving doses of 1.0 and 2.0 kGy, it was observed 11 and 9% of fruits, respectively, with evident symptoms of decay, to be compared with control fruits exhibiting a total of 38% with advanced disease (Figure 1A). However, it was observed that the colour of rot disease contaminated control fruits remained uniform, whereas irradiated fruits showed a slight unevenness in skin color. Similar results were found by Wani et al. (2007), who work with pear (*Pyrus communis* L.) stored at a temperature of 25ºC, which reported that doses of 2.0 and 2.5 kGy could reduce the microbiological inoculum charge and the delay of fruit ripening, but the color of the fruit was affected.

At the end of the experiment after a 12-d storage period, fruits that received dose of 1.0 kGy showed 21% beginning rot contamination, and those who received the dose of 2.0 kGy showed 15% beginning rot. However, 48% of non-irradiated fruits (control) presented a very advanced rot attack (Figure 1A). The results indicate a direct effect of ionizing radiation in reducing the amount of fruits affected by phytopathogenic microorganisms and also limiting the intensity of damage they caused. This sanitation effect can be attributed to the action of radiation in preventing or slowing the reproduction of quickly dividing cells, such as in the case of bacteria, fungi and yeasts (Jessup et al., 1988). Other studies have demonstrated the effect of radiation to extend the shelf life of fruits. Baghel et al. (2006), when they worked with different doses of ionizing radiation applied to lime (*Citrus* sp.) found that small dose of 100 Gy resulted in maintaining the physical-chemical composition of the fruit up to 22 days compared to control fruits.

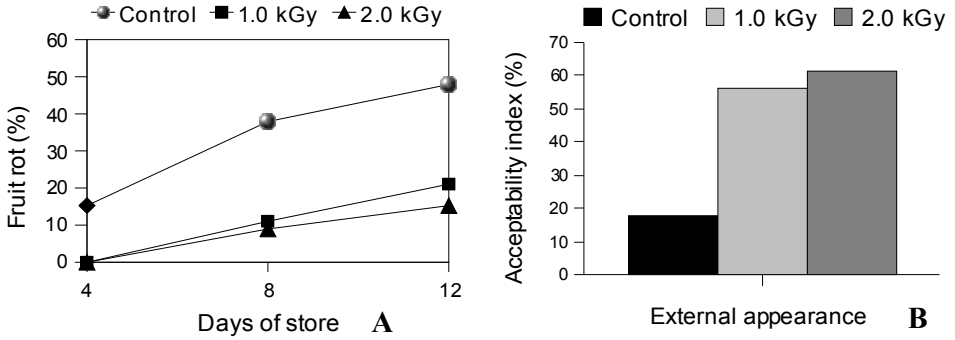

Figure 1 Percentage of fruit with rot (FIG. A) and rate of acceptance (FIG. B) of caja (*Spondias spp*), irradiated and stored for 12 days at 23ºC.

Sensory analysis of external appearance of control fruit showed a significant decrease of 68 and 71% in the acceptability over fruits that received doses of 1.0 and 2.0 kGy, respectively (Figure 1B). These levels of reduction in acceptability of non-irradiated fruits was significant in the analysis of test ordering by Newell & Mac Fairlane, when compared with irradiated fruits (Table 1).

Table 1 Statistical averages of the chemical analysis of caja (*Spondias spp*) irradiated and stored for 12 days at 23º C.

Variable	Dose (kGy)		
	Control	**1.0**	**2.0**
External appearance	1.24 b*	3.92 a	4.27 a
Total soluble solids (ºBrix)	7.85 a	8.25 ab	8.80 b
Total titra. acidity (% of ac. citric)	1.45 a	1.45 a	1.53 a
Potential of hydrogen (pH)	3.32 a	3.34 a	3.24 a
Soluble /acidity ratio	5.43 a	5.71 a	5.78a
Ascorbic Acid (mg/100g)	14.63 a	11.38 a	11.7 a
Colour of the pulp (L*)	53.42 b	47.36 a	46.27 a
Colour of the pulp (a*)	6.13 a	4.03 a	5.02 a
Colour of the pulp (b*)	30.06 b	23.28 a	23.77 a

For a given row, averages followed by the same letter do not differ statistically for the Duncans multiple range test ($P < 0.05$).

The amount of soluble solids was slightly higher in fruits irradiated with values of 8.25 and 8.80 °Brix in fruits with doses of 1 and 2 kGy, respectively, and 7.85 °Brix for control fruits (Table 1). Lira Junior et al. (2005), studying 19 genotypes of Spondias spp., found increased values of soluble solids, with a mean of 14.84 °Brix. Mata et al. (2005) found values closer to the present study who reported average value of 9.10 °Brix in the pulp of *Spondias lutea* L.

The values of total acidity showed little variation, and no significant difference between the different doses of radiation can be observed. With fruits irradiated at a dose of 2.0 kGy, it it was observed an increase in total acidity values, with an average of 1.53 g of citric acid per 100 g of fresh pulp values in agreement with the values obtained for pH, since these irradiated fruits have the lowest pH measured at 3.24 on average (Table 1). Silva et al. (1997) also found values of total acidity similar to the values reported in the present study, i.e. 1.47 g of citric acid per 100 g of fresh pulp of Spondias lutea L.

The ratio solids/acidity represents a good characteristic for quality assessment for caja fruit species (Spondias spp.), because it highlights the flavor of the pulp and may even be more representative than a separated measurement of sweetness (total solids) and acid (acidity). The ratio values ranged from 5.43 to 5.78 for control fruits and fruits that received the dose of 2.0 kGy (Table 1), respectively. These values, considered relatively low, are associated with the high value of the acidity found in fruits. Lira Junior et al. (2005) found slightly higher values, with an average of 9.05 in *Spondias* spp.

The amount of ascorbic acid is one of the properties of the fruits that is the most exposed to environment conditions changes depending on various factors external and internal to the fruit. The results found for this variable revealed that control fruits resulted in a greater amount of ascorbic acid, with a mean value of 14.63 mg per 100 g of fresh pulp, whereas irradiated fruits with 1.0 and 2.0 kGy showed average values of 11.38 and 11.70 mg per 100 g of fresh pulp, respectively. However, this reduction in ascorbic acid content after irradiation was not significant between the two studied doses (Table 1). Similar values have been found by Oliveira et al. (1999), which reported an average of 10.29 mg per 100 g of fresh pulp in their work with *Spondias lutea* L.

The color index f fruits pulp of control fruits showed the highest values for parameters L*, a*, b*, with a significant difference observed for L* and b* between control fruits and irradiated fruits (Table 1). The results for flesh color indicated a more yellow color, more red and higher luminosity for the pulp of control fruits, which may represent a greater degree of maturity in these fruits.

4. Conclusion

From the results found in this study it can be concluded that ionizing radiation has proven effective in controlling phytopathogenic microorganisms of caja fruits (Spondias spp.), without significant changes in the chemical characteristics of the fruit.

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Antifungal activity of extracts of *Ocimum gratissimum* and *Aframomum danielli* against moulds isolated from stored rice

Bankole, S.A.[1], Somorin, Y.M.*#[2],
[1] Department of Microbiology, Olabisi Onabanjo University, P.M.B 2002, Ago-Iwoye, Nigeria
[2] Department of Microbiology, University of Agriculture, P.O Box 851, Ota, Ogun State, Nigeria.
Email: yinkasomorin@yahoo.com,

* Corresponding author
# Presenting author

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Abstract

The fungitoxic effect of extracts of *Ocimum gratissimum* and *Aframomum danielli* on some moulds isolated from rice grains were determined *in vitro*. Aqueous extracts of *Aframomum danielli* inhibited the radial growth of the moulds at different levels between 46.4 - 56.7%. *Aspergillus niger* (56.7%) was the most sensitive to *Aframomum danielli* while *Cladosporium sphaerospermum* (46.4%) was the least sensitive. *Ocimum gratissimum* extract inhibited the radial growth of the moulds between 46.4 – 59.7% with *Penicillium citrinum* showing the highest sensitivity and *C. sphaerospermum* being the least sensitive. There was no significant difference ($p>0.05$) in the effect of *Ocimum gratissimum* and *Aframomum danielli* on all the moulds. *Ocimum gratissimum* showed the greater antifungal activity against the storage fungi (mean = 53.4%) compared to *Aframomum danielli* (mean = 51.9%). However, there was no significant difference ($p>0.05$) in the effect of *Ocimum gratissimum* and *Aframomum danielli* on the storage fungi. Both plant products showed varying levels of fungitoxic activities and could be potentially used in the storage of *Ofada* and *Abakaliki* rice against moulds.

Keywords: Plant extracts, Antifungal activity, Storage fungi, Moulds, Rice,

1. Introduction

Rice (*Oryza sativa* L.) is a cereal grain that meets many nutritional needs. Rice is a major source of income and nutrition in many developing countries where there is insufficient food supply (Janick, 2002). Rice is a predominant staple food in about eight African countries including Nigeria (FAO, 2004). Although the largest producer of rice in West Africa, the yield of rice in Nigeria is still very low and is reducing due to the problems of storage (WARDA, 2002). Storage of rice is done traditionally in baskets, sacks and bamboo-made structures and this exposes rice to many pests, parasites and microorganism causing the loss of a considerable proportion of stored grains. Fungi have been found to be one of the principal causes of grain loss and deterioration (Greer, 1990). The presence of moulds in stored grains may lead to various forms of deterioration (Aboaba and Amasike, 1991), decreased nutritive value (Maxiya-Dixon, 2004) and mycotoxin production (Bankole et al., 1999).

Over the years, control of pathogenic fungi in foods have drawn considerable attention with the use of industrial chemicals such as propionic acid and ammonia in the storage of grains against fungal attack (Frazier and Westhoff, 1998). These chemicals have shown to be effective in preventing fungal growth. However, when they are concentrated on the grains there could induce chemical poisoning, environmental toxicity and development of resistance by fungi to the chemical agent. Some tropical aromatic plants have shown to exert high antimicrobial activities and since they are natural products, mostly consumed by man, there is little or no fear of poisoning even at very high concentrations (Adegoke et al., 2002). Some of these plants include *Azadirachta indica* (neem) (Bankole and Adebanjo, 1995), *Cymbopogon citratus* (Bankole et al., 2005) and *Thymus vulgaris* (Nguefack et al., 2004).

Ocimum gratissimum L. has been shown to have several medicinal uses (Ojeifo and Denton, 1993). Leaves of *O. gratissimum* have been found to exhibit high antifungal activities against *Fusarium moniliforme*, *Aspergillus flavus* and *Aspergillus fumigatus* (Nguefack et al., 2004) and based on convincing *in vitro* evidence, it is said to be a potential food preservative (Tagne et al., 2000). *Aframomum danielli* has been reported to show antimicrobial activities and also to reduce aflatoxin concentration in maize (Adegoke et al., 2000a, b; Ikehorah and Okoye, 2005). It has also been used in the storage of maize and soybean (Adegoke et al., 2002). The present study assesses the fungitoxic activity

of *O. gratissimum* and *A. danielli* on some moulds isolated from stored rice grains *in vitro*. This is to serve as an indicator of the potential of these natural plant products for use in the storage of rice.

2. Materials and methods

2.1. Collection of plant materials

Seeds of *A. danielli* and leaves of *O. gratissimum* were collected from Ibadan, Nigeria. Botanists in the Department of Plant Science and Applied Zoology of the Olabisi Onabanjo University, Ago-Iwoye, Nigeria confirmed the identity of the plant materials. The plant materials were air-dried under the shade at 25-29ºC until they became dry and crispy after five days. The dried leaves of *Ocimum gratissimum* were ground using a vegetable blender and sieved to remove coarse particles. Also, the dried seeds of *A. danielli* were grounded into powder using a sterile mortar and pestle.

2.2. Extraction

The powder of both plants was extracted as follows: 250 g of the powdered leaves were put in separate round bottom flasks. One L of sterile distilled water was added into each flask, covered with aluminum foil and allowed to stand for about five days. The mixture was thoroughly shaken and filtered.

2.3. Concentration

The filtrate was then concentrated by heating over a water bath until all the moisture had evaporated. The crude extract was then obtained in a beaker. The crude extracts of both plants were kept in the refrigerator at 4ºC until it was used.

2.4. Re-propagation of fungal isolates

Fungal isolates from *Ofada* and *Abakaliki* rice preserved at the culture bank of the Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Nigeria were used for the study. A sterile needle was used to pick spores of the stock culture of each organism and inoculated on a fresh Potato Dextrose Agar (PDA) plate and incubated for seven days. Sterile cork borer was used to make discs on the culture of each isolate. The mycelial discs were then used to inoculate fresh PDA plates. The plates were incubated at 28°C for seven days. The radial growth of each isolate was recorded at 24 hours interval for the seven days period.

2.5. Bioassay

The antifungal property of the extracts on the isolates was determined using the procedure described by Tagne et al. (2000). The crude extracts were diluted with distilled water at 5:10 w/v. Five mL of each extract solution was dispensed into 10 mL molten agar and poured into sterile Petri dishes. The agar was then allowed to solidify. Sterile cork borers (6 mm diameter) were used to cut each isolate culture which was about one week old. Mycelial disc of each isolate was inoculated on separate plates. The plates were then incubated at 28°C for seven days. The radial growth of each isolate was measured at 24 h interval for the seven-day period. Two controls were set up, one with chloramphenicol and the other with PDA only. The inhibition was calculated as percentage of the difference between the radial growth of the isolate when inhibited and the radial growth when uninhibited, for each isolate. The percentage of inhibition (PI) for radial growth was calculated

$$PI = \frac{(a - b)}{a} \times 100$$

a - radial growth when uninhibited
b - radial growth when inhibited

3. Results and discussion

Both extracts of *O. gratissimum* and *A. danielli* inhibited the radial growth of the fungi on PDA after seven days of incubation at different levels. The extracts of *A. danielli* exhibited the highest activity on *Aspergillus niger* (56.7%) while the lowest activity was observed on *C. sphaerospermum* (46.4%) (Table 1). This result is similar to a study by Adegoke et al. (2000b) who reported that *A. danielli* inhibited the growth of *A. flavus*, *A. fumigatus* and some other food spoilage yeasts. Lipid extracts of the spice was also reported to reduce aflatoxin B_1 in maize even at concentration as low as 50 ppb (Ikheorah and Okoye, 2005).

Table 1 Percentage Inhibition of moulds by extract of *Aframomum danielli.*

Fungi Type	Isolates	Radial growth (mm) * a	Radial growth (mm) * b	Percentage inhibition (%)
Storage	*Aspergillus flavus*	72	38	47.2
	Aspergillus niger	83	36	56.7
	Aspergillus tamarii	80	35	56.3
	Penicillium citrinum	72	35	51.4
	Penicillium oxalicum	75	37	50.7
	Rhizopus nigricans	74	35	52.7
	Rhizopus oryzae	66	34	48.5
	Mean			51.9
Field	*Cladosporium sphaerospermum*	69	37	46.4
	Fusarium compacticum	77	35	54.5
	Fusarium oxysporum	78	38	51.3
	Fusarium proliferatum	80	35	56.3
	Mean			52.1

* - values given are mean values of triplicates; a - radial growth when uninhibited; b - radial growth when inhibited

The aqueous extracts of *O. gratissimum* showed its highest antifungal activity on *Penicillium citrinum* (59.7%) while it was least on *C. sphaerospermum* (46.4%) (Table 2). This is similar to the report by Tagne et al. (2000) that essential oils of *O. gratissimum* at 10 ppm inhibited the growth of *Fusarium moniliforme* whereas at 200 ppm, the growth was completely inhibited. In another study by Nguefack et al. (2004) essential oils of *O. gratissimum* at 800 ppm completely inhibited the growth of *F. moniliforme, A. flavus* and *A. fumigatus*.

Table 2 Percentage Inhibition of moulds by extract of *Ocimum gratissimum.*

Fungi Type	Isolates	Radial growth (mm) *a	Radial growth (mm) * b	Percentage inhibition (%)
Storage	*Aspergillus flavus*	72	36	50.0
	Aspergillus niger	83	39	56.0
	Aspergillus tamarii	80	35	56.3
	Penicillium citrinum	72	29	59.7
	Penicillium oxalicum	75	37	50.7
	Rhizopus nigricans	74	35	52.7
	Rhizopus oryzae	66	34	48.5
	Mean			53.4
Field	*Cladosporium sphaerospermum*	69	37	46.4
	Fusarium compacticum	77	35	54.5
	Fusarium oxysporum	78	38	51.3
	Fusarium proliferatum	80	35	56.3
	Mean			52.1

* - values given are mean values of triplicates; a - radial growth when uninhibited; b- radial growth when inhibited

From the result of this study, there is a great similarity in the activities of *A. danielli* and *O. gratissimum* extracts, and there was no significant difference ($p > 0.05$) in the activities of the two extracts on the radial growth of the moulds. Though the aqueous extracts inhibited the growth of the fungi, there are usually greater efficacies recorded when essential oils are used. It could hence be thought that the active ingredients causing the inhibition of growth are concentrated in the essential oil. Based on the fungitoxic activity observed in this study, *A. danielli* and *O. gratissimum* can be potentially used in the protection of rice grains against storage fungi. Although *A. danielli* has already been used successfully to preserve maize and soybean in storage (Adegoke et al., 2002), *O. gratissimum* had more inhibitory effects on the storage fungi used in this study (53.4%) compared to *A. danielli* (51.9%) thus suggesting that *O. gratissimum* could have more potential in the control of moulds in stored rice.

4. Conclusion

Natural plant products which have shown to be useful in protecting agricultural commodities against fungal infection and consequent mycotoxin production were shown to retard fungal growth in this study. If these plants are used in the storage of rice, they could reduce the loss of grains in storage and also the consumption of mycotoxin-contaminated foods especially in populations where grains constitute a major portion of the diet. The use of natural plant products in storage will also eliminate the problem of chemical poisoning that could arise from use of synthetic chemicals in the storage of grains. However, the best form of the plant product which could offer the best protection against moulds should be determined and exploited in the storage of agricultural produce.

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Can ozone fumigation effectively reduce aflatoxin B_1 and other mycotoxins contamination on stored grain?

Songxue, W.*#, Han, L., Jiayong, L., Yang, C.
Academy of State Administration of Grain No.11 Baiwangzhuang Street, Beijing, 100037, P. R. China. Email: wsx@chinagrain.org

* Corresponding author
# Presenting author

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Abstract

Mycotoxins are secondary metabolites produced by filamentous fungi that have deleterious effects on human and animal consumers. Cereals are probably the most important source of intake of mycotoxins, because they are susceptible to fungal attack either in the field or during storage and consequently contaminated by mycotoxins under adverse conditions favouring fungal invasion and growth. As a powerful oxidant, ozone has been successfully used for controlling stored-product pests, fungi, and sometime degrading mycotoxins in solution or in vitro (present as pure standards). Despite the above fact, information on the use of gaseous ozone as a mycotoxin-degradation agent for cereal grain preservation is still very limited. Also, it is not clear whether ozone fumigation can effectively degrade mycotoxins on stored grains. In the paper, we put forward wet method of ozone fumigation to degrade mycotoxin contamination on stored grains, and compared the degradation efficacy of three ozone gas treatments-dry method, wet method and aqueous method on aflatoxin B_1 and other mycotoxins in grains. Our results showed that the wet method of ozone fumigation could better degrade mycotoxin-spiked grain sample than the dry method, and aqueous method also had a good mycotoxin-degradation efficacy. To our knowledge, it is the first time to specially report the wet method of ozone fumigation to degrade mycotoxins on stored grains.

Keywords: Ozone gas, Mycotoxin degradation, Stored grain, Wet method

1. Introduction

Ozone, or triatomic oxygen (O_3), is a powerful disinfectant and oxidising agent (McKenzie et al., 1997). Since 1997, it has been considered as a GRAS (generally recognized as safe) substance and used in a number of applications in the food industry for destruction or detoxification of chemicals or micro organisms (Silva et al., 2001; Raila et al., 2006; Inan et al., 2007). Ozone gas also could effectively degrade mycotoxins in solution or in vitro (present as pure standards), such as aflatoxins, cyclopiazonic acid (CPA), fumonisin B_1, ochratoxin A (OTA), patulin, secalonic acid D (SAD) and zearalenone (ZEN) (McKenzie et al., 1997), and degradation products are generally harmless. For example, ozone would react across the 8, 9 double bond of the furan ring of aflatoxin through electrophilic attack, causing the formation of primary ozonides followed by rearrangement into monozonide derivatives such as aldehydes, ketones and organic acids (Proctor et al., 2004).

But information on the use of gaseous ozone as a mycotoxin-degradation agent for cereal grain preservation is still very limited. Application of ozone gas in stored grains to decompose the mycotoxins has been reported. Traditionally ozone gas generated by an ozone generator is directly into the spiked or contaminated grains, and then the mycotoxin content is analyzed for determining the treatment efficacy. But in practice, it was found that its efficacy of mycotoxin degradation for mycotoxin-spiked grain sample was not always satisfactory using this traditional method, and sometimes the higher doses of ozone and longer treatment duration were needed to achieve a good result (Luo jianwei et al., 2003; Proctor et al., 2004). For instance, 10 to 12 % (by weight) of ozone gas was used in the study on efficacy of ozonation to degrade aflatoxins in maize reported by Prudente and King (2002).

Our previous work showed that aflatoxin B_1, OTA, ZEN and DON in aqueous solution could be rapidly and completely degraded within a few tens of seconds using ozone gas. It may be implied that water-vaporized ozone gas (called wet ozone gas by us) also has the high ability of degrading the mycotoxins. Therefore, in this paper, we firstly divided the ozone fumigation into a dry method and a wet method, and compared the two methods of the efficacy of degrading AFB_1 in cereal grain substrates. To our

knowledge, it was the first to specially report the wet method of ozone fumigation to degrade mycotoxins on stored grains. In addition, for better understanding the ability of different ozone application ways, we also investigated the efficacy of aqueous ozone solution on degrading mycotoxins in grains.

2. Materials and methods

2.1. Grains and sample preparation

Wheat, maize and paddy rice samples were collected from Chinese state grain storage bin. The moisture level in the samples was determined in triplicate by Chinese National Standard Method (GB/T) No. 5497-85 Inspection of grain and oilseeds-Methods for determination of moisture content. All samples were cleaned to remove impurities and stored at room temperature before use.

Prior to ozonation experiments, grain samples were artificially contaminated with aflatoxin B_1 at a level of 25 $\mu g.kg^{-1}$ with wheat, 50 $\mu g.kg^{-1}$ with paddy rice and 100 $\mu g.kg^{-1}$ with maize according to 5 folds of Chinese hygienic MRLs in grains prescribed in hygienic standard for grains (GB 2715-2005). All spiked grain samples were thoroughly mixed and stored overnight before ozonation. Unspiked grain samples served as negative controls while spiked but untreated samples served as positive controls. To ensure complete recovery of aflatoxin, each sample was spiked with aflatoxins in the same container in which ozonation was carried out. After ozonation, each grain batch was homogenized entirely in 8:2 methanol / water immediately and then ground in a blend jar (Laboratory blender, 8010S, Waring Commercial®, USA) at high speed to ensure complete extraction of residual aflatoxins. This approach was used to reduce unsystematic variations typically associated with sampling of naturally contaminated grain samples and to provide a direct measure of treatment method efficiency (Proctor et al., 2004).

2.2. Ozonation experiments

2.2.1. Ozone gas generator

Ozone gas was generated from laboratory corona discharge ozone generator (Tonglin Corporation, P.R. CHINA) using pure oxygen. The pure oxygen was used because it could produce higher doses of ozone gas than air, and air could produce unfriendly substances such as NOx compounds during corona discharge process (Franco et al., 2008). In this experiment, ozone gas concentrations produced were determined by I_2 method (The industrial standard of the people's republic of China: CJ/T 3028.2-94 for the measure of ozone concentration, output, and specific energy consumption for ozone generator). At selected conditions of 30% generator power and 0.4 $L.min^{-1}$ of oxygen gas, the ozone gas concentrations produced were about 4.8 $mg.L^{-1}$.

2.2.2. Dry method

In the dry method, ozone gas was put directly into the glass reactor. Fifty grams of whole grain samples were placed in the ozone reactor, the check valve of the oxygen cylinder was opened and the flow-rate of oxygen gas was adjusted. Oxygen entered at the bottom of ozone generator and the ozone-rich stream was fed out from the top to the ozone reactor. Grains were exposed to ozone gas which concentrations were 4.8 $mg.L^{-1}$ and exposure time was set 12 h at room temperature. After these treatments, grain samples were extracted as described above.

2.2.3. Aqueous method

In the aqueous method, the reactor was added water, and grain samples were soaked in water, and ozone-rich steam was directly entered into the water where the reactions occurred between aqueous ozone and mycotoxin-spiked grains. Other setups were the same to the dry method. After these treatments, grain samples were air-dried and extracted as described above.

2.2.4. Wet method

In wet method, there was one container full of water between the ozone generator and ozone reactor, and ozone-rich steam produced was firstly put into water and then fed to the ozone reactor where water-vaporized ozone gas reacted with mycotoxin-spiked grain samples. After these treatments, grain samples were extracted as described above.

2.2.5. Aflatoxin B_1 and other mycotoxins including DON, OTA and ZON analysis

After ozone treatment, the entire grain samples of fifty grams were used for aflatoxin B_1 analysis. Each batch of treatment sample was ground using a blend jar (Waring 8010S) and extracted with 200 mL methanol – water (MeOH-H_2O) (8+2) solution containing 5 g NaCl. The combined mixture was blended for 3 min at high speed, and an aliquot of each suspension was then passed through a Whatman No. 4 paper in a porcelain filter under vacuum, collecting in a measuring cylinder. And the sequent analytical procedures were performed according to AOAC official method 2005.08. The determination of other mycotoxins- OTA, DON and ZEN in grains was also performed using HPLC methods with immunoaffinity column clear up. The analysis procedures of OTA, DON and ZEN were respectively performed according to GB/T No. 23502-2009, GB/T No. 23503-2009, and GB/T No. 23504-2009.

2.3. Quality checking

Some physical and biochemical characteristics including water content, color, fatty acid value and germination before and after dry and wet ozonation were examined according to the corresponding national standard methods. Among these, water content of wheat, paddy rice and maize was determined by Chinese National Standard Method (GB/T) reference No. 5497-85. Color and odour were checked using GB/T No. 5492-85. Fatty acidity of paddy rice was determined using GB/T No. 15684—1995. Germination test was determined using GB/T No. 5520－85.

2.4. Statistical analysis

All experiments were replicated three times. The results were evaluated using a Statistical computer program. Differences were considered significant at $P< 0.05$.

3. Results

3.1. Degradation efficacy of ozonation by three ways of ozone treatments

The degradation results of three treatment methods for paddy rice were shown in Fig. 1. Aflatoxin B_1 in paddy rice could be degraded by any method. But wet method gave the highest degradation efficacy, and at 12 h exposure time, the aflatoxin B1 content was reduced 94.4%, the residual content was 2.8 ppb, which was lower than maximum trace tolerable limits (MTLs) of aflatoxin B_1 in paddy rice, which is 10 ppb. The second was the aqueous method and its degradation ratio was 87.4%, and the dry method gave least degradation efficacy, its degradation ratio was 70.8% in the experiment. The degradation results of three treatment methods for maize are shown in Fig. 2. As shown above that the dry method only reduced aflatoxin B_1 52.4%, and aqueous method could reduce it 78.1%, and the wet method could reduce 85.0%. After wet method treatment, the residual content was 15 ppb with 12 h exposure time, and met the grain hygiene standard for maize which is 20 ppb. The degradation results of three treatment methods for wheat are shown in Fig. 3. In this experiment, the aflatoxin B_1 could be reduced more using the aqueous method which degradation level was 92.2%, and the second was the wet method, which degradation level was 85.5%. The dry method only reduced 56.8% with the same treatment conditions. After treatment using wet method and aqueous method, and the residual aflatoxin B_1 was 3.6 ppb and 1.95 ppb respectively and was also below the national grain hygiene standard which is 5 ppb.

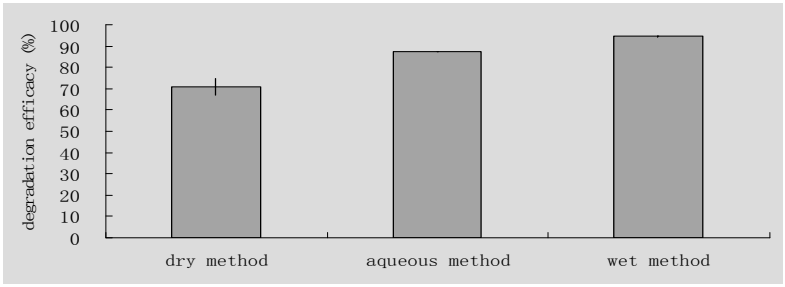

Figure 1 Degradation effect of aflatoxin B_1 in paddy rice using different ozonation methods.

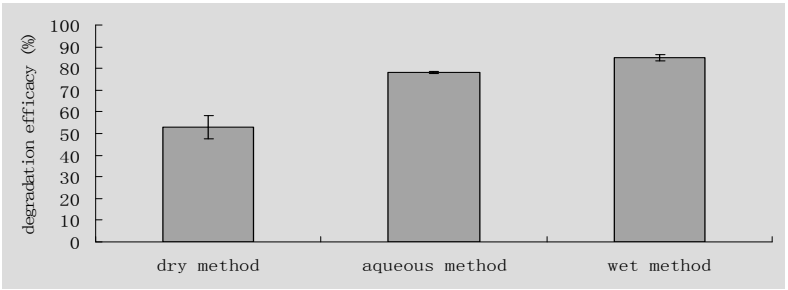

Figure 2 Degradation effect of aflatoxin B_1 in maize using different ozonation methods.

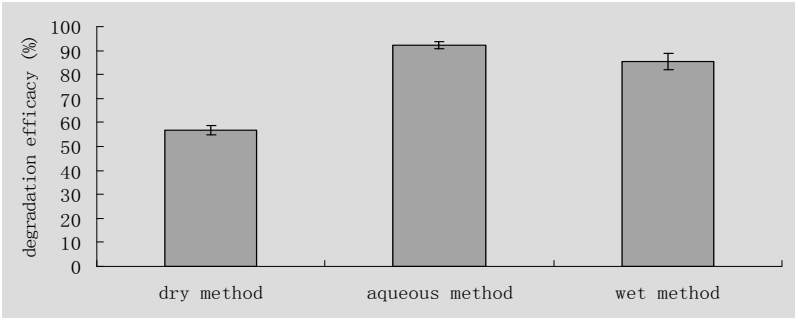

Figure 3 Degradation effect of aflatoxin B_1 in wheat using different ozonation methods.

The efficacy of different ozone treatment methods on DON, OTA and ZEN in grains was also investigated (data not shown). Similar to the results obtained from AFB_1, the wet method or aqueous method degraded mycotoxins in grains more than dry method.

3.2. Effect of dry and wet methods of ozone gas on the characteristics of cereal grains.

Table 1 showed the changes of some physical and biochemical characteristics of wheat, paddy rice and maize after dry and wet ozone treatment, and no ozone treatment grains were served as control samples. Different treatment methods had distinct effects on grain water content. Compared with control samples, water content of wheat, paddy rice and maize was reduced 1.78-2.7% after dry ozone method, and increased 0.44-1.62% after the wet method. Germination tests showed that grain germination was reduced after ozonation whatever wet or dry method. Compared with the dry method, the wet method seems to decrease more the germination capability. Few effects were observed for grain odour after wet and dry treatment method. However, the color appeared somewhat white after wet method compared with dry method and no obvious color changes were observed between grain treated by dry method and control sample.

Table 1 Some physical and biochemical characteristics of grains after dry and wet ozone treatment methods.

Treatment method	Grain type	Water content %	Germination rate %	Fatty acidity (mg KOH/100g)	color	odour
	wheat	11.80	97	/	normal	normal
No ozone treatment	Paddy rice	10.76	97	7.10	normal	normal
	corn	15.56	99	/	normal	normal
	wheat	9.90	30	/	normal	normal
Dry ozone treatment	Paddy rice	8.98	86	7.20	normal	normal
	corn	12.86	60	/	normal	normal
	wheat	12.24	34	/	somewhat white	normal
wet ozone treatment	Paddy rice	12.38	79	7.38	somewhat white	normal
	corn	16.08	38	/	somewhat white	normal

4. Discussion

As a promising treatment method, ozone gas had been used in decontamination of fungi and mycotoxin contaminated stored grain. Generally ozone gas treatment could significantly prevent fungi development and consequently reduce mycotoxin production (Raila et al., 2006). As we have showed that aflatoxin B_1 in stored grain also could be directly degraded by ozone gas. This result was in agreement with other studies. Traditional application of ozone gas, called dry method by us, was that directly feeding it into stored grain after ozone production without any pretreatment. In this experiment, aflatoxin B_1 was not effectively degraded within limited ozone concentrations and exposure time using dry method. The initial level of approximately 5 fold of aflatoxin B_1 MTLs in Chinese national grain hygiene standard in the ozone treated contaminated sample was only further reduced 50-70% after 12 h exposure, and the residual concentrations were still higher than Chinese national grain hygiene standard. This may indicated that dry method has low ability of degrading AFB_1 in stored grain with the relative low ozone concentrations and limited exposure time.

Aqueous ozone was another application way of ozonation, which was widely applied in food processing industry and water treatment (Wei Kuangji et al., 2007; Franco, et al., 2008; Suarez et al., 2007). When ozone gas as a water additive was dissolved in water, the oxidation of organic and inorganic compounds in water during ozonation could occur via ozone or OH radicals or a combination thereof (von Gunten, 2003). It was found that aqueous ozone was very effective in significantly reducing organic pollutants in water or microbial loads on live catfish entering the plant. Mycotoxins in solutions also could be greatly degraded using aqueous ozone (Young et al., 2006), therefore the decontamination efficacy of aqueous ozone on mycotoxin-contaminated grains was expected. Herein we investigated the efficacy of aqueous ozone water on degradation of mycotoxin contaminated grains, and the results showed that aqueous ozone was very effective in significantly reducing mycotoxins in grains. Generally the efficacy of aqueous ozone method was superior to the dry method and the AFB_1 in the three types of grain could all be reduced about 80% or above, especially for wheat. We believed in that aqueous ozone would provide a good solution to processing the contaminated-mycotoxin grains in the grain processing industry.

For storage environment, aqueous ozone was not suitable, but the strong ability of degrading mycotoxins by the aqueous method seemed to indicate that the oxidation ability of ozone gas may be related with water, in other words, the water or water vapour appeared to play an important role in the reaction between mycotoxin-contaminated grains and ozone. Young et al. (2006) also mentioned the role of water in ozone reaction with DON. So the water-vaporized ozone gas was investigated about its ability of degrading mycotoxins in stored grains. The water-vaporized ozone gas was produced by being bubbled through water, and the application method of ozone gas was named the wet method, which was used to differentiate with traditional method-dry method. The results above showed that wet method proved to be

an effective method. Generally the efficacy of the wet method was superior to the dry method and close to or better than the aqueous method. The possible mechanism was attributed to the reaction of water with ozone gas. When ozone gas contacted with water, the reactions might occur as below.

$$O_3+H_2O\rightarrow HO_3^{+}+OH- \quad (1)$$
$$HO_3^{+}+OH-\rightarrow 2HO_2 \quad (2)$$
$$O_3+HO_2\cdot\rightarrow\cdot OH+2O_2 \quad (3)$$
$$HO\cdot+HO_2\cdot\rightarrow H_2O+O_2 \quad (4)$$

In these reactions, the free radicals were produced such as OH which had the stronger oxidation ability than ozone gas. So the oxidation ability of ozone gas would be greatly enhanced after water-vaporization.

We also investigated the water content of grain after dry and wet method, and from the above results we could see that the water loss was significant after dry method, and after wet method, the grain water content did not also be elevated significantly as expected. Generally the water loss should be avoided during grain storage because of its direct relation with economy loss, but the higher water content would affect the grain storage safety because this would significantly increase possibility of mould development. Wet method could not lead to the water loss of grains and also did not lead to the significant increases of grain water content. Germination tests showed that ozonation significantly affected the capability of grain germination. Germination ability is very important for grains used as seed, and if a seed grain loses its ability to germinate, it has little value as a seed to be planted. Therefore ozone fumigation could only be used for the preservation of the stored grains, but could not be used as storage of seeds. Ozone fumigation, wet method or dry method, has little effect on grain odour, but has somewhat effect on grain color especially for the wet method. Other biochemical characteristics such as crude protein and RVA profile characteristics were also determined (data not shown), and detrimental effects were not observed. The effects of ozone treatment on physical and biochemical characteristics were also studied by other researchers. In general, there were no detrimental effects on grain characteristics after ozone treatments with certain treatment conditions. For example, the results observed by Mendez et al. (2003) showed that treatment of grains with 50 ppm ozone for 30 d had no detrimental effect on popping volume of popcorn, fatty acid and amino acid composition of soybean, wheat, and maize, milling characteristics of wheat and maize, baking characteristics of wheat, and stickiness of rice. Desvignes et al. (2008) also found that application of ozone treatment (10 g/kg) of common wheat before milling, the required energy at breaking stage whatever the grain hardness was significantly reduced (by 10-20%) and without changes in the flour yield.

In a word, ozone in managing stored grains has potential usefulness, and as a promising method for degradation of mycotoxins used in storage environment of grains, the wet method should be further developed in the future.

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Contribution of the light filth method to the Integrated Pest Management of a flour mill

Trematerra, P.*#, Catalano, S.
Department of Animal, Plant and Environmental Science, University of Molise, via De Sanctis, I-86100 Campobasso, Italy. E-mail address: trema@unimol.it

* Corresponding author
# Presenting author

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Abstract

An important contribution to Integrated Pest Management in stored-product protection can be provided by the light-filth method since it gives particular attention to the extraneous particles contaminating food (such as insects, insect fragments, mites, hairs, feather barbules, etc.), extending to the identification of the material from which they have originated or the animals and vegetables from which they derive or have been part of in the past. In this regard, semolina produced by an industrial mill (processing 500 t of durum wheat per day) located in South Italy was examined for light filth according to the method established by Italian regulation. During the investigations we verified the presence of insect fragments in 250 semolina samples collected from June 2008 to July 2009. Our results show that the number of insect fragments found in the samples (from 0 to 15 fragments per 50 g semolina) remained below the limit of 75 fragments per 50 g flour established by the Italian regulation. The fragments of arthropods found in the semolina samples had different origins. Numerous fragments came from both immature and adult insects infesting plants of wheat in fields (thrips and aphids); many other fragments belong to internal feeding insects and external feeding insects (*Sitophilus* spp., *Rhyzopertha dominica*, *Tribolium* spp., *Cryptolestes* spp., *Oryzaephilus* spp., and *Nemapogon granellus*) which are able to infest cereals during post-harvest processing or to colonize mill-rooms in which dusts, cereal debris, and flour residues are present. We also found fragments associable to structural pests like flies and psocids that are present in environments contaminated by mould spores and fungal hyphae. The results revealed that the fumigation of the mill realized in August 2008 did not modify the number of fragments contaminating the semolina, which remained at the same level during the 14 months of the experiment.

Keywords: Light filth, Semolina, IPM, Flour mill, Italy.

1. Introduction

In the last years, the European food industry has faced the need to give convincing answers to requests for qualitative standards of excellence in alimentary products with respect to both nutritional characteristics and hygienic-sanitary aspects. Therefore, the requirement for exclusion of arthropods from food has become a general exigency due to not only the comprehensible sense of repugnance generated by their presence but also the related health risks for people who ingest them. In fact, if any of the operators involved in the production chain starting from the fields up to the development are careless, insects and mites can proliferate, proceed to infest products ready to be packaged, and end up directly in the shops. There, if neglected, they can invade other wrappings and food and find their way inside the final consumers' houses.

To mitigate such problems, several active ingredients have been developed which are able to eliminate a large part of the pests for alimentary products, both in fields and in the subsequent phases of storage and food processing. As is known, this has involved abundant employment of insecticides, with the consequent development of pest resistance to various chemicals and furthermore serious environmental damage and risks for the health of the consumers.

With the aim of reducing the use of such substances, the concept of Integrated Pest Management (IPM) has recently found space in the food industry and spread in agriculture. IPM works as a multidisciplinary approach and introduces the concept of integration of the different methodologies to control infestations. In this way the tendency is to obtain long lasting results and certain advantages for the environment and final products. This multidisciplinary approach consists of: i/ *cognitive interventions*, through monitoring, identification of the infesting pests, and verification of the obtained results; ii/ *preventive interventions*, with the purpose of eliminating the favourable conditions for the development of the

infesting pests; iii/ *corrective interventions*, through methods of direct attack and strengthening of preventive interventions (Trematerra and Gentile, 2008).

In such a context, an important contribution to the integrated management of the infestations can be provided by the light-filth method, as *cognitive intervention* in the IPM, since it pays particular attention to the extraneous particles contaminating food, extending to the identification of the material by which they have been originated or the animals and vegetables from which they derive or they have been part of in the past (Italian G.U., 1999). Much information to this matter can be found in Brader et al., 2002; Perez-Mendoza et al., 2005; Atui et al., 2006; Stejskal and Hubert, 2006; Neethirajan et al., 2007; Toews et al., 2007; Hubert et al., 2009; Trematerra and Catalano, 2009.

The main purpose of the present work was to evaluate the hygienic-sanitary quality of the semolina flour obtained by an industrial mill during one year, through isolation and identification of the extraneous particles contained in it, with particular attention to the fragments of insects.

2. Materials and methods

The observations were carried out in an industrial semolina-mill located in the Apulia region, South Italy. The mill was a large building of 18000 m^3, with seven floors processing 500 t of durum wheat, *Triticum durum* Desfontaines each day. The sampling was done for 14 consecutive months, from June 2008 to July 2009, focusing on four storage silos in the structure (identified by the numbers 23, 24, 27, and 28). Semolina to be analysed was collected with weekly frequency or, in any case, each time when one of the four monitored silos was filled with new semolina. In August, because of a fumigation treatment which involved the structure during the second week of the month, the operation of the plant stopped for about 10 d.

The analysed semolina was obtained from national and international (coming from different continents) grinding wheats, taken individually or as a mixture. Solid impurities were isolated, identified and analysed using the official light-filth method as given in the Regulation of the Italian Policy Agricultural Office, 12 January 1999, "Official Methods of Cereals Analysis – Supplement No. 5 'Determination of solid impurities in flour and transformed products' and 'Identification of substances of biological origin and mineral extraneous substances in cereal flours'".

For each sample, a quantity of semolina varying from 1.5 to 2.5 kg was collected from which a portion weighing at least 600 g was obtained using the standard sample division method in force, and homogenized inside their containers using a spatula. From these samples, 50 g of semolina was collected at different points, weighed for analysis, and introduced into a flask through a glass funnel used for dust handling.

Following the official methodology, the sample analysed was submitted to acetic-nitric digestion until ebullition, the impurities separated by flotation inside specific proportions of alcohol and gasoline in a Wildman trap flask, before being collected on a paper filter through deep-bed filtration with a Buchner funnel. The material gathered on the filter was observed through the microscope using lower magnification. In many cases, impurities were pulled out and placed on a slide in Faure's inclusion liquid and observed by the compound microscope for identification. According to the regulation, isolated and identified fragments were classified into different categories: whole insect (adult and/or larva); cephalic capsule of insects; fragment of arthropods; moth scale; hair of mammals (rodents, man, other); textile fibres; other fragments (metal, plastics, glass, combustibles).

3. Results

According to the results obtained during our analyses, fragments of insects identified through the light-filth method had different origins. Numerous fragments come from both immature and adult insects infesting wheat from the field. They belonged to phytophagous insects active on plants or on the ears of wheat (adult body or leg of aphids; and head, leg, and distal abdominal portions of immature and adult Thysanoptera). Many other fragments belong to specimens infesting post-harvest cereals (mainly head, mandible, and cuticular fragments of larvae; elytra and leg of adults; also whole larvae or adults). There were also internal-feeding insects (mainly *Sitophilus* spp. (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), which are active on grains during storage but in some circumstances are able to begin their infestation in the field.

Represented in smaller quantities were external-feeding insects (for example: fragments of cuticle, leg, mandible, and also adults and larvae of coleopteran *Tribolium* spp. (Tenebrionidae), *Cryptolestes* spp. (Laemophloeidae), *Oryzaephilus* spp. (Silvanidae), and a lepidopteran *Nemapogon granella* (L.) (Gelechiidae), which are able to colonize stored cereals, mainly the machineries of mills in which dusts, cereal debris, and flour residues accumulate. Finally, among the observed semolina samples there were also fragments associable to environmental pests like flies (Diptera) and structural pests such as psocids (Psocoptera) that can proliferate in humid environments that facilitate fungus mycelium growth.

During our investigations, a total of 250 semolina samples were examined using the light-filth method (Figure 1). Different categories of solid impurities were found: i/ many of synthetic origin; ii/ some impurities of vegetable origin, related to the raw material, and iii/ some insect fragments. In this last category we found 599 fragments belonging to insects of different families. More specifically: i/ 152 insect fragments from 63 samples from semolina silo No. 23; ii/ 154 insect fragments from 63 samples from semolina silo No. 24; iii/ 157 fragments from 63 samples from silo No. 27; iv/ 136 insect fragments from 61 samples analysed from silo No. 28 (Table 1). Various semolina samples occasionally contained natural textile fibres, crystalline particles, and burned particles, but rodent hair was never found.

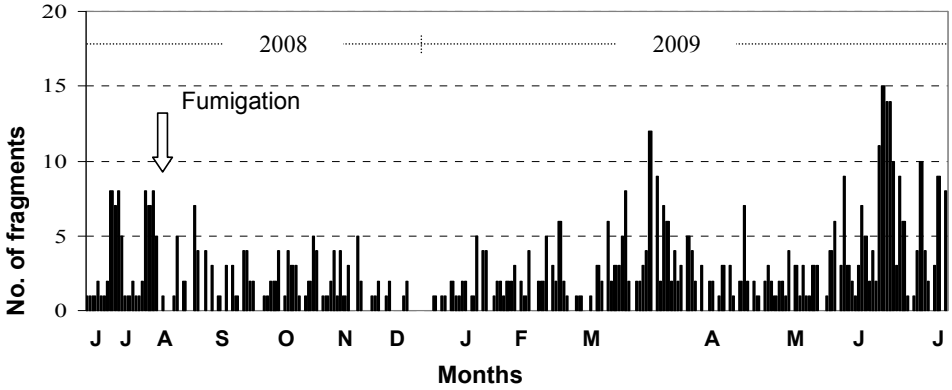

Figure 1 Total number of insect fragments recovered in semolina samples during 2008 and 2009.

Table 1 Insect fragments recovered in semolina samples.

Insect fragments	Silo 23	Silo 24	Silo 27	Silo 28	Total
Abdomen	8	9	5	5	27
Adult cuticular fragments	28	19	24	15	86
Antennas	0	4	6	9	19
Cephalic capsulae	6	6	2	3	17
Larvae cuticular fragments	8	7	8	6	29
Legs	25	30	30	32	117
Mandibles	42	50	51	43	186
Pronotum	4	2	2	1	9
Rostrum	1	1	0	1	3
Sternum	2	0	0	1	3
Torax	2	1	3	1	7
Moth scales	2	1	2	2	7
Unidentified fragments	9	6	7	5	27
Whole adults	5	4	5	3	17
Whole larvae	10	14	12	9	45
Total	152	154	157	136	599

Figure 2 shows the number of insect fragments found in each semolina sample, separated for each silo considered. Insect fragments were found in 142 samples (56.8% of cases) of the inspected semolina. Altogether, the number of insect fragments found in the 250 samples ranged from 0 to 15 fragments per 50 g semolina. This was below the average defect action level of 75 fragments per 50 g flour established by the Italian regulation (Italian G.U., 1999).

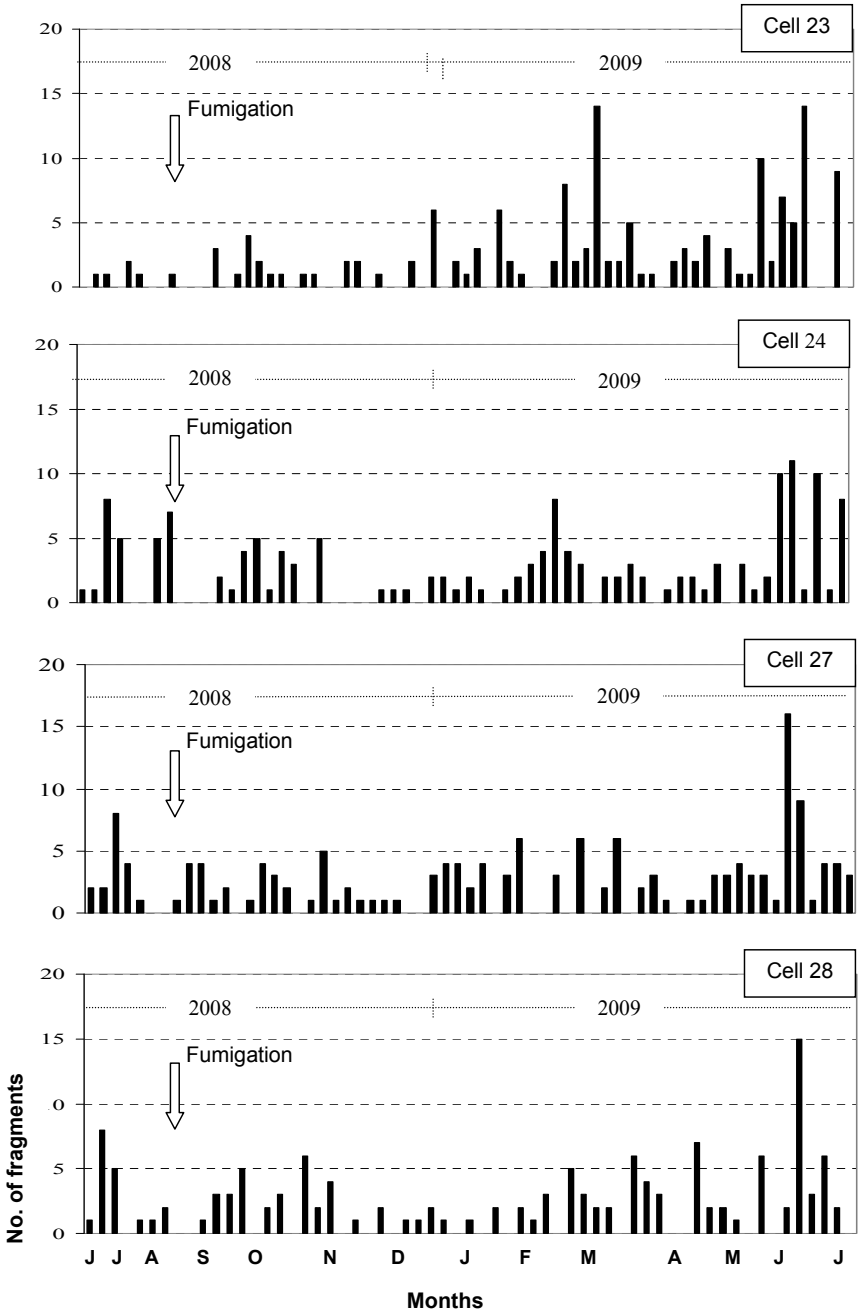

Figure 2 Number of insect fragments recovered in semolina samples separated for each silo considered.

4. Discussion

Our findings obtained during the present light-filth survey work are different from those observed using a monitoring programme based on insect food-trap capture that studied the spatial distribution of insects on the different floors of the same mill (Trematerra et al., 2007). In fact, on that occasion *Tribolium confusum* Jaquelin du Val followed by *Typhaea stercorea* (L.) (Coleoptera: Mycetophagidae) and *Tribolium castaneum* (Herbst) were the most recurrent species found, while *Sitophilus oryzae* (L.) and *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) were found occasionally; moths were almost entirely absent.

In this regard, it must be remembered that the light-filth method and monitoring realized with traps have different purposes and are used at different points of the grain chain. In fact, in this case the former allows only showed the presence of impurities to be found in the semolina, and therefore, the control that is performed indirectly involves both the stored cereals to be transformed and the milling equipments, including the silos used for semolina storage. On the other hand, traditional monitoring with an insect trap network essentially checks the presence of insects that are in the mill environment and outside the machineries.

Nevertheless, the two different results obtained by monitoring and by the light-filth method point out a diversified and complex situation that suggests a multidisciplinary approach in the control of the infestations in closed circuit of industrial mills, with a need for their integration.

As is known, insect-pest management in Italian and southern European mills is generally founded on structural fumigation with annual or half-yearly frequency and on numerous treatments with contact insecticides achieved especially in the summer period. The monitoring is normally carried out because it is considered useful to get information on the presence of the insect pests. The monitoring data obtained are not often used as support to individualize the actions to be taken to control risky situations before they become real emergencies but are read only as a measurement of the problems to be solved by periodical treatments. Furthermore, the correct importance is not always given to preventive measures against the proliferation of pests (Trematerra and Gentile, 2008).

In our case, the general fumigation of the mill was done on the second week of August, using sulfuryl fluoride with 62 h of exposure. Because of this fumigation treatment, the mill suspended its activities for about 10 d, the period considered necessary for the toxic effects of the gas to decay.

Despite this, observing the temporal distribution of the fragments in the 250 analysed samples, it was underlined that the hygienic and sanitary quality of semolina produced by the mill was nearly unchanged despite the different origins of the cereals processed. This points out that the structural fumigation of the plant did not have positive effects on semolina contamination, considering that although the number of fragments found (and their typology) decreased for several weeks after a fumigation treatment, the number remained almost unchanged from June 2008 to July 2009 (Figure 1).

From a managerial point of view, the qualitative and quantitative indications obtained from the results of the light-filth method put into discussion both the utility of the structural fumigation and the opportunity to effect the fumigation treatment in August, not only due to the negative economic aspects and direct costs of the treatment, but also due to the indirect costs related to the days when the mill did not operate.

Traditionally, the people responsible for mills remedied the missed production time by carrying out the structural fumigation when the mill operations were already stopped, during the summer holidays of the personnel.

In the light of our results, the expenses of the fumigation could be optimized better in the logic of IPM, for instance, by improving the cleaning procedures and carrying out localized insecticide treatments in only the areas affected by infestation, or by adopting alternative technical measures to chemical biocides (Schöller et al., 1997; Mourier and Poulsen, 2000; Fields and White, 2002; Trematerra and Gentile, 2008).

In cereal flour, insects and their fragments originated from primary infestation of the grain before milling and/or from secondary infestation during the storage of flour and flour products on the food processing line. Field pest fragments can be removed from grain by cleaning, immature stages and pre-emergent adults of internal grain-feeding insects may not be removed by cleaning before milling. As a result, these

stages are one of the main source of insect fragments in wheat flour. In this regard, several methods have been developed to detect hidden insects in whole kernels. Infestation of grains may be detected by staining of kernels to identify entrance holes for eggs, floatation, radiographic techniques, acoustic techniques, uric-acid measurement, nuclear magnetic resonance imaging and immunoassays (Neethirajan et al., 2007).

Incoming wheat in commercial facilities can be cleaned with entoleters, scalpers, and fluidized bed aspiration before milling. Wheat thrown by centrifugal force inside an entoleter during the cleaning process would likely break hollow kernels, such as those housing large larvae. Broken kernels and newly exposed insects resulting from entoleters would be easily separated from sound kernels. Therefore, no insect fragments from these sources would be evident in the final mill-stream. Unfortunately, not all mills use this type of cleaning equipment. Relative to the abundance of species infesting the mill-rooms (secondary infestation), a possible fumigation treatment should be decided upon monitoring various aspects within the mill to safeguard the health of personnel and operators, and to preserve the quality of finished products. Furthermore, monitoring plans should be implemented opportunely not only to check for presence of infesting animals, but also to furnish valid help with respect to population abundance and spatial-temporal distribution. This information is of extreme importance when it is necessary to decide where and how manage infestations, with the purpose of avoiding periodical chemical treatments, reducing the area and frequency of treatments, and implementing prevention methodologies or alternative methods to the application of insecticides (Athanassiou et al., 2005; Trematerra et al., 2007). In this regard, the critical points of access to the mill, the areas with accumulation of food debris, and the micro-climatic conditions favourable to the development of the infesting pests should be identified.

For a good IPM approach, in situations similar to the ones investigated here, it is necessary to adopt a permanent monitoring activity which will be useful for a meaningful reduction in the use of chemicals. These will be directed localized treatments in areas of higher pest density based on accurate cleaning of the transformation departments, and above all, on a careful choice and inspection of the cereals on their arrival and during the storage period in silos.

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Section: Non-Chemical Control

Biological control of stored-product insects in commodities, food processing facilities and museums

Schöller, M.*#
Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Section Stored Product Protection; Königin-Luise-Str. 19, D-14195 Berlin.
Email: Matthias.Schoeller@jki.bund.de

* Corresponding author
# Presenting author

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Abstract

Non-chemical control methods have gained importance in integrated pest management, as policies aiming to minimize the application of residual chemical insecticides are being adopted by many companies, and a growing market of organic produce. The associations of organic farming have established self-restrictions concerning chemical control. Examples are given of how organically producing farms and processing companies function without synthetic chemical pesticides. Both non-chemical control methods for complete disinfestations and for suppression of re-infestation or residual infestations are needed. For complete disinfestations, heat treatment of buildings is now more widely used. Data on heat-tolerance of stored-product pests and an example for a heat treatment of a mill will be given. For high-value products such as spices, tea or medical plants, deep freezing is applied. Temperature data are needed to apply product-specific freezing conditions to obtain complete control at the core of the bulk. An integrated management strategy is needed to keep products free from infestation following disinfestations, along the whole chain from the storage of raw products to the consumer. Biological control is a part of that strategy. A new branch of the biological control industry is developing in Europe. Natural enemies for stored-product pests are now produced in The Netherlands, Germany and Switzerland. Traditional pest control companies are using insect parasites and predators more and more to control stored-product insects, indicating an adoption of biological control. Homeowners throughout Europe are purchasing online *Trichogramma* sp. to control moth pests. Biological control is especially attractive to processing facilities that are not willing to stop production for pest control operations. Small farms with bulk grain stores that are not gas-tight apply parasitoids as well. Recommendations for the application of natural enemies are presented for these examples. Finally, recent developments on natural enemies both for stored-product pests, e.g. flour beetles, and museum and wood boring pests are presented.

Keywords: Stored-product Pests, Material Destroying pests, Temperature modification, Biological control

1. Introduction

Non-chemical control methods have gained importance in integrated pest management, as policies aiming to minimize the application of residual chemical insecticides are being adopted by many companies, and a growing market of organic produce. Governmental and international regulations, and loss of synthetic insecticides such as methyl bromide (Fields and White, 2002) and greater restrictions on the use of dichlorvos, have left few alternatives for even non-organic food processors. In this review, non-chemical control strategies are reviewed focusing on biological control.

2. Non-chemical disinfestations

In many cases, the control of stored-product pests requires the control of large numbers of pest individuals hidden in large amounts of product or structurally complex buildings. For complete disinfestations, temperature modification is the method of choice. Heat treatment of buildings is becoming more widely used in North America and Europe (Fields, 1992; Burks et al., 2000; Dosland et al., 2006; Adler, 2007). Typically rooms are heated and the temperature is kept constantly at about 50 to 60°C for 24 h. Facilities up to 100,000 m^3 are treated (Hofmeir, 2002). For high-value products like

spices, tea and medicinal plants, deep freezing is applied. Product-specific temperature data is needed for the freezing conditions to obtain complete control at the core of the bulk (Burks et al., 2000). For bulk grain, aeration is a basic component of non-chemical pest control (Reed and Arthur, 2000). Additional options for the processing industry are vacuum (Calderon et al., 1966; Mbata et al., 2004), steam and vacuum (Prozell and Schöller, 2004), impact (Plarre and Reichmuth, 2000), and last but not least, sanitation and exclusion (Imholte and Imholte-Tauscher, 1999; Mullen and Pederson, 2000).

3. Biological control

Several reviews have been published on the use of pathogens, and insect parasitoids and predators to control stored-product insect pests (Arbogast 1984 a,b; Haines, 1984; Brower, 1990; Nilakhe and Parker, 1990; Brower, 1991; Burkholder, 1981; Burkholder and Faustini 1991; Brower et al., 1996; Schöller et al., 1997; Schöller, 1998; Schöller and Flinn, 2000; Schöller and Prozell, 2006; Schöller et al., 2006). In this contribution, some results are summarized that were published since the last review, especially for fields that were identified as major challenges for stored-product biological control; control of *Tribolium* spp. in food processing facilities and material destroying pests.

3.1. Parasitoid biology

A number of films on the biology of parasitoids attacking stored-product pests are now available online (http://www.entofilm.com), namely *Habrobracon hebetor* (Say) vs. *Ephestia kuehniella* Zeller, *Cephalonomia tarsalis* (Ashmead) vs. *Oryzaephilus surinamensis* (L.), *Lariophagus distinguendus* (Förster) vs. *Sitophilus granarius* (L.) and *Trichogramma brassicae* Bezdenko vs. moth eggs. These films revealed interesting insights into the behaviour of the parasitoids, for example it was shown that *H. hebetor* builds a short feeding-tube when feeding on the body content of the host larva (Fig. 1).

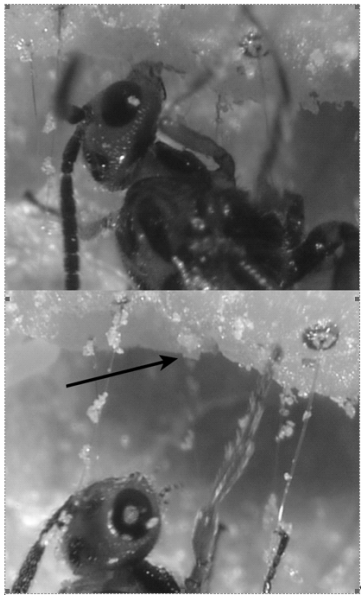

Figure 1 *Habrobracon hebetor* host feeding tube (Wyss et al., 2007).

3.2. Long-term control with natural enemies

A number of retail shops, wholesale stores, bakeries, mills and food processing companies have used parasitoids against stored-product moths continuously for 10 to 15 years in Germany. Even so monitoring for moths was documented with the help of pheromone traps, it is not easy to obtain comparable data rows for such long periods. Larger facilities are generally more suitable for data collection, because more traps are used and bias due to invasion of pests from outside as well as the impact of few infested packages is generally less pronounced. However, mills, bakeries and food processing plants typically undergo many changes in their production units, machinery or even floor

plans, resulting in the need to adapt the monitoring plan and making it difficult to compare from one year to the next. There are few studies in facilities that have remained unchanged, and these offer a unique opportunity to study the population dynamics. An example is a bakery where *Trichogramma evanescens* Westwood was released at a rate of 25,000/week and *H. hebetor* at a rate of 100/month (Schöller et al., 2006). The number of *E. kuehniella* caught in pheromone baited funnel traps decreased over time and was reduced below 15 moths/trap-month during the last four years (Fig. 2). This example shows that it is possible to manage stored-product moths in a bakery without synthetic chemical insecticides.

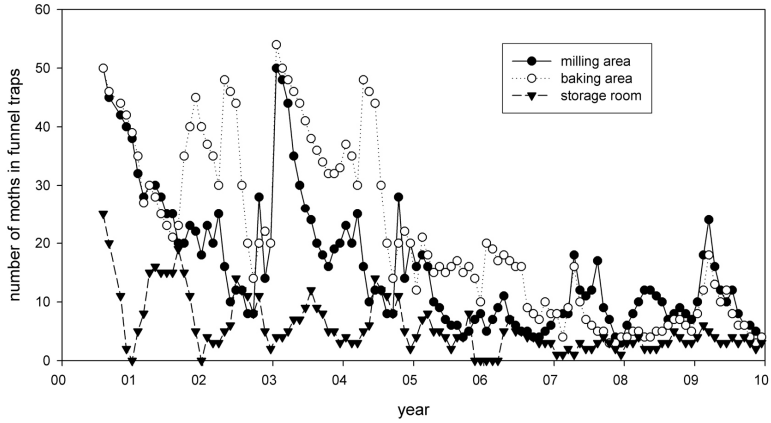

Figure 2 Number of moths in funnel traps in three areas in a bakery in Rhineland-Palatinate, Germany, from 2000 to 2010 (Schöller et al., 2006).

3.3. Future of biological control in stored-products revisited

The evaluation of biological control of stored-product pests in Europe was supported by a five-year COST (European Cooperation in Science and Technology) project. This project allowed a group of researchers to meet on a regular basis and also funded research projects (Hansen and Wakefield, 2007). Proceedings from the meetings are available at http://cost842.csl.gov.uk. A final resolution (Hansen, 2007) was prepared and signed by twenty-six researchers from sixteen European countries that identified the following situations where biological control hold most promise:

1. Empty room treatment using predatory mites, parasitic wasps and entomopathogenic fungi against stored-product mites, beetles and moths,
2. Preventative treatment of bulk commodities, in particular grain, using parasitic wasps and predatory mites and,
3. Preventative application of egg-parasitoids of *Trichogramma* spp. to protect packaged products from infestation by moths.

In the last extensive review of biological control of stored-product pests (Schöller et al., 2006), a number of challenges were listed in the paragraph “future of biological control in stored products”. The following two paragraphs will address recent developments concerning these issues.

3.3.1. Release guidelines for natural enemies

The development of effective release guidelines for natural enemies has been an important issue in biological control. Because the same species of pest may be found among a wide variety of storage systems, this area of research is especially important for management of stored-product pests. The number of beneficial insects to be release and correct timing with host phenology is an area that requires additional study. Both host and natural enemy phenologies need to be studied under a variety of environmental conditions in order to optimise the timing of release. Biological control of stored-product moths was the first area of commercial application of parasitoids. Both egg and larval parasitoids have been used. *Habrobracon hebetor* is a gregarious ectoparasitoid of Pyralid moth larvae. Female *H. hebetor* parasitises the larvae of several species of stored-product moths, including Indianmeal moth

Plodia interpunctella (Hübner), Mediterranean flour moth *E. kuehniella*, warehouse moth *Ephestia elutella* (Hübner), and the tropical warehouse moth *Cadra cautella* Walker. *Trichogramma* spp. are small endoparasitoids of lepidopteran eggs. The major advantage of *Trichogramma* species is their extremely small size; with adult egg parasitoids measuring only 0.3 mm in length, making them virtually invisible to the casual observer. *Trichogramma* spp. lay their eggs in lepidopteran eggs, killing the developing moth embryo prior to hatching, and therefore preventing the damaging larval stage. The parasitoid larva consumes the contents of the moth egg, pupates, and emerges as an adult wasp in 7 to 14 d (Grieshop et al., 2007). *Trichogramma* spp. are usually released as pupae glued to egg cards at the rate of at least 500 females per card, and one card per linear meter of shelving. Higher release rates may be needed for situations where shelving is more than 2 m in height. Because *Trichogramma* usually does not become established, release units should be applied in a way that guarantees continuous presence of the parasitoids. To facilitate this, commercial insectaries offer release cards containing overlapping cohorts of insects resulting in a staggered emergence of *Trichogramma* spp. parasitoids over a 2-wk period, a 3-wk period, or even a 4-wk period when released from a new release card developed in 2009. Figure 3 shows the number of releases per year for these systems. It is advantageous because store-keepers or pest control operators spend less time for changing the release units. Moreover, in many cases pest control operators visit stores monthly for pest monitoring and rodent control, so the new release unit fits to this schedule . Moreover, Figure 3 shows the recommended monitoring for the timing of the releases. Pyralid moths are monitored with the help of pheromone-baited traps. In temperate regions, the Indianmeal moth enters diapause, usually from November to April, consequently no *Trichogramma* should be released during this period. During the period of diapause, the larval parasitoid *H. hebetor* can be released to obtain a further reduction of the moth population.

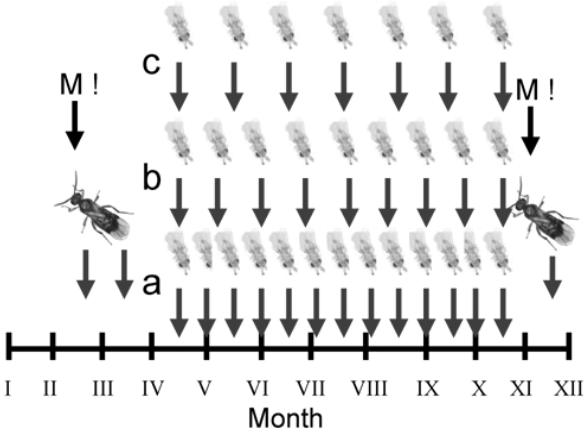

Figure 3 Management strategies for biological control of diapausing stored-product pyralid moths in temperate regions; large wasp = *H. hebetor*, small wasp = *Trichogramma evanescens,* a = *T. evanescens*, 2-week release unit (RU), b = 3-week RU, c = 4 week RU; "M !" indicates monitoring-action needed to time schematic release of parasitoids.

Discussion is still going on whether *L. distinguendus* or *Anisopteromalus calandrae* (Howard) is more suitable for control of *Sitophilus* spp. in bulk grain. For temperate regions, *L. distinguendus* was thought to be more effective due to its comparatively greater tolerance towards cooler temperatures, and the penetration into the grain column was shown to be very effective (Steidle and Schöller, 2001). Hansen and Steenberg (2007) found greater suppression of *S. granarius* in units with 9 kg wheat and *L. distinguendus* compared to units treated with *A. calandrae* at 20°C. Recently Niedermayer and Steidle (2007) suggested that *A. calandrae* should be more effective in summer, due to its shorter generation time. Moreover, *Theocolax elegans* (Westwood) was released against *S. granarius* in Central Europe (Schöller and Prozell, 2007). More field studies are needed to find the optimum release strategy for pteromalid wasps against weevils in bulk grain.

Release guidelines have been developed for other storage situations (Schöller and Prozell, 2006), but much more research, especially field studies are necessary to explore the potential of natural enemies for biological control of stored-product pests. Moreover, there are no official guidelines for the application of natural enemies to durable stored products, although the first studies in this field date back to the 1920's.

3.3.2. Progress in promising fields of application: Tribolium spp.

The discovery and development of natural enemies for the flour beetles *Tribolium castaneum* (Herbst) and *Tribolium confusum* Jaquelin du Val has been identified as a major challenge for stored-product biological control (Schöller et al., 2006). This is of particular interest for flour mills that have recently lost the use of methyl bromide, their major tool for control of this insect. Future biological control of flour beetles was thought to depend on foreign and domestic exploration for new species of natural enemies as well as trying to find effective rearing methods for the parasitoid wasp *Holepyris silvanidis* (Brèthes, 1913). In fact, an improved rearing method for *H. silvanidis* was found. Currently two approaches are followed, the release of *H. silvanidis*, and the application of the pirate warehouse bug *Xylocoris flavipes* (Reuter). The bethylid wasp *H. silvanidis* is an ectoparasitoid of larvae of *Tribolium confusum* Jacquelin du Val 1868, eventually it is even monophagous on this beetle. Some life-history data on the biology of *H. silvanidis* is available. Recently Frielitz (2007) found that female *H. silvanidis* are comparatively long-lived if provided with honey as a food source (Fig. 4). Even if no hosts are present (or found), at 25-26°C females live 51 d. Other parasitoids of stored-product pests typically live for 4 to 14 d at this temperature (Eliopoulos, 2007). Extended longevity is a promising trait because foraging is not time-limited in situations of low pest densities, e.g. after a heat treatment. The honey can easily be provided within the release unit. There is little information on host-finding behaviour, and this information is crucial for successful application for biological control. Lorenz et al. (2010) showed that female *H. sylvanidis* attacked *T. confusum* larvae down to 4 cm depth in fine grist (main particle size: < 0.2 mm) and down to at least 8 cm depth in coarse grist (main particle size: 1.4 - 3mm).

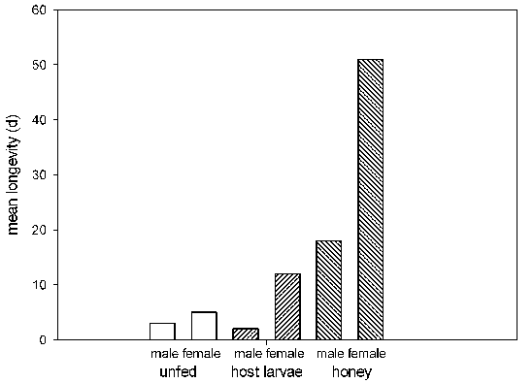

Figure 4 Longevity of *Holepyris silvanidis* at different nutritional conditions at 25-26°C and 55 ± 2.5 % r.h. (from Frielitz, 2007).

The pirate warehouse bug *X. flavipes* is a polyphagous predator of eggs and early developmental stages of many stored-product pests. *Xylocoris flavipes* can suppress *T. confusum* in presence of a thin flour layer or small amounts of flour (Schöller and Prozell, 2010), a result that was not expected because LeCato (1974) found no suppression in bulk wheat flour on *T. confusum,* even though the beetles were completely controlled in rolled oats and whole wheat, and partially in cracked maize. In bakeries and mills, flour is not only present in bulk in silos or in bags, but thin flour layers can be found in processing areas and flour residues accumulate in cracks or little heaps. In such places, *T. confusum* can frequently be found and would be the target of foraging *X. flavipes*.

4. Biological control of museum and structural pests

Stored-product pests may destroy materials as well, either on their way to pupation sites or because the materials contain ingredients suitable for development. In Halle / Saale, Saxony-Anhalt, Germany, a historic library became infested by *Stegobium paniceum* (L.). The beetles were thriving both below the

floorboard on wheat straw used as insulation, and in book covers. The books originated from the 16th to 18th century, when the book covers were filled with pulp made from linen scraps. *Stegobium paniceum* developed in the pulp, produced the characteristic exit holes and therewith destroyed irreplaceable cultural heritage (Fig. 5). The books were moved to a fumigation-chamber and treated with nitrogen. However, some re-infestation was detected after the books were moved back to the library, presumably originating from the floorboard. The parasitoid *L. distinguendus* was released on the shelves, 2000 in October and 2000 in June. The release was evaluated to have successfully suppressed the re-infestation of the library. Another trial on host-finding in boxes containing books was carried out in an Israeli library, where *L. distinguendus* was shown to find host larvae both between and inside infested books (Wilamowski et. al., 2008).

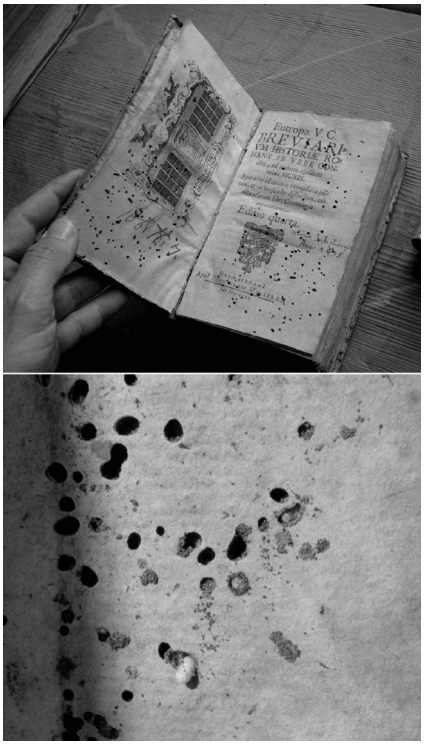

Figure 5 Book from the 17th Century infested by the drugstore beetle *Stegobium paniceum* (top), detail with larvae (bottom).

Lariophagus distinguendus has also been released to control the hump beetle *Gibbium psylloides* (Czenpinski) and the golden spider beetle *Niptus hololeucus* (Faldermann) in historic buildings in Germany. These beetles infest the fillings made by plant material and mud. While the larvae develop hidden with in the walls and in dead floors, the beetles appear in the rooms, sometimes in large numbers. Field applications during the last three years were promising and will be continued (Kassel, 2008).

A number of laboratory and field studies addressed the biological control of the common furniture beetle *Anobium punctatum* (L.). The attempt to control *A. punctatum* with *L. distinguendus* failed: even though the larvae of *A. punctatum* were physiologically suitable as hosts for *L. distinguendus*, the parasitoids they were not able to reach the larvae within wood and/or had not enough space within the galleries for parasitisation (Steidle et al., 2007).

Early detection of material destroying pests is essential to prevent damage, especially when irreplaceable objects of cultural heritage are concerned. However, monitoring of these pest species is often difficult. For example, pheromone traps for detection of *A. punctatum* resulted in very poor trap catches in field trials in Germany. During the course of study of natural enemies, it turned out that some natural enemies are more easily detectable and give indirect evidence of the presence of the pest. Paul et al. (2007)

studied *A. punctatum* and its natural enemies in a church closed for restoration in Erfurt, Germany. Yellow dish traps, a monitoring technique used in outdoor ecological field studies was used here in the context of protection of museum artefacts and wood. Yellow dishes are filled with water and a bit of detergence in order to attract flower-visiting insects (Mühlenberg, 1989). These traps are especially attractive for parasitoids that do no host-feeding and rely on nectar for adult nutrition. Other arthropods are trapped by chance, the number caught in the trap is affected by the number of insects present and temperature. Table 1 shows the number of *A. punctatum* and three natural enemies trapped in yellow dish traps. Few *A. punctatum* were trapped, mostly between mid of June and mid of July. The braconid parasitoid *Spathius exarator* (L.) was trapped throughout the trapping season from mid of May to mid of July in relatively large numbers, the peak coinciding with that of *A. punctatum*. The presence of *A. punctatum* could therefore be proven before adult beetles became active. The bethylid parasitoid *Cephalonomia gallicola* Ashmead and the beetle predator *Korynetes caeruleus* (DeGeer) were trapped before *A. punctatum*, too, but numbers were too low to make them promising candidates for indirect monitoring for *A. punctatum*.

Table 1 *Anobium punctatum*, common furniture beetle and its parasites and predotors trapped in yellow dish traps in the Allerheiligen church in Erfurt, Germany, 2006.

Species	**Insects trapped**						
	Date of collection						
	May 19	**June 2**	**June 16**	**June 23**	**June 30**	**July 7**	**July 14**
Anobium punctatum	0	1	0	5	10	4	1
Spathius exarator	5	10	9	51	87	31	25
Korynetes caeruleus	4	0	4	0	1	0	0
Cephalonomia gallicola	2	4	3	2	1	1	1

A monitoring method currently used for *A. punctatum* is the count of frass piles composed of faecal pellets and fine wood fragments. The absence of new frass piles after injection of insecticides into the galleries of *A. punctatum* was previously thought to be an indicator of effective control of *A. punctatum*. However, Paul et al. (2007) showed that the frass piles are caused by the beetle predator *K. caeruleus* foraging for *A. punctatum*-larvae. The injection of chemical insecticides into gallery of *A. punctatum* consequently negatively affects the predator *K. caeruleus* and stops its digging activity, but did not control *A. punctatum* (Paul et al., 2007). In Germany, the predator was shown to be active all year round except for the coldest period around February in an unheated church (Fig. 6). Unfortunately, rearing of the predator for biological control seems to be too difficult due to the extended larval period and larval mortality at high temperatures (Haustein et al., 2010).

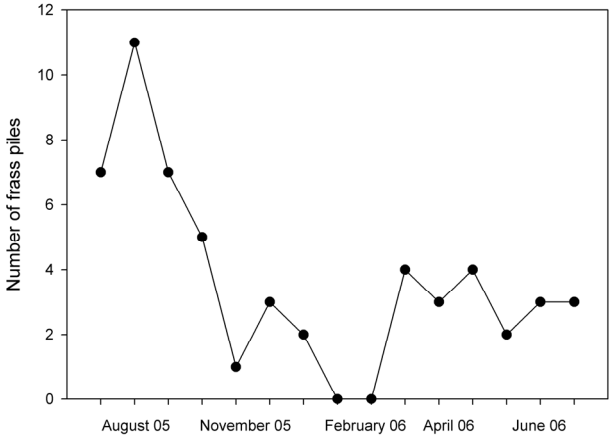

Figure 6 Frass heaps due to *Korynetes caeruleus* below a kneeler in the Allerheiligen-church in Erfurt, Germany (after Paul et al., 2007).

Several studies focused on the biological control of the webbing clothes moth *Tineola bisselliella* (Hummel) with the help of egg- and larval parasitoids (Plarre et al., 1999; Zimmermann et al., 2003). No mass-production technique has been developed yet for larval parasitoids, and efficiency of the mass-release of *Trichogramma* spp. has been variable. However, recent promising results have been obtained for the mass-release of *T. evanescens* in museums. Historic cars in museum exhibition rooms were treated in Vienna (Austria) and Munich and Bochum (Germany). Felt mats within the cars were infested by *T. bisselliella*. The surface area of felt is relatively small compared to other woolen materials, and monitoring of the moths with the help of pheromone-baited sticky traps showed a breakdown of the moth population after parasitoid release. The number of *T. evanescens* released per week on a total of 60 cars was 45,000 (Biebl, 2009).

These examples show that there are practical applications for inundative biological control strategies for pests in materials and museums that meet the requirements for low or zero tolerance of pests in these environments. The reaction of the staff to the release of wasps has been positive, and the cost has been competitive in the cases presented here. However, biological control of material destroying pests is in its very beginnings. More information on naturally occurring enemies of material destroying pests can be found in Becker (1954).

5. Commercial production of natural enemies

The release of *T. evanescens*, *H. hebetor*, *L. distinguendus* or *A. calandrae* and *C. tarsalis* has proven to be economically feasible for pest control companies. On the one hand, they can get new clients in the organic food market, on the other hand they can offer a wider range of control techniques and brand competence.

Natural enemies against stored-product pests are now produced in Germany, The Netherlands and Switzerland. Biological control is especially attractive to processing facilities that are not willing to stop production for pest control operations. Small farms with bulk grain stores that are not gas-tight apply parasitoids as well. For moth control in private households, internet shops sell *Trichogramma* sp. throughout Europe. The release units are sent several times to the consumer to cover at least eight weeks of parasitoid activity.

In a way, the application of natural enemies is an open-source-technology. Organisms cannot be patented, and hopefully this will be the case in the future. Registration is either not required for macro-organisms for biological control, or registration cost is comparatively low. Consequently, potentially every pest-control company can start the production of beneficials. Producers of beneficials may be more competitive by the development of innovative release units, the development of cost-effective rearing methods, or the development of rearing methods for beneficials that were hitherto not commercially produced. As long as beneficials do not become part of food, there are many opportunities to apply beneficials in stored-product environments.

6. Conclusions

Both non-chemical control methods for complete disinfestations and methods for suppression of re-infestation or residual infestations are needed. Specific control strategies have to be developed that provide rapid and effective suppression of pest populations. Even though non-chemical methods are currently widely applied, more examples are needed for cost-effective management methods that have been tested extensively under commercial conditions. An integrated management strategy is needed to keep products free from infestation along the whole storage chain from the raw products to the consumer. Biological control is a part of that strategy.

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Bio-rational control of red flour beetle *Tribolium castaneum* (Herbst) in stored wheat with Calneem® oil derived from neem seeds

Adarkwah, C.*[1,2], Obeng-Ofori, D.[3], Büttner, C.[1], Reichmuth, C.[2], Schöller, M.#[2]
1 Humboldt University of Berlin, Institute for Horticultural Sciences, Section Phytomedicine, Königin-Luise-Str. 19, D-14195 Berlin, Germany. Email: lesadark@yahoo.com
[2] Federal Research Centre for Cultivated Plants – Julius Kühn - Institut, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Königin-Luise-Str. 19, D-14195 Berlin, Germany
[3] University of Ghana, Department of Crop Science, School of Agriculture, College of Agriculture & Consumer Sciences, Legon, P.O. Box 68, Accra, Ghana

* Corresponding author
# Presenting author

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Abstract

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleloptera: Tenebrionidae) is one of the most serious secondary pests that feeds on a wide range of durable stored products including cereals, cereal products and other high value produce such as cocoa beans and dried fruits. Toxicity and protectant potential of Calneem® oil (derived from the seeds of the neem tree *Azadirachata indica* (A. Juss)) to *T. castaneum* were evaluated in stored cracked wheat in the laboratory using contact toxicity, grain treatment, persistency, progeny emergence and repellency assays. Calneem® oil is a biopesticide produced, registered and marketed in Ghana by AQUA AGRIC Community Projects (AACP), Tema, Accra. Calneem® contains about 0.3% azadirachtin as its major active ingredient. The Calneem® was applied at six concentrations (0.1, 0.2, 0.5, 1.0, 2.0 and 3.0%). The oil was emulsified with water using 0.07% soap. Different doses of Calneem® oil were toxic and highly repellent to *T. castaneum* with an overall repellency in the range of 52-88%. The highest concentration of 3.0% of Calneem® oil killed at least 90% of the beetles within 72 h on grain and 88% on filter paper. Beetle mortality was dose-dependent. The development of eggs to adults on cracked wheat was significantly (P<0.05) inhibited by Calneem® oil treatments. The effectiveness of Calneem® oil was significantly reduced by the length of storage after application.The results obtained suggest good potential for the practical use of Calneem® oil as a grain protectant for stored-product pest control. The use of plant materials such as neem oil may be a safe, cost-effective method of grain preservation against pest infestation among poor subsistent farmers who store small amounts of grain.

Keywords: *Tribolium castaneum*, *Azadirachta indica*, Cereals, Repellency, Contact toxicity

Low temperature to control *Plodia interpunctella* and *Stegobium paniceum*

Adler, C.*#
Federal Research Centre for Cultivated Plants, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Königin-Luise-Str. 19, 14195 Berlin. Email: cornel.adler@jki.bund.de

* Corresponding author
# Presenting author

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Abstract

Plodia interpunctella (Indianmeal moth) and *Stegobium paniceum* (drugstore beetle) are two of the most common insects in dried fruits, nuts, grain products, herb teas or spices. All the stages of both these insects were held at -10, -14 and -18°C for 5 to 480 min and the survival noted. The following times and temperatures were required to control all stages: *P. interpunctella*; 480 min at -10°C (1% survival of eggs), 240 min at -14°C, 60 min at -18°C; *S. paniceum*; over 480 min at -10°C, over 240 min at -14°C, 60 min at -18°C. For *P. interpunctella,* eggs were the most cold hardy stage. For *S. paniceum*, adults were the most cold hardy stage with the exception of –14°C, where about 10% of the eggs but no adult beetles survived 240 min of exposure.

Keywords: Cold disinfestation, Freezing, Control, *Plodia interpunctella, Stegobium paniceum.*

1. Introduction

The Indianmeal moth *Plodia interpunctella* (Huebner) and the drugstore beetle *Stegobium paniceum* (L.) are among the most common stored product pests, and they often occur in grain and grain products, nuts, dried fruit, fruit, herb teas and spices. If the products are dried in the open, it is difficult to prevent infestation. To control infestation in storage and processing, fumigation has been a common control option for decades. After the phase-out of methyl bromide (CH_3Br), world-wide dependency on phosphine (PH_3) increased dramatically. At the same time, reports on the development of resistance to phosphine in various stored product insects (Chaudry, 1999; Collins, 2006) have lead to concerns and increased the need to search for alternatives to fumigants.

Looking at non-chemical techniques, the utilization of extreme temperatures may also lead to complete control of pests, and it does not pose a risk to workers or involve the application of potentially hazardous agents (Fields, 1992; Burks et al., 2000).

Artificial cooling or freezing has been described as a means of pest control by various authors (David et al., 1977; Evans, 1987; Hagstrum and Flinn, 1994; Lasseran and Fleurat-Lessard, 1990; Dohino et al., 1999). Freezing dry stored products between - 10°C and - 20°C is an option for rapid disinfestation of high-value goods. While this process is fast, leaves no residues, has little or no negative effect on product quality and is comparatively safe for workers, the costs for the construction of a cooling chamber and fairly high energy costs are drawbacks that limit widespread adoption of this method. On a commercial scale, an organic dried fruit processing company with locations in Turkey, the USA, and Germany, places all dried fruits in a cooling chamber at -20°C for 24 h, prior to processing. Furthermore, a herb tea and spice producer in Germany has utilized a cooling chamber for more than 15 years to freeze all products upon reception, until a core temperature of -18°C is reached. Cooling is achieved by adding liquid nitrogen and chamber temperatures are allowed to drop as low as -90°C. Product is held for 12 to 36 h depending on the heat conductivity, the bale size and the volume of the treated product. Depending on product and treatment time, 3000 – 5000 L of liquid nitrogen are needed for a treatment, and costs are roughly estimated to be 1000 Euro/t (Tallafus, personal communication).

The aim of this study was to test which exposure times are required at -10, -14 and -18°C to control all the developmental stages and adults of *P. interpunctella* and *S. paniceum*.

2. Materials and methods

2.1. Insects

Insects came from cultures kept for many years at the Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection at 25 ± 1°C and 65 ± 5 % r.h. *Plodia interpunctella* was cultivated weekly by placing approximately 500 eggs into a mixture of 500 mL wheat bran and approximately 15 g of broken almonds. *S. paniceum* was cultivated by placing 200 adult beetles for two wk onto 150 mL wheat bran. After removing the beetles a further 200 mL of wheat bran were added together with a dried white bread roll. The insects used were not pre-adapted to cold prior to treatment.

To provide bio-test samples, eggs, larvae, pupae and adults were counted into separate cages. Fifty moth eggs (0-2 d old) were counted into small glass tubes (diameter: 13 mm, length: 13 mm) with a nylon mesh (0.3 mm width) at one end and closed with a rubber cork at the other end. This small glass tube was then fitted into a round cage made of wire mesh gauze (diameter: 14 mm, length: 50 mm) filled with 5 mL of wheat bran in a way that the nylon gauze touched the wheat bran. This was done to keep eggs separate from substrate for the evaluation of hatch while providing them with insulation comparable to the other stages. Fifty larvae of *P. interpunctella* were counted into 5 mL of wheat bran and filled directly into the wire mesh cages. The larvae were third instar, approximately 4 wk after placing eggs onto fresh substrate. Fifty pupae were taken from cultures 6 wk after placing eggs onto fresh substrate, and they were placed with substrate into cages just like the larvae. Young adult moths were counted in batches of 50 into a wide glass tube (diameter: 40 mm, length: 105 mm) without substrate and closed on both ends with cotton cloth fixed onto the tube with a rubber band (Fig. 1).

Figure 1 Glass tubes for exposure of adult moths after treatment with -14°C.

In the case of *S. paniceum*, 50 eggs (0-1 d old) were counted into a glass tube (diameter:13 mm, length:17 mm) in which one end was sealed with a nylon mesh (0.3 mm width), covered from inside with a 2 mm thick layer of black felt, from the other end with a rubber cork. As in the case of moth eggs, the glass tube was inserted into a wire mesh cage with substrate before treatment (Fig. 2). Larval and adult drugstore beetles were counted into batches of 50 individuals that were filled together with 5 mL of wheat bran into a wire mesh cage that was then closed with a rubber cork. In preliminary tests, *S. paniceum* pupae had shown high mortality in untreated controls when removed from the webbings for counting. This is why in just one replicate 80 eggs, 0-1d old, were placed into the wire mesh cages with 5 mL of substrate and the cages were kept for 34 d at 25°C and 65% r.h. until the majority of control samples showed development into pupal stages. After treatment, hatching of adult beetles was counted as survival.

Figure 2 Small glass tubes for exposure of drugstore beetle eggs, and egg cages enclosed into wire mesh cages for testing.

2.2. Testing and evaluation

Test were carried out in a laboratory freezer (Rumed 3501, Rubarth Apparate GmbH, Laatzen, Germany) equipped with a PT 100 thermo couple and an accuracy of ±0.5°C. In order to minimize the loss of cold air when opening the door, the freezing chamber had been sealed with a plastic sheet leaving just sufficient space to fit in the tray with test samples and data loggers for temperature recording.

Temperatures and exposure times tested were:

- -10°C for exposure times of 30, 60, 120, 240 and 480 min,
- -14°C for exposure times of 10, 30, 60, 120 and 240 min, and
- -18°C for exposure times of 5, 10, 30, 60, and 120 min.

After the given exposure times, the samples were removed from the freezer. The minute cages with *P. interpunctella* eggs were placed into small glasses with 2 mL of wheat bran and covered with an insect-proof plastic cap allowing gas exchange. Hatch of larvae was counted as survival while darker colouration and shrinking of eggs due to water loss were signs of mortality after 7 d at 25°C and 65±5 % r.h. Moth larvae and wheat bran were transferred into Petri dishes, pupae and their substrate into 200 mL glass jars. Both were checked for hatch of adult moths. *P. interpunctella* adults retrieved from the freezer were kept in the glass vials. They like all other test insects were transferred to a temperature-controlled chamber with 25°C and 65±5 % r.h. to be checked for survival 2 d after exposure (Fig 1).

Stegobium paniceum eggs were transferred into a small plastic Petri dish (diameter 35 mm) without substrate and checked for larval hatch after 7 and 14 d at 25°C and 65±5 % r.h. For untreated controls, batches of 50 eggs were directly counted into Petri dishes. Larvae and their substrate were transferred into small Petri dishes and checked for survival after 2 and 7 d. Pupal stages were transferred from cages into a 200 mL glass jar with wheat bran and checked after 7 and 14 d for hatch of adults. Adult beetles and their substrate were placed into small Petri dishes and checked for survivors after 2 and 7 d. All data were collected and calculated into % mortality correcting for natural mortality in untreated controls according to Abbott (1925). Mean values were calculated and plotted against exposure time.

3. Results

At -10°C, all stages of *P. interpunctella* except eggs could be controlled in 480 min. In *S. paniceum* none of the tested stages was controlled completely at this temperature and exposure time. Results of the treatment of *P. interpunctella* or *S. paniceum*, respectively, are given in Figures 3 and 4. Pupal stages of *S. paniceum* at -14°C were controlled within 120 min and at -18°C within 60 min. As recorded by data loggers, 10-15 min were required to reach target temperatures after the insect samples had been placed into the freezer. In untreated pupae of *S. paniceum*, an average of 58 ± 7 adult beetles emerged from 80 eggs in 11 replicates that had been placed with 5 mL of substrate into wire mesh cages.

4. Discussion

At -10°C, *P. interpunctella* was controlled at the longest exposure time of 8 h. In one of three replicates few eggs survived the treatment. Therefore, possibly 10-12 h could be a safe treatment time. Stage and cold acclimation can greatly increase the cold tolerance. Under similar conditions, Fields and Timlick (2010) found that fifth instar *P. interpunctella* had 98% mortality after 48 h at -10°C. For *P. interpunctella* that were cold acclimated and in diapause, after 14 d there was 88% mortality. In comparison, *S. paniceum* was not controlled at -10°C, and the adult beetles seem to be least affected by cold. In this stage there is no obvious dependency between mortality and exposure time with zero mortality in two of three replicates after 480 min exposure, while in egg and larval stages mortality increased with exposure time (Fig. 3). It is thus obvious that much longer times are required to achieve control at -10°C. Ryan (1995) reports on practical treatments of tobacco infested with the closely related tobacco beetle *Lasioderma serricorne* (F.) that 100% mortality was achieved at -10°C within 28 d. From literature data available, Reichmuth and co-workers (2007) calculated for this species a lethal time of approximately 18-20 d at -10°C.

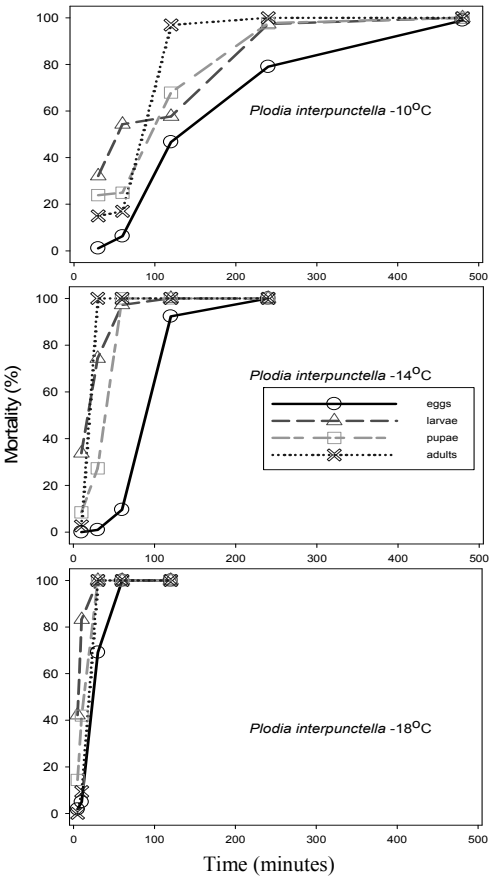

Figure 3 Mortality of eggs, larvae, pupae and adults of *Plodia interpunctella* after exposure to –10, -14, and –18°C.

At -14°C, eggs proved to be the most cold-hardy stage in both tested species. Eggs of *P. interpunctella* have the lowest supercooling points (-24°C) of all stages (Carillo and Cannon, 2005). At shorter exposure times of 60 and 120 min, adult *S. paniceum* were at least as or even more tolerant than eggs, but

no survivors were found at the longest exposure time (Fig. 4). This may hint to several and different factors being responsible for chill injury and mortality in eggs or beetles, respectively.

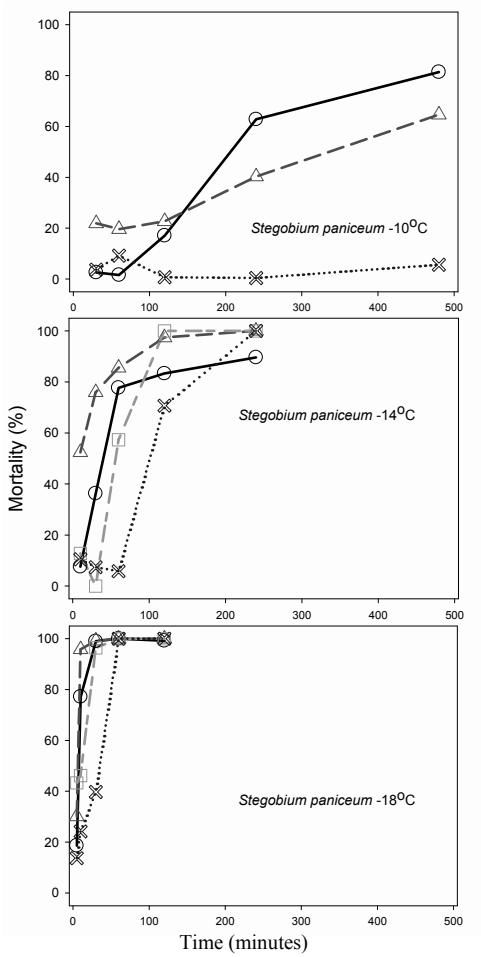

Figure 4 Mortality of eggs, larvae, pupae and adults of *Stegobium paniceum* after exposure to –10, -14, and –18°C.

At -18°C, eggs of *P. interpunctella* were controlled after an exposure time of 60 min the other tested stages could be controlled in half this time. Eggs of *S. paniceum* did not survive 60 min of exposure, but two survivors were found after 120 min exposure in one of five replicates. This effect may have been caused by a variation in genetic predisposition to cold hardiness. But also an artefact could be possible and further experiments are needed to add more precision to these data. More than 50% of the beetles survived up to 30 min exposure at -18°C. This is much more than in any other stage, while no survivors were found at longer exposure times. Dohino et al (1999) compared the efficacy of freezing at -18°C between various stored product beetles and moths including *P. interpunctella* and stated that the egg stage was most tolerant. Imai and Harada (2006) tested freezing treatments at various temperatures against *L. serricorne* and found that at -15°C eggs of unacclimated individuals were most tolerant and 100% mortality was reached after 5 h. On the contrary, Abdelghany et al. (2010) found that adults not eggs to be most cold tolerant stage. Variations in the cold hardiness of eggs may depend on age. The eggs used in this study were 0-24 h old and thus comparatively young.

For pupal stages of *S. paniceum*, just one replicate could be completed at this time, but together with other preliminary results, these data indicate that pupae are not the most tolerant stage. From the presented data one may conclude that *S. paniceum* is more tolerant to low temperatures than *P. interpunctella*. Compared to other developmental stages, young moth eggs are fairly tolerant to low temperatures at -10, -14, and -18°C. The same seems to be true for *S. paniceum* eggs that appeared less uniform in their tolerance to cold than adult beetles. At -10°C adult beetles are more cold-hardy. At this temperature, exposure times much longer than 8 h will be needed to control beetles.

Freezing with liquid nitrogen adds safety because most of a product is cooled to even lower temperatures until the core reaches -18°C. Subsequently, the bales are stored untouched. This prolongs treatment time in the core of a product and leaves little chance for insect survival. Temperature profile recordings and bio-assays could help to prove the reliability of this method.

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Repellent activity of two medicinal plant essential oils on *Tribolium castaneum* and *Ephestia kuehniella*

Akrami, H.*#[1], Moharramipour, S.[2] , Imani, S.[1]
[1] Department of Entomology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
Email: hd.akrami@yahoo.com
[2] Department of Entomology, Faculty of Agriculture, Tarbiat Modares University

* Corresponding author
# Presenting author

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Abstract

Red flour beetle (*Tribolium castaneum* (Herbst)) and Mediterranean flour moth (*Ephestia kuehniella* (Zeller)) are important stored product pests that contaminate and cause substantial loss of stored products. Volatile oils are secondary metabolites of plants for defending against insects and other herbivores. Some of them are very repellent for insects. The intent of the present study is to examine effect of *Thymus kotschyanus* Boiss and Hohen. and *Mentha longifolia* L. essential oils on Red flour beetle and Mediterranean flour moth. Essential oils were extracted by hydrodistillation. Experiments were done by RZR olfactometer model. Food plus essential oil was put in one arm and only food in the other side. Each insect was left for an hour let to choose one arm. In addition, another experiment was done to ten insects in each group. The results showed that, at 0.4 µL/L air, *T. kotschyanus* and *M. longifolia* had 83.33 and 93.33% repellent effect on *T. castaneum* and 90 and 100% repllenency on *E. kuehniella*, respectively. These essential oils could have potential to prevent infestation of the stored product pests in the warehouses.

Keywords: *Tribolium castaneum, Ephestia kuehniella, Thymus kotschyanus, Mentha longifolia*, Repellency

Potential of plant products as protectants of stored maize against *Sitophilus zeamais* Motschulsky (Coleoptera:Curculionidae)

Conceição, C.[1], Barbosa, A.*#[3], Matos, O.[2], Mexia, A.[4]
[1] Universidade José Eduardo dos Santos, UJES, Huambo, Angola
[2] Instituto Nacional de Recursos Biológicos, IP, INRB-INIA, Oeiras, Portugal
[3] Instituto de Investigação Científica Tropical, IICT, Lisboa, Portugal
[4] Instituto Superior de Agronomia, ISA, Lisboa, Portugal

* Corresponding author
# Presenting author

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Abstract

Laboratory studies were carried out to evaluate the effects of some formulations from *Mentha pulegium*, *Lonchocarpus sericeus*, *Daphne gnidium*, *Laurus nobilis*, *Momordica charantia*, *Nerium oleander* and *Ptaeroxylon obliquum* as protectants against adult insects of *Sitophilus zeamais* on stored maize. The dusts from leaves of *L. nobilis* at 30% w/w caused 86% mortality and reduced F1 progeny emergence up to 57%. At the same concentration, dusts of pink flowers from *N. oleander* and leaves from *L. sericeus* reduced the F1 progeny up to 68% and 70%, reduced the developmental index and prolonged the developmental period by 4 and 6 d, respectively.

The suspensions (2% v/v) from *M. charantia*, *N. oleander* and *P. obliquum* reduced the F1 progeny emergence up to 58, 91 and 94% and the number of holes in grains by 75, 91 and 97%, respectively. The methanol extracts were more effective than n-hexane extracts and affected the F1 progeny emergence and the developmental index.

Keywords: *Sitophilus zeamais*, Botanical insecticides, Repellence, Insect control agents.

1. Introduction

The use of natural products from plant origin as insecticides for defence against phytophagous insects can be traced back over many centuries to written documents of the early civilizations in China, India, Near East and over 150 years in Europe and North America (Matos, 2004; Isman, 2006). Nevertheless, in the early 19th century, emphasis shifted away from insecticides of plant origin with the tremendous development of synthetic insecticides industry after the Second World War. Plant metabolites were mainly investigated from a phytochemical and chemotaxonomic point of view during that period. Over the last two decades, however, interest in substances of plant origin and their potential in the pest management as environmentally friendly insecticides has been growing steadily. Since pyrethrum and rotenone, two of the first commercial natural insecticides, hundreds of new natural substances, including the volatile oils, are isolated and identified every year and their potential as plant protection agents is now of particular interest (Amaro, 2003; Isman, 2006), Such is the case of the triterpenoid azadiractin, extracted from the neem tree *Azadiracta indica* (A. Juss.), that is useful for pest control due to its properties; antifeedant, ovipositional deterrence, repellent, growth disruption, reduction of fitness and sterility.

The present study was inspired from the use of plant products as protectants of stored maize at the farm level in some rural communities in Angola. The main objective was to investigate the effects of *Laurus nobilis* L., *Nerium oleander* L., *Daphne gnidium* L. and *Mentha pulegium* L. from Portugal, species of tropical origin, *Ptaeroxylon obliquum* (Thumb.) from Angola, *Lonchocarpus sericeus* (Poir.) Khunt. and *Leonotis nepetifolia* (L.) R. Br. from São Tomé and Principe, against the maize weevil, *Sitophilus zeamais* Motschulsky.

2. Materials and methods

2.1. Maize, insects and plant products

The maize grain was yellow and was acquired in local trade with an average moisture content of 14 ± 0.5%. The unsexed adult insects aged from 1 to 7 d. The stock cultures of insects and all the biological tests were carried out in a single incubator at 27ºC and 75 ± 5% r.h. During the total period of the

bioassays it was necessary to collect the plant material several times from the same locations and during flowering. The harvested plant material was washed and dried out at room temperature in the darkness for two weeks, to reduce the water content of the structures and to optimize the concentration of bioactive substances. Then the plant material (leaves and flowers) was lyophilised, grounded and the dusts were kept in a desiccator protected from light. Due to shortage of plant material, the methanol suspensions were prepared only from dusts of *L. nobilis*, *M. charantia*, *N. oleander* and *P. obliquum* at a single concentration of 2% w/v (Table 1). From the lyophilised dusts methanol and n-hexane extracts were also produced (macerating 0.4 g of plant in 20 mL of solvent, for 24 h at room temperature, followed by filtration under vacuum).

Table 1 Plants, vegetal structures, type of formulation and dosage rates used in the bioassays with *Sitophilus zeamais*.

Plant species	Structure	Formulation	Concentrations of the formulation(% w/v)	Dosage rate
D. gnidium	Leaves	Extract	2.0. 3.5. 5.0 6.5. 8.0	15 mL/300 g maize (5% v/w)
L. nobilis	Leaves	Dust	—	15 and 30% w/w
		Suspension	2.0	20 mL/80 g maize
		Extract	2.0. 3.5. 5.0 6.5. 8.0	15 mL/300 g maize (5% v/w)
L. sericeus	Leaves	Dust	—	15 and 30% w/w
M. pulegium	Leaves	Dust	—	15% w/w and 30% w/w
		Extract	2.0. 3.5. 5.0 6.5. 8.0	15 mL/300 g maize (5% v/w)
M.charantia	Leaves	Dust	—	15 and 30% w/w
	Leaves	Suspension	2.0	20 mL/80 g maize
N. oleander.	Leaves and flowers	Dust	—	15 and 30%w/w
		Suspension	2.0	
		Extract	2.0. 3.5. 5.0 6.5. 8.0	15 mL/300 g maize (5% v/w)
P. obliquum	Leaves	Dust	—	15 and 30%w/w
		Suspension	2.0	20 mL/80g maize
		Extract	2.0. 3.5. 5.0 6.5. 8.0	15 mL/300 g maize (5% v/w)

2.2. Bioassays

The maize and the dusts were mixed in glass jars for 10 min with a mechanical mixer. After mixing, samples of 30 g of treated maize were transferred into glass jars (4 replicates per dosage rate) and 10 adult insects were used per replicate. After 168 h of exposure the parent adult insects were removed for observation of mortality, and the grains with the immature stages were incubated for the F1 emergence. The reduction in emergence (RE) of progeny (F1) was evaluated by using the formula RE (%) = [(Cn-Tn) / Tn] x100, where Cn is the number of insects present in control samples and Tn is the number of insects present in the treated samples (Tapondjou et al., 2005). The development index was calculated based on the emergency (F1) and average duration of development (ADD), which reflects the average number of days between the middle of the laying period and the emergence of 50% of F1 (Dobie, 1974).

The criterion from Haryadi and Rahayu (2002) was adopted for calculating the development index, using the following formula: DI = (ln F1/ADD) x 100, where DI = development index of *S. zeamais*, F1 = number of adult insects emerged from F1, ADD = average duration of development.

To evaluate the effect of the suspensions of *L. nobilis, M. charantia, N. oleander* and *P. obliquum*, a multiple-choice device was used (Fig. 1) with some modifications, adapted from Wesolowska and Ignatowicz (1994).

In the center of the Petri dishes (9 cm in diameter) was placed a central ring with 14 mm high and 20 mm diameter, to which were engaged in three equidistant points, three tabs of cardboard hydraulic of 32 mm long and 10 mm height (Fig. 1). The central ring and the tabs did not entirely touch on the bottom of the Petri dish. The ring had in the bottom three cuts of 4 mm height where the insects could pass. The tabs were stuck just above the bottom of the ring, also allowing the movement of insects between the sections. With the aim of providing ventilation inside the Petri dishes, a circular portion of 75 mm was removed from the lid and replaced by screening, which ensured the retention of insects and allowed the desired ventilation and observation.

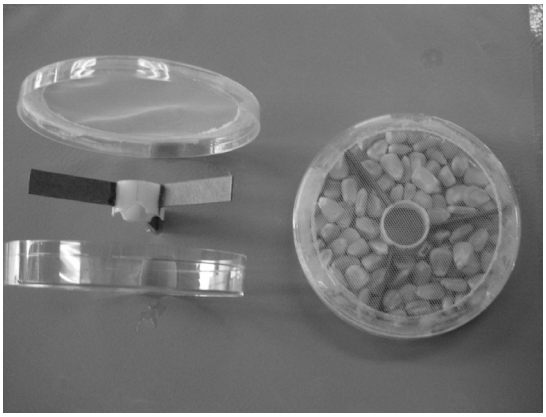

Figure 1 Multiple choice device for testing suspensions; a - lid with screening, b – central ring and cardboard tabs; c - base of the Petri dish; d - Petri dish with maize grain.

Ten adult insects were placed in the central ring. Mortality was assessed 24 h, 48 h and 168 h after exposure to treatments and repellency after 1, 24, 48 and 168 h. The parent adults were removed from the maize after 168 h. Maize seeds infested with eggs and immature stages were transferred to 60 mL glass jars, which were placed in the incubator for 35 d for observation and counting of progeny (F1). The count of insect damaged grains as an indicator of the feeding-inhibition was carried out 7 d after exposure of insects to treated maize. In the tests performed with extracts the species *D. gnidium, L. nobilis, M. pulegium, N. oleander* and *P. obliquum* were used at concentrations of 2% w/v, 3.5% w/v (0.7 g/20 mL), 5% w/v (1 g/20 mL), 6.5% w/v (1.3 g/20 mL) and 8 % w/v (1.6 g/20 mL) (Table 2).

Table 2 Mortality, average duration of development (ADD), emergency (F1), development index (DI), and life cycle of *Sitophilus zeamais* in maize treated with lyophilised dusts, n=4.

Plant	Dose (%w/w)	Mortality after 168 h (%)	*ADD* (d)	Mean F1	DI (d)	F1 reduction (%)	Life cycle (d)
L. nobilis	0	8	34.1 ± 1.0a	47.3 a	10.8 a		31.5 ± 0.9 b
	15	31	38.3 ± 5.5 a	25.5 ab	8.4 ab	46	33.8 ± 2.6 ab
	30	86	37.3 ± 5.6 a	15.3 b	6.8 b	68	35.5 ± 2.5 a
L. sericeus	0	3	32.4 ± 1.5b	41.5 a	10.6 a		31.8 ± 1.5 b
	15	8	36.2 ± 1.8 b	16.0 ab	7.1 a	65	36.0 ± 2.3 a
	30	8	38.1 ± 4.3 a	12.5 b	5.4 a	70	37.8 ± 2.1 a
M. pulegium	0	0	32.2 ± 1.1 a	47.8 ab	10.9 a		30.3 ± 0.5 a
	15	3	33.9 ± 2.7 a	54.0 a	11.6 a	-29	30.3 ± 0.5 a
	30	5	32.2 ± 7.4 a	22.0 b	8.8 a	14	30.0 ± 0.0 a
N. oleander	0	5	36.8 ± 4.1 a	28.3 a	8.9 a		33.5 ± 2.9 a
flowers	15	8	37.3 ± 4.4 a	30.5 a	8.2 a	-14	35.8 ± 6.6 a
	30	3	37.2 ± 5.9 a	16.3 a	6.1 a	57	33.3 ± 2.1 a
N. oleander	0	0	32.2 ± 1.3 a	48.3 a	11.5 a		30.3 ± 0.5 a
leaves	15	3	33.3 ± 2.6 a	26.3 a	9.8 a	46	31.0 ± 0.8 a
	30	5	37.2 ± 4.1 a	35 a	9.6 a	28	31.3 ± 1.0 a
P. obliquum	0	0	35.6 ± 1.4 a	48.3 a	11.6 a		30.3 ± 0.5 a
	15	5	36.8 ± 2.6 a	39.3 a	10.8 a	16	30.5 ± 0.6 a
	30	8	40.6 ± 4.1 a	32.8 a	9.3 a	8	32.0 ± 2.3 a

For a given plant, values followed by same letter do not differ significantly at $P \leq 0.05$.

The extracts were mixed with maize in the jars by manual shaking and then using a mechanical mixer for 10 min. The treated maize was placed in Petri dishes and / or glass jars and kept at room temperature in a laminar air flow for one hour to remove excess solvent, leaving the active substances of the extract impregnated maize. There was ten replicates for each concentration and 10 adult insects in each replicate. Mortality was observed after 24, 48 and 168 h of exposure of adult insects to treated maize. After 168 h exposure the adult insects were removed and the treated maize containing the immature stages was kept in the incubator over 3 wk and the emerging progeny were counted. The Development Index (DI) and the life cycle (LC) were also evaluated. The results were analyzed by Statistica ® 6.0 and SPSS 11.5 for Windows.

3. Results and discussion

The dusts of natural products require, in general, the use of high concentrations, sometimes exceeding 20%, which have to be applied to submerge the grain kernel in order to have protective effects (Golob, 1997; Golob et al., 1999). For that reason the concentrations of 15 and 30%, were used in the present tests. The most effective treatment of lyophilised dusts was *L. nobilis* at concentrations of 15% and 30% with mortalities of 31 and 86%, respectively (Table 2). There was a large reduction in the F1 progeny when treated with 30% dust; 68% reduction with *L. nobilis*, 70% reduction with *L. sericeus* and 57% reduction with flowers of *N. oleander* (Table 2). The average duration of development (ADD) and life cycle of insects were affected at a concentration of 30% of *L. nobilis* and *L. sericeus*. However, the difference between 15 and 30% in each plant is not significant. Boeke et al. (2001) showed that most of the effects of formulations of dust plants applied against adult insects are revealed in the reproduction and, consequently, on the embryonic development of insect larvae. In this study, the progeny production and the development index (DI) were affected in all plants, although the differences are significant only in *L. nobilis* and *L. sericeus* (Table 2).

Table 3 Repellency, damage and F1 production of seed treated with just methanol or methanol suspensions of plant dusts treated maize and exposed to *Sitophilus zeamais.*

		Multiple choice test (Mean number of insects/section)								
		Duration (h)				**Damage**				
Plant	**Treatment**	**1**	**24**	**48**	**168**	**Grain with holes (%)**	**Mean number of holes**	**Reduction of holes (%)**	**F1**	**F1 reduction (%)**
L. nobilis	Untreated	5a	5a	3a	4a	50a	26.0a		17a	
	Methanol	2a	3b	2a	2b	56a	32.1a		19a	
	Methanol Suspension	3a	2b	5b	4a	29b	13.5b	48	15a	13
M. charantia						47a	25.2a		21a	
	Untreated	1a	3a	2a	2a					
	Methanol	5b	4b	4a	4b	47a	24.6a		19a	
	Methanol Suspension	4b	3a	4a	4b	14b	6.2b	75	9a	58
N. oleander	Untreated	5a	2b	3ab	2b	48a	29.5a		17a	
	Methanol	3ab	3b	3b	4ab	48a	20.2a		19a	
	Methanol Suspension	2b	5a	4a	4a	4b	0.9b	91	2a	91
P. obliquum	Untreated	3a	4a	5a	3b	53a	21.6a		18a	
	Methanol	4a	3b	1b	4a	49a	23.9a		20a	
	Methanol Suspension	3a	3b	4a	3a	2b	1.9 b	97	1a	94

Values with the same letter for the species in the same column do not differ significantly ($P \leq 0.05$), n=8.

The methanol suspensions were highly variable in their repellency. In the case of *M. charantia* the effect of repellency is not clear, although the number of holes and the F1 progeny has been reduced, the number of insects present in treated maize was higher than in the control (Table 3). However, the mortality of *S. zeamais* with methanol suspensions was below 15% for all mixtures tested (results not shown), the percentage of perforated grains reveals the intensity of attack of *S. zeamais*. There were

significant differences in perforated grains and F1 production. The maize seed treated with *M. charantia*, *N. oleander* and *P. obliquum* had reduced the number of perforations in 75, 91 and 97%, respectively. For the same treatments the reduction of the F1 progeny was 58, 91 and 94%.

Table 4 Mortality of *Sitophilus zeamais* adults after 24, 48 or 168 h of exposure to maize treated with plant extracts of methanol and hexane, n=10.

		Methanol			**Hexane**		
		Mortality (%)			**Mortality (%)**		
		Duration (h)			**Duration (h)**		
Plant	**Treatment**	**24**	**48**	**168**	**24**	**48**	**168**
D. gnidium	Untreated	0	0	0	0	0	0
	Solvent	33	33	36	0	0	0
	2.0	-	-	-	0	0	2
	3.5	61	62	66	0	0	1
	5.0	63	67	72	0	0	2
	6.5	68	75	81	-	-	-
	8.0	73	79	85	-	-	-
L. nobilis	Untreated	0	0	0	0	0	1
	Solvent	48	50	62	0	0	2
	2.0	-	-	-	0	0	0
	3.5	72	72	76	0	0	0
	5.0	78	80	89	1	1	1
	6.5	93	94	96	0	0	0
	8.0	94	95	96	0	0	4
M. pulegium	Untreated	0	2	2	2	2	2
	Solvent	43	43	48	0	0	6
	2.0	2	8	17	0	2	8
	3.5	17	18	24	0	5	8
	5.0	20	24	30	-	-	-
	6.5	26	39	43	-	-	-
	8.0	-	-	-	-	-	-
N. oleander	Untreated	0	0	1	0	0	0
	Solvent	39	39	40	0	0	3
	2.0	27	37	39	0	0	0
	3.5	45	50	55	0	0	0
	5.0	47	53	61	-	-	-
	6.5	49	67	69	-	-	-
	8.0	50	69	73	-	-	-
P. obliquum	Untreated	0	0	0	0	0	0
	Solvent	48	48	60	0	0	4
	2.0	-	-	-	-	-	-
	3.5	78	78	79	0	0	5
	5.0	83	83	87	0	0	11
	6.5	56	64	71	0	0	25
	8.0	-	-	-	-	-	-

- Not available

Methanol suspensions of *L. nobilis, M. pulegium, M. charantia, N. oleander* and *P. obliquum* probably contained various substances dissolved in the solvent, for example, linalool, geraniol, cineol and ethanol in the case of *L. nobilis* (Saim and Meloan, 1986, Fiorini et al., 1997), psitosterol and several proteins in *M. charantia* (Golob et al., 1999), glycosides and proteins in *N. oleander* (Golob et al., 1999) or coumarins and diterpenoids in *P. obliquum* (Mulholland et al., 1999). However, the identification of active substances was not the objective of the present study.

The results obtained by counting the number of insect damaged grains and holes showed that, on average, the suspensions of *P. obliquum, N. oleander* and *M. charantia* have inhibited the feeding

activity and the reproduction of *S. zeamais* . Throne (1990) and Baker et al. (1991) demonstrated that non-lethal inhibition of insect populations can result in significant reductions in populations of stored product insects. We found that the suspensions of *M. charantia, N. oleander and P. obliquum* at 2% w/v reduced the F1 progeny in 58, 91 and 94%, respectively.

Table 5 Effects of methanol and hexane plant extracts on the F1 progeny, average duration of development (ADD), developmental index (DI) and life cycle (LC) of *Sitophilus zeamais* n=10.

		Methanol [a]					Hexane				
Plant	**Treatment**	**F1**	**F1 Reduction (%)**	**ADD (d)**	**DI**	**LC (d)**	**F1**	**F1 Reduction (%)**	**ADD (d)**	**DI**	**LC (d)**
D. gnidium	Control	42.3 a	-	36.3	10.2 a	33 b	59.0 a	-	37.6	10.7 a	33.9 a
	Solvent	10.0 b	76	39.3	7.3 ab	37 ab	49.8 ab	16	38.5	10.1a	35.1 a
	2.0	-	-	-	-	-	-	-	-	-	-
	3.5	3.7 b	91	37.5	5.8 b	35 ab	31.5 b	47	41.2	8.1 a	34.2 a
	5.0	11.1 b	74	39.4	7.3 ab	34 ab	28.5 b	52	40.4	7.9 a	35.6 a
	6.5	4.6 b	89	36.4	6.0 b	35 ab	-	-	-	-	-
	8.0	7.2 b	83	38.4	4.4 b	38 a	-	-	-	-	-
L. nobilis	Control	32.7 a	-	37	8.7 a	35 c	59.0 a	-	34.6	11.6 a	33.9 b
	Solvent	11.1 b	66	39.4	5.0 b	40 b	49.8 ab	16	33.9	11.4 a	35.1 b
	2.0	-	-	-	-	-	33.1 b	44	35.5	8.8 ab	35.7 b
	3.5	10.5 b	68	41	4.1 b	42 ab	23.7 bc	60	35.5	9.6 ab	33.2 b
	5.0	1.3 b	96	41.1	2.1 bc	44 ab	17.4 bc	71	35.5	7.5 b	33.9 b
	6.5	0.4 b	99	41.5	0.5 c	45 a	32.9 bc	44	41.1	7.1 bc	36.2 ab
	8.0	1.8 b	94	43.1	3.5 bc	45 ab	11.6 c	80	43.1	5.0 c	39.3 a
M. pulegium	Control	46.7 ab	-	33.9	10.8 a	33 b	47.9 a	-	34.7	11.1 a	28.6 b
	Solvent	26.1 bc	44	36.6	7.5 b	37 a	28.7 ab	40	33.9	9.1 a	31.7 a
	2.0	59.0 a	126	33.2	11.4 a	32 b	26.5 ab	45	36.1	8.8 ab	32.6 a
	3.5	32.6 ab	30	41	8.9 ab	36 ab	19. 6 b	59	35	7.9 b	32.4 a
	5.0	31.7 b	32	36.4	9.6 ab	34 ab	-	-	-	-	-
	6.5	19.2 b	59	38	8.3 ab	37 a	-	-	-	-	-
	8.0	-	-	-	-	-	-	-	-	-	-
N. oleander	Control	33.5 a	-	32.9	10.1 a	33 b	59.0 a	-	37.6	10.7 a	33.9 a
	Solvent	4.2 b	87	36.5	4.2 b	39 ab	49.8 a	16	38.5	10.1 a	35.1 a
	2.0	9.2 b	73	34.5	5.8 b	37 b	44.6 a	24	40.7	11.4 a	34.1 a
	3.5	7.2 b	79	32.9	7.7 ab	35 b	40.0 a	32	38.1	11.3a	34.7 a
	5.0	3.7 b	89	32.6	5.4 b	34 b	-	-	-	-	-
	6.5	2.6 b	92	39.8	3.7 b	45 a	-	-	-	-	-
	8.0	5.9 b	82	38.9	5.9 b	37 b	-	-	-	-	-
P.obliquum	Control	32.7 a	-	38	8.7 a	35 c	41.4 a	-	34.7	9.8 a	35.7 a
	Solvent	12.3 b	62	37	5.1 b	40 ab	40.3 a	3	33.9	9.6 a	35.6 a
	2.0	-	-	-	-	-	-	-	-	-	-
	3.5	2.8 b	91	41.4	2.4 b	43 a	41.5 a	0	33.3	11.0 a	35.1 a
	5.0	3.4 b	90	39.5	4.0 b	40 ab	25.1 a	39	-	-	35.7 a
	6.5	4.5 b	86	36.9	4.0 b	38 bc	22.7 a	45	-	-	37.1 a

Values followed by same letter do not differ significantly; - not available

The maize treated with methanol alone caused mortalities ranged from 33% to 60% (Table 4), probably due to vapour effect caused by methanol inside the closed glass jars. However, the results obtained with most concentrations are higher than those of methanol alone. At the concentration of 3.5% plant extract after 168 h the order of effectiveness was *P. obliquum* (79%) > *L. nobilis* (76%) > *D. gnidium* (66%) > *N. oleander* (55%) > *M. pulegium* (24%), at 5% plant extract the mortality was; *L. nobilis* (89%) > *P. obliquum* (87%) > *D. gnidium* (72%) > *N. oleander* (61%) > *M. pulegium* (30%) and at 6,5% plant extract the mortality was *L. nobilis* (96%) > *D. gnidium* (81%) > *P. obliquum* (71%) > *N. oleander* (69%) > *M. pulegium* (30%).

For the methanol extracts the best results on the reduction of the F1 progeny were obtained with *D. gnidium* (89%), *L. nobilis* (99%), *M. pulegium* (59%) and *N. oleander* (92%) at 6.5% and *P. obliquum* (91%) at 3.5%. The average duration of development (ADD) of the insect increased with the concentration of both methanol and hexane extracts (Table 5).

The results of the development (ADD) and the life cycle on maize treated with both solvents alone have shown higher values when compared to the untreated maize grains. There were significant differences in the life cycle between the extract treatments. The life cycle was delayed from 3 to 10 d and from 2 up to 6 d for methanol and hexane extracts, respectively, when compared to the untreated control maize. There was also a decrease in DI with the increase in concentration. The DI values obtained for concentrations above 3.5% in extracts of both solvents of *L. nobilis, N. oleander* and *P. obliquum* are significantly lower than those of the untreated control samples.

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Structural heat treatments against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae): effect of flour depth, life stage and floor

Brijwani, M.[1], Subramanyam, B.*#[1], Flinn, P.W.[2], Langemeier, M.R.[3]
[1] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, U.S.A.
Email: sbhadrir@k-state.edu
[2] USDA-ARS, Center for Grain and Animal Health Research, Manhattan, KS 66502, U.S.A.
[3] Department of Agricultural Economics, Kansas State University, Manhattan, KS 66506, U.S.A.

* Corresponding author
# Presenting author

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Abstract

The effect of high temperatures (50-60°C) and two levels of sanitation (~0.5 and 43 g of flour), on mortality of eggs, young larvae, old larvae, pupae, and adults of the red flour beetle, *Tribolium castaneum*, were evaluated during heat treatment of a pilot flour mill at Kansas State University. The mill was heated once during 13-14 May 2009 and once during 25-26 August 2009. Each of the heat treatments lasted 24 h. Bioassay boxes, with life stages of *T. castaneum* and temperature sensors confined in small compartments, were placed in 25 locations across all five mill floors. Temperature data showed that the mean time to 50°C based on the two treatments ranged from 10.39 to 17.18 h, and the mean time above 50°C ranged from 6.01 to 13.63 h. The mean maximum temperatures attained ranged from 50.7 to 61.4 °C. In general, temperatures were lower in compartments with 43 g of flour when compared with compartments with 0.5 g of flour. Temperatures were also lower on the first floor than on the remaining floors. In box bioassays, essentially none of the life stages survived the 24 h heat treatment (99-100% mortality), except on the first floor. The survival of insects, especially on the first floor, is related to how quickly temperatures reached 50°C and how long temperatures were held between 50 and 60°C, and the maximum temperatures attained at a given location. There were only small differences in mortality between the two levels of sanitation. These results show that heat treatment of flour mills can control all life stages of *T. castaneum* in 24 h.

Keywords**:** *Tribolium castaneum*, Heat treatment, Sanitation, Life stages, Methyl bromide alternatives

1. Introduction

Extreme temperatures cause significant natural mortality in insect populations thus offering a potential tool that can be used as an environmentally benign management strategy (Hallman and Denlinger, 1998). Heat treatment of structures is an age-old technology for managing insects associated with grain-processing facilities such as flour mills (Dean, 1911). Heat treatment involves raising the ambient temperature of a food-processing facility to 50-60°C and holding these temperatures for 24 h or less to kill all life stages of stored-product insects (Dosland et al., 2006). Some companies have been known to extend this time to at least 36 h. There is renewed interest in heat treatments because methyl bromide, a structural fumigant, was phased out in the USA as of 2005 except for certain critical uses. The phase out of methyl bromide is related to its adverse effects on stratospheric ozone (Makhijani and Gurney, 1995).

Grain-processing facilities such as flour mills are dusty, because of continuous production. Flour beetles (*Tribolium spp.)* are the most abundant insect species in flour mills and their life stages are found throughout the milling process (Good, 1937). The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is a major pest of flour mills worldwide (Sinha and Watters, 1985; Hagstrum and Subramanyam, 2009).

Previous research at Kansas State University has shown that the susceptibility of stored-product insects to heat varies among species and within a species among the life stages (Mahroof et al., 2003a; b; Boina and Subramanyam, 2004). In general, it is now well recognized that the minimum temperature for effective disinfestation should be at least 50°C (Dosland et al., 2006). Very little is known about how sanitation influences effectiveness of heat in killing life stages of *T. castaneum*. Flour is a good insulator, and before heat treatments flour mill staff do extensive cleaning to remove flour that can serve as refugia for insects to escape the heat. Therefore, in the present investigation, mortality of eggs, young

larvae, old larvae, pupae, and adults of *T. castaneum* during two heat treatments of a pilot flour mill were evaluated. Additionally, during the heat treatment of the flour mill, the effect of different sanitation levels on mortality of *T. castaneum* life stages was assessed.

2. Materials and methods

2.1. Pilot flour mill

The Hal Ross Flour Mill of the Department of Grain Science and Industry, Kansas State University, is a state-of-the-art pilot scale mill. It has five floors and a capacity of 9626 m^3. The mill is used for student and faculty research and teaching, and occasionally for use by grain industry stakeholders.

2.2. Box bioassays

Cultures of *T. castaneum* were reared on wheat flour plus 5% (by wt) Brewer's yeast diet at 28°C and 65% r.h. Eggs, young larvae, old larvae, pupae, and unsexed adults of mixed ages were collected from cultures following procedures described by Mahroof et al. (2003a;b). Each life stage was confined in plastic compartments inside a rectangular plastic box that was 27 cm long, 17.5 cm wide, and 4.2 cm high. There were a total of 12 small compartments within this box, and each compartment measured 8.3 cm long, 4.2 cm wide, and 3.7 cm deep. The top row of the compartments had 2 cm deep flour (43 g/compartment), and the bottom row had a dusting of flour (~0.5 g/compartment). Ten of the 12 compartments were used for confining insects and the other two were used for placing small sensors (SmartButton; ACR Systems Inc., Surrey, Canada) for measuring temperatures during heat treatment. In each compartment, 50 individuals of a life stage were introduced. Compartments with 0.5 g of flour represented or simulated "good" sanitation in food-processing facilities and the 43 g of flour simulated "poor" sanitation. Infested boxes were placed in 25 preselected mill locations. There were five boxes on each floor. Boxes among floors were placed within equipment or on the floor. The control bioassay box, with all life stages of *T. castaneum* was placed in a growth chamber in the laboratory at 28°C and 65% r.h. There were four such control boxes. After 24 h of heat treatment, all bioassay boxes were brought to the laboratory and incubated at 28°C and 65% r.h. The mortality of adults was measured 24 h after incubation at 28°C and 65% r.h. Immature stages were reared until emergence of adults and the number of adults counted.

2.3. Mill heat treatment

The structural heat treatments were conducted on 13-14 May 2009 and on 25-26 August 2009. Each treatment was performed for 24 h using forced-air gas heaters and the treatments were done by a professional heat treatment service provider (Temp-Air, Burnsville, MN, U.S.A.). Propane was used as the fuel for the gas heaters. Uniform distribution of heat was ensured by placing 8 fans on each of the five floors. The real-time temperature distribution during heat treatment within the mill was monitored by the facilitator using Temp-Air wireless sensors, and these data are not reported here.

Two SmartButton sensors were used in each bioassay box. One sensor was placed in a compartment with 0.5 g of flour and the other was used in the compartment with 43 g of flour. In compartments with 43 g of flour, the sensor was placed at a depth of 1 cm. Sensors recorded temperature every 2 min during a heat treatment. The 24 h time-dependent temperature profile for the two treatments was averaged by location and flour depth to determine time to reach 50°C, time above 50°C, and the maximum temperature.

2.4. Data analysis

The mean mortality data of *T. castaneum* life stages from the two treatments was subjected to factorial analysis of variance (ANOVA) to determine differences among the main (stage, flour depth, and floor) and interactive effects using the GLM procedure of SAS (SAS Institute, 2002). The relationship between mean mortality of each life stage and time to 50°C, time above 50°C, and maximum temperature was determined by subjecting data to the CORR procedure of SAS. All differences were considered significant at the $\alpha = 0.05$ level.

3. Results

The outside temperature during May and August within the bioassays ranged from 20.5 to 25.5°C and 24.5 to 33.5°C, respectively. During the May treatment 5,306 L of propane was consumed, and the total

heat energy generated was 37,748 kW. During August treatment 4,889 L of propane was consumed, and the total heat generated was 34,782 kW. The total heat energy per cubic meter of the mill space per hour during the May and August treatment was 0.16 kW/m^3/h and 0.15 kW/m^3/h, respectively.

The mean time to reach 50°C based on the two treatments ranged from 10.39 to 17.18 h (Table 1). Similarly, temperatures above 50°C were maintained for a mean time period of 6.01 to 13.63 h. The mean maximum temperatures attained ranged from 50.6 to 61.4°C. In general, the time to reach 50°C took longer in compartments with 43 g of flour when compared with compartments with 0.5 g of flour (Fig. 1), whereas the time above 50°C and maximum temperatures attained were slightly lower in compartments with 43 g of flour when compared with compartments with 0.5 g of flour. Temperatures in general were lower on the first floor than on the remaining floors above it.

Table 1 Summary of temperatures observed in bioassay boxes by mill floor based on May and August, 2009 heat treatments of a small flour mill.

	0.5 g of flour			43 g of flour		
Floor	**Time to 50°C (h)**	**Time above 50°C (h)**	**Maximum temp. (°C)**	**Time to 50°C (h)**	**Time above 50°C (h)**	**Maximum temp. (°C)**
First	17.17	6.85	51.95	17.18	6.01	50.65
Second	10.39	13.63	59.55	13.45	10.57	57.70
Third	10.54	13.49	61.35	12.20	11.83	60.05
Fourth	12.56	11.46	58.85	15.39	8.63	56.15
Fifth	12.02	11.99	57.70	14.94	9.07	55.35

Each mean is based on $n = 2$ replications.

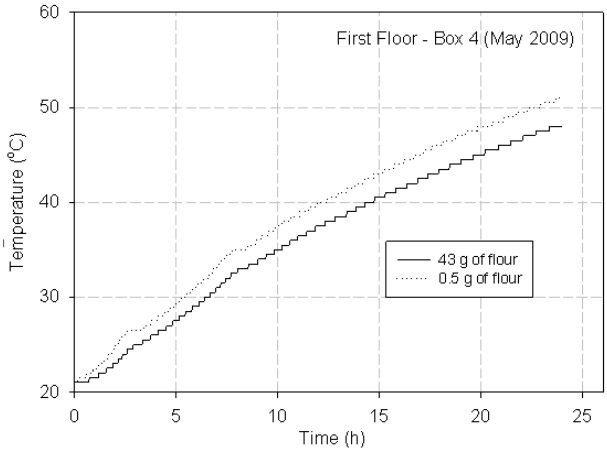

Figure 1 Temperature profiles in compartments with 0.5 g and 43 g of flour. These data are from a bioassay box placed on the first floor of the mill during May heat treatment.

The mean mortality of eggs in unheated compartments with 43 g of flour and 0.5 g of flour was 24 and 57%, respectively. High mortality (37%) was also observed with young larvae in compartments with 0.5 g of flour. Mortality of all other stages in unheated compartments ranged from 0 to 7%. The lack of food in compartments with 0.5 g of flour may have contributed to the high mortality observed.

The mortality of life stages of *T. castaneum* ranged from 99 to 100% on second through fifth floors of the mill (Table 2). On the first mill floor, only the egg stage was completely controlled, especially in compartments with 0.5 g of flour. Mortality of all other stages on this floor ranged from 51 to 99%, with greater survival in compartments with 43 g of flour than in compartments with 0.5 g of flour. In second through fifth floors, very few young larvae and occasionally old larvae in compartments survived, however in all these cases, mortality ranged from 99.4 to 99.8%.

Table 2 Mortality of *T. castaneum* life stages exposed to elevated temperatures during May and August, 2009 heat treatments of small flour mill.

Floor	Life stage	Mean mortality (%)	
		0.5 g of flour	43 g of flour
First	Egg	99.2	98.0
	Young larva	99.0	84.4
	Old larva	89.8	83.4
	Pupa	89.8	90.8
	Adult	90.0	50.6
Second	Egg	100.0	99.8
	Young larva	99.4	100.0
	Old larva	100.0	100.0
	Pupa	100.0	100.0
	Adult	100.0	100.0
Third	Egg	100.0	100.0
	Young larva	99.4	99.8
	Old larva	100.0	99.6
	Pupa	100.0	100.0
	Adult	100.0	100.0
Fourth	Egg	100.0	100.0
	Young larva	100.0	100.0
	Old larva	100.0	99.8
	Pupa	100.0	100.0
	Adult	100.0	100.0
Fifth	Egg	100.0	100.0
	Young larva	99.8	99.4
	Old larva	99.2	99.5
	Pupa	100.0	100.0
	Adult	100.0	100.0

Each mean is based on $n = 2$ replications.

Mortality observed in bioassay boxes varied significantly ($P < 0.05$) among *T. castaneum* life stages, floors, and between compartments with 0.5 and 43 g of flour. Except for stage x depth interaction, all other two and three way interactions were all significant (Table 3).

Table 3 Analysis of variance statistics showing factors affecting *T. castaneum* mortality.

Source of variation	df	Mean square	*F*-value	*P*-value
Stage	4	87.36	3.82	0.0088
Floor	4	614.39	26.84	<0.00001
Depth	1	146.41	6.40	0.0146
Stage*Floor	16	89.64	3.92	0.0001
Stage*Depth	4	53.21	2.32	0.0692**
Floor*Depth	4	147.03	6.42	0.0003
Stage*Floor*Depth	16	53.91	2.36	0.0108
Error	50	22.89		

**Not significant ($P > 0.05$); all other main and interactive effects are significant ($P < 0.05$).

The mortality of each life stage was inversely related to how quickly temperatures in box bioassays reached 50°C, but positively related to time above 50°C and the maximum temperature. All correlation coefficients were significantly different from 0 ($P < 0.05$), except the coefficients relating mortality of adults and young larvae and time to reach 50°C, and a coefficient relating young larvae and time above 50°C (Table 4).

Table 4 Correlation between percentage mortality of *T. castaneum* life stages and temperature variables.

Variables	Adult	Pupa	Old larva	Young larva	Egg
Time to 50°C (h)	-0.61 (0.060)	-0.76 (0.011)	-0.74 (0.014)	-0.52 (0.124)	-0.69 (0.027)
Time above 50°C (h)	0.68 (0.032)	0.78 (0.008)	0.78 (0.007)	0.59 (0.073)	0.75 (0.013)
Maximum temperature (°C)	0.74 (0.014)	0.85 (0.002)	0.86 (0.001)	0.66 (0.039)	0.81 (0.004)

Value in parenthesis is probability that the correlation coefficient is significantly different from zero. All *P*-values are significant ($P < 0.05$), except for the coefficient between adult/young larval mortality and time to 50°C, and for the coefficient between young larval mortality and time above 50°C.

4. Discussion

The mill at Kansas State University is a new facility that opened in 2006, and it is relatively an air tight building. Therefore, distributing the hot air by using fans was not as efficient, especially on the first floor which has large pieces of equipment which hindered air movement. Also, hot air rises up and this vertical stratification resulted in lower temperatures on the first floor. As a result mortality of *T. castaneum* life stages was less than 100% on this floor.

There were mortality differences among *T. castaneum* life stages, and most of these differences were obvious on the first floor. Laboratory tests at constant temperatures between 50 and 60°C (Mahroof et al., 2003b) revealed young larvae of *T. castaneum* to be the most heat-tolerant stage. However, during treatment of a pilot flour mill, where temperatures are dynamic (change over time), pupae were found to be the most heat-tolerant stage (Mahroof et al., 2003a). No consistent trend is obvious in our data, but on second through fifth floors, very few young larvae survived.

Significant differences among floors in mortality of life stages can be attributed to differences found between the first floor and all other floors. On second through fifth floors mortality was essentially between 99 and 100%. Differences in mortality between the two flour depths/quantities were more evident on the first floor, and generally mortality was slightly lower in compartments with 43 g of flour than in compartments with 0.5 g of flour. This effect is related to three factors. The mortality of each stage was influenced by how quickly temperatures reached 50°C, and the number of hours temperatures were held above 50°C within the 24 h window of time, and the maximum temperature.

In summary, data from the two heat treatments proved that heat is a viable alternative to methyl bromide, and effective heat treatments can be conducted within 24 h, provided lethal temperatures (50-60°C) are attained quickly. In our mill, lethal temperatures were attained within 15 to 16 h. Typically, lethal temperatures should be attained within 8 to 10 h so that these lethal temperatures can be held for 14 to 16 h for effective disinfestation. Our results also reinforce the need for sanitation in the mill environment for effective kill of *T. castaneum* life stages.

❖ Mention of product or trade names in this paper do not constitute an endorsement for its use by Kansas State University or USDA.

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Non-chemical alternative in rice storage: the use of refrigeration for insect control and quality maintenance of paddy rice

Barbosa, A.#[1], Carvalho, M.O.*[1], Barros, G.[1], Pires, I.[2], Riudavets, J.[3], Navarro, S.[4]
1 Instituto de Investigação Científica Tropical, Lisbon, Portugal. Email: motiliac@netcabo.pt
[2] Saludães, Produtos Alimentares, SA., Oliveira de Azeméis, Portugal
[3] Institut de Recerca I Tecnologia Agroalimentàries, Barcelona, Espanha
[4] Food Technology International Consultancy, Israel

* Corresponding author
# Presenting author

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Abstract

Trials were conducted to evaluate the potential effects of refrigeration in controlling insect infestations of *Sitophilus zeamais* and *S. oryzae* on stored rice. In the rice storage and processing industry use of aeration systems during winter and refrigeration units under summer conditions can provide a good solution to reduce paddy temperature for control of insects and maintenance of paddy quality; though it is restricted by costs of machinery and electric power. Trials were conducted in a rice mill in Portugal close to the Mondego Valley. A granary containing 140 t of paddy (variety Ripallo) was cooled using refrigerated aeration because during the trials ambient temperatures were too high for successful insect control by ambient air aeration. *Sitophilus zeamais* and *S. oryzae* adults reared on brown rice at 28°C and 68±3% r.h. were used as bioassays. Paddy stored in the granary was cooled to below 18ºC from 27 June 2008 to 19 September 2008. The mean ambient temperature during the total period of the trial varied from 12ºC to 27ºC. The moisture content of the paddy in the granary ranged from 12.1% to 13.9% during the same period of time. The total F_1 of adult emergence population of *S. zeamais* and *S. oryzae* was counted after the experiments. The F_1 adult from parent adults of *S. zeamais* was reduced up to 77%; the F_1 adult from eggs of *S. zeamais* and *S. oryzae* were also reduced up to 71% and 45%, respectively, when compared with corresponding untreated controls. Refrigeration of the paddy allowed storage for almost three months at about 18ºC and caused delay in rice weevils development. As a consequence, this negated the necessity for fumigation. Although the energy consumed during refrigeration doubled the cost when compared with cooling using an ambient-air, aeration system during the cold season, refrigeration provided an environmentally sound and user-friendly treatment during warm months of the year.

Keywords: Refrigeration, *Sitophilus zeamais*, *Sitophilus oryzae,* Paddy, Rice mill, Storage

1. Introduction

Currently the most common non-chemical alternative in the rice storage and processing industry is the use of aeration systems that can be effectively run during winter to reduce paddy temperature. Under summer conditions use of refrigeration units provides a good solution for quality maintenance of paddy, but is restricted by costs of machinery and electric power. The objective with aeration and refrigeration systems is to achieve temperatures of less than 18ºC (Navarro, 2007) which significantly reduces insect activity. Aeration using ambient air may not be sufficient to control fungi on moist grain, control mites and insects, prevent self-heating of grain, preserve germination capacity and quality of stored grain in warm climates, or when warm grain is stored immediately after harvest. To address these situations, refrigerated air units for chilling grain have been developed for commodities that can justify the added expense of refrigerated aeration cooling. In this type of aeration process, ambient air is conditioned by passing it through the evaporator coil and a secondary reheat coil of the refrigeration unit, then the chilled air is blown into the grain bulk via the existing aeration system (Navarro, 2007).

Grain chilling is accepted as a grain-conditioning technology in much of Western Europe; currently most new units appear to be marketed in Southeast Asia. In the 1960s grain chillers were primarily used as a means of preserving high moisture (moist, damp) grain. Later, grain chilling was applied to improve storability of sensitive commodities subject to development of heat foci (hot spots), i.e., for soybean and

maize, and preserve the quality of high value dry grain, seeds and edible beans, primarily against mites and insects (Navarro and Noyes, 2001).

Consumer and regulatory agencies for environmental protection demand chemical-free and contamination-free products. This is a general tendency that industry finds difficult to conform to because insecticides are often necessary to prevent economic damage. Additionally, in many countries, insects have been developing resistance to contact insecticides and to the fumigant, phosphine.

This project was carried out integrating environmentally sound and sustainable technologies to replace conventional chemical treatments needed for protecting the quality of rice at different phases of post-harvest handling and storage to meet European standards. Trials were conducted to evaluate the potential effects of refrigeration in controlling insect infestations of *Sitophilus zeamais* Motschulsky (Coleloptera: Curculionidae) and *S. oryzae* L. on stored rice.

2. Materials and methods

Trials were conducted in a rice mill in Portugal close to the Mondego Valley. A granary (metal bin) containing 140 t of paddy (variety Ripallo) was cooled using refrigerated aeration. Refrigeration was used for cooling the paddy because ambient temperatures were too high for successful insect control by aeration with ambient air. *Sitophilus zeamais* adults reared in polished rice at 28°C and 68% r.h. were used as bioassays. One-week-old adults were placed in clean rice for one week to lay eggs, at the IICT laboratory, in Lisbon. Then the infested rice with immature stages was also used in bioassays. Infested grain with eggs of *S. oryzae* were of integral husked rice. Adults were removed and only eggs or early stage larvae that remained in the rice kernels were used.

Four separate replicates of infested rice with eggs of *S. zeamais* and *S. oryzae* were placed in tubes-type metal cages which had a capacity to contain 16 g of rice; four replicates of 20 one-week-old adults of *S. zeamais* were placed in box- type metal cages which had a capacity to contain 40 g of rice. The location of these metal cages in the granary and big bag is shown in Table 1.

Table 1 Operational details of the bioassay cages used in the refrigeration trial.

Bioassay cage	Layout	Location
4 tube type metal: eggs of *S. Oryzae* 4 tube type metal: eggs of *S. Zeamais* 4 box type metal:20 adults of *S. zeamais*	6 cages 0.5 m from top + 6 cages 1.5 m from bottom	Granary
4 tube type metal: eggs of *S. Oryzae* 4 tube type metal: eggs of *S. Zeamais* 4 box type metal:20 adults of *S. zeamais*	3 cages from each type at four different depths	Big bag
4 glass jars: eggs of *S. Oryzae* 4 glass jars: eggs of *S. Zeamais* 4 glass jars: 20 adults of *S. zeamais*	------	Incubator

Each replicate of both types of infested rice cage was placed in the granary at two depths; 50 cm from the top and 1.5 m from the bottom. Four replicates of each type of infested rice were also placed in four different depths in the big bag containing 1 t stored rice used as control. Another separate group of four replicates in glass jars (Table 1) was maintained in an incubator at the laboratory to observe insect development at 28ºC and 65-70% r.h.

Temperature and r.h. inside the grain bulk and outside the granary were monitored during the experiment using HOBO data and three temperature probes with two points of recording data. The big bag was kept at ambient conditions of the factory and was also equipped with a HOBO within the bag to monitor humidity and temperature throughout the trial. A refrigerated-air unit was connected to the granary and the stored paddy was cooled to below 18ºC. This temperature was maintained from 27 June 2008 to 19 September 2008. At the end of the experiment, insect development was monitored and compared in the granary under refrigeration, the big bag stored at ambient temperature and at laboratory control conditions.

3. Results and discussion

During the months (June-September) that trials were carried out, the ambient temperatures fluctuated between 24ºC and 30ºC. The paddy mean temperature, under the influence of the aeration with refrigerated air, was reduced to 17.8±0.4ºC (Table 2). Paddy temperature close to each probe had an average of 17.2±0.3ºC in probe 2, and 18.2±0.4ºC in probe 1. But high paddy temperatures occurred following the cessation of refrigeration, when the refrigeration unit had to be replaced monthly. As a result, there was a rapid increase of paddy temperature up to 27ºC. This increase in temperature may be explained by the warm ambient air blown into the paddy due to aeration with ambient air and without the assistance of the refrigeration unit.

In addition, the limited insulation capabilities of the relatively small bulk of paddy containing only 140 tones also contributed to the rapid increase in temperature, but to a lesser extent. According to Navarro and Noyes (2001) larger bulks of cereal grain of about 1000 t could maintain winter temperatures throughout the summer of the Mediterranean climate with only slight increase in temperature from 15 to 20ºC when ambient day temperatures reached 30ºC. In the present study, temperatures in probe 1 reached 27ºC. On the contrary, in non-aerated big bags the temperature was more stable with a mean of 22ºC and very short interval (minimum 21.6ºC and maximum 21.9ºC).

Table 2 Mean, standard error of the mean, minimum and maximum temperature registered inside the granary, during refrigeration and inside the big bag, from 27 June to 18 September 2008.

	Granary				*Big Bag*
Data	***Probe 1***	***Probe 2***	***Probe 3***	***Mean***	***Mean***
Mean temp. (ºC)	18.2	17.2	17.9	17.8	22.0
Standard error (±)	0.4	0.3	0.4	0.4	0.0
Minimum	13.0	12.0	13.0	13.0	21.6
Maximum	27.0	24.0	26.0	25.7	21.9
Counts	66	66	66	66	4198

At the beginning of the trials samples were collected to determine moisture content. Both paddy stored either in the big bag or granary were 12.1% m.c. During experiments the mean moisture content of paddy inside the big bag was 12.2±0.1% and ranged between 11.9 and 12.5% (in August and July-September, respectively). The mean moisture content of paddy stored under refrigeration was 13.1±0.2% and ranged between 12.1% (at beginning of the experiments) and 13.9% (at the end of experiments).

The increase in moisture content of the paddy probably reflected the collection of samples from sections close to the aeration duct where air with high relative humidity was blown into the granary, and not necessarily the inner layers of the stored paddy bulk. On the contrary, moisture content of paddy stored in the big bag maintained the same moisture content.

The total F_1 of adult emergence population of *S. zeamais* and *S. oryzae* was counted after the experiments and numerical differences are shown in Figure 1. The F_1 adult from parent adults of *S. zeamais* was reduced up to 77%; the F_1 adult from eggs of *S. zeamais* and *S. oryzae* were also reduced up to 71% and 45%, respectively, when compared with corresponding untreated controls from the big bag.

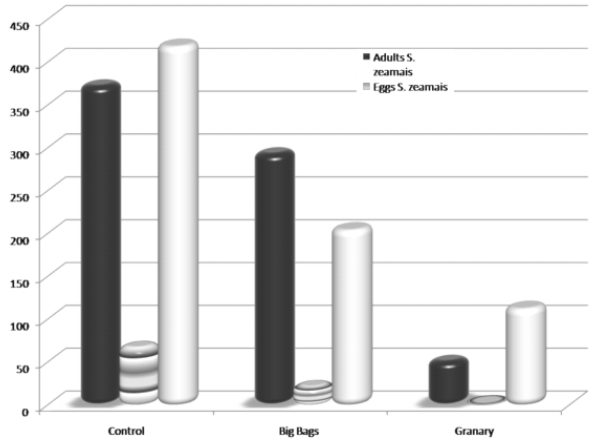

Figure 1 Total number of adults of *Sitophilus zeamais* and *Sitophilus oryzae* emerged after the experiments: from the control incubator at laboratory conditions; from control big bag under ambient conditions in the rice mill, and from the granary under refrigeration.

Refrigeration enabled to store the paddy during almost three months at around 18ºC and caused the delay of rice weevil development. As a consequence, fumigation applications were not required. Although the energy consumed during the application doubled the cost when compared to ambient-air aeration systems used during the cold season, refrigeration provided an environmentally sound and user friendly treatment.

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The effect of ultraviolet C radiation on stored-product pests

Collins. D.A.*#, Kitchingman, L.
Food and Environment Research Agency (FERA), Department for Environment, Food and Rural Affairs, Sand Hutton, York, YO41 1LZ, U.K. Email: debbie.collins@fera.gsi.gov.uk

* Corresponding author
# Presenting author

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Abstract

The potential of using ultraviolet C (UVC) radiation as an alternative treatment and hygiene measure in storage premises was investigated in the laboratory. The effect of UVC on development and progeny production was assessed for pest species of the storage beetles *Oryzaephilus surinamensis* and *Tribolium castaneum*, and the mites, *Acarus siro* and *Tyrophagus putrescentiae*. Photo-reactivation and the effect of indirect exposure were also investigated, as was the effect on spore germination of a mycotoxin-producing fungus *Penicillium verrucosum*. The ED_{95} values for *O. surinamensis*, *T. castaneum*, *T. putrescentiae* and *A. siro* were 96,549, 59,069, 22,014 and 3,802 μJ cm^{-2} respectively, when incubated in lighted conditions. There was an indication of a photo-reactivation effect with *T. putrescentiae*. Limited penetrative ability through substrates was observed at the doses assessed. Complete prevention of spore germination and complete spore destruction of *P. verrucosum* was achieved at 20,000 and 25,000 μJ cm^{-2} respectively. There was no significant difference in the numbers of *O. surinamensis*, *T. castaneum* and *T. putrescentiae* progeny produced by untreated females and females treated with a sub-lethal dose of UVC. However, there was a large degree of variation in the number of progeny produced by individual females. There was a significant reduction in the numbers of *A. siro* progeny produced by UVC treated females compared to untreated females; however, the majority of females died during the incubation period before any eggs had been laid. Practical applications of UVC within a storage environment may lie in the treatment of structural and equipment surfaces, such as conveyor systems. However, cleaning prior to treatment is an important consideration as UVC has limited penetrative ability.

Keywords: Ultraviolet C radiation; Storage beetle, Mite and fungal pests; Structural treatment; Hygiene measure.

1. Introduction

An integrated pest-management strategy is critical for the safe storage of post-harvest commodities. The use of effective hygiene measures and chemical protectants are integral parts of this strategy. However, the number of pesticides currently approved for the protection of stored commodities is very limited. Efficacy may also be affected by the development of pesticide resistant populations. Alternative non-chemical control measures are sought which can be incorporated into this pest management strategy.

In principle, ultraviolet C (UVC) radiation may provide an effective means of combating pest infestations associated with the structure of a building and may serve as a potential new hygiene measure. UVC is short wavelength (100-280 nm) radiation and is primarily used for the disinfection of air, surfaces and liquids from microbial contaminants. The UV destroys the DNA of bacteria and other microbial contaminants, thereby preventing further replication and growth. The use of UVC radiation as a method of pest control has not been extensively investigated due to the perceived risks to human health and the lack of penetration through substrates (Bruce and Lum, 1978). The limited penetration therefore precludes its use as a treatment on bulk commodities. It may, however, offer potential as a surface hygiene treatment in empty stores.

The efficacy of UVC has been previously demonstrated against house dust mites and some stored-product beetle and mite pests (Calderon and Navarro, 1971; Bruce, 1975; Bruce and Lum, 1978; Calderon et al., 1985; Needham et al., 2006; Ghanem and Shamma, 2007; Faruki et al., 2007) with sensitivity varying with species and lifestage (Beard, 1972). It is, however, difficult to make direct comparisons between studies as the level of UV dose achieved is not always stated and UV intensities vary with light sources.

Sensitivity to UVC is determined by the transmittance of surface membranes and the presence of sensitive substrate (Beard, 1972). An increased sensitivity has been demonstrated in the eggs of stored-product moths and beetles (Bruce, 1975; Calderon and Navarro, 1971; Calderon et al., 1985), with moth eggs less sensitive than beetle eggs (Faruki et al., 2007). The effect of UVC on fungal spores is also known to vary among genera, with spores that are thin walled and have a lighter pigmentation being most sensitive (Begum et al., 2009).

Sensitivity has also been found to increase with age, with eggs older than 24 h more sensitive than younger eggs (Bruce, 1975, Calderon and Navarro, 1971; Calderon et al., 1985). Eggs aged between 72 and 96 h undergo a development phase which renders them much more sensitive to UV-induced damage (Calderon et al., 1985), which may reflect changes in the nucleic acid composition or other biochemical and physiological states during embryonic development (Beard, 1972). There has also been a suggestion of a delayed effect of UV radiation on eggs, with mortality increasing when assessed 2 wks post-exposure, compared to when determined by egg hatch (Calderon et al., 1985; Faruki et al., 2007).

Photo-reactivation is an important consideration with UV treatments. Eggs placed in lighted areas after exposure to UVC radiation require a longer exposure period to produce an equivalent lethal effect than those placed in the dark (Bruce and Lum, 1978). Other indirect effects include pheromone degradation (Bruce and Lum, 1978).

The aim of these laboratory experiments was to assess the effect of UVC on the development and progeny production of two species of beetle and mite pests. Photo-reactivation and the effect of substrate were also investigated, as was the effect on spore germination of a mycotoxin-producing fungus.

2. Materials and methods

2.1. Pest species

Laboratory organophosphate (OP) susceptible strains of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), *Tribolium castaneum* (Herbst) (Coleoptera : Tenebrionidae), *Acarus siro* L. (Astigmata: Acaridae) and *Tyrophagus putrescentiae* (Schrank) (Acaridae) were used. All were reared in the laboratory in constant conditions of 25 ± 2ºC and 70 ± 5% r.h. and 15 ± 2ºC and 90 ± 5% r.h. for the beetle and mites, respectively, without exposure to pesticides. Spores of the storage fungus *Penicillium verrucosum* Dierckx (Trichomaceae) were also used.

2.2. UVC light source

A UVP CX-2000 crosslinker was used to generate the UVC at a wavelength of 254 nm. The crosslinker was calibrated using a UVP radiometer with UVX-25 sensor and found to deliver a light irradiance of approximately 9 mW cm^{-2}. The test samples were placed approximately 9 cm from under the light source.

2.3. Effect of UVC on development

Twenty eggs of each beetle (aged 24 h) and mite (aged 72 h) species were put into separate glass petri dishes (48-mm diameter, 18-mm high) and mite cells (Thind and Muggleton, 1998), respectively. Six replicates were prepared for each UVC dose, including untreated controls. The dishes and cells containing the eggs were placed singly under the light source (without lids) and exposed to five different energy levels (10,000, 20,000, 40,000, 60,000 and 120,000 µJ cm^{-2} for the beetles; 2000, 4000, 8000, 16,000 and 20,000 µJ cm^2 for *T. putrescentiae* and 500, 1000, 1500, 2000 and 4000 µJ cm^2 for *A. siro*). After exposure, the beetle eggs were transferred into glass bioassay jars (120 mL) half-filled with laboratory diet and mite food was added to the mite eggs in the cells. The jars and cells were put into the appropriate controlled conditions and the eggs incubated until adult emergence.

To assess the potential effects of photo-reactivation and substrate, experiments were set up as described above, but the eggs were either incubated in darkness or covered with food (1 g for beetles, 0.03 g for mites) prior to treatment. The effective doses (ED) required to produce 50% and 95% mortality of each pest species were calculated from the dose-mortality data using the probit analysis program PROBIT (version 7). The linear relationship between the logarithm of the dose and the probit of the percentage mortality was estimated using the maximum likelihood method (Finney, 1971).

2.4. Effect of UVC on progeny production

Single virgin pairs of each species were left for fixed periods of time (6 d for beetles, 24 h for mites) to allow mating, but before egg laying commenced. Males and females were then separated and the female insects and mites were put singly into petri dishes and mite cells, respectively. Ten replicates were prepared for each pest species and treatment (including untreated controls). Females were then exposed to UVC for sub-lethal periods of time (2 h for beetles, 12 s for mites). Food was added and test samples were incubated in the appropriate controlled conditions until F_1 development. The numbers of progeny were statistically compared to those produced by untreated females using linear regression with 95% confidence levels.

2.5. Effect of UVC on Penicillium verrucosum

Begum et al. (2009) have found that fungal spores are most susceptible to UVC when spread in a monolayer onto an agar surface. Therefore, agar plates were inoculated with approximately 1,000 spores per plate. The spores were carefully spread around the centre of the plate taking care to avoid the plate edges to ensure that no spores were shielded from the UV treatment. Five replicates were prepared for each treatment including untreated controls. The plates were placed singly and uncovered under the light source and exposed to five different energy levels (5,000, 10,000, 15,000, 20,000 and 25,000 μJ cm^{-2}). The plates were then incubated for 24 h at room temperature (~20°C) and 100 spores per plate were assessed microscopically for germination. A spore was classed as germinated if the germ tube was longer than the length of the spore. Germination counts were repeated after 2 d. The EDs required to reduce spore germination by 50% and 95% were calculated using probit analysis.

3. Results

3.1. Effect of UVC on development

Table 1 shows the ED values required to produce 50% and 95% mortality of each beetle and mite species when exposed to five doses of UVC and incubated in the different conditions. The ED_{95} values varied with species with 96,549, 59,069, 22,014 and 3,802 μJ cm^{-2} calculated for *O. surinamensis*, *T. castaneum*, *T. putrescentiae* and *A. siro*, respectively, when incubated in the light. A significant difference (P<0.05) in mortality of those incubated in light and dark was only observed with *T. putrescentiae*, with ED_{95}s of 22,014 and 14,290 μJ cm^{-2} respectively.

Table 1 UVC doses required to provide 50% and 95% mortality of each pest species.

Species	Incubation periods	ED_{50} (μJ/cm^{-2})	95% fiducial limits for ED_{50}	ED_{95} (μJ/cm^{-2})	95% fiducial limits for ED_{95}	Slope ± S.E.	Chi-square	D.F.	P-value
O. surinamensis	In light	8456	5041, 11671	96549	68750, 167571	1.56 ± 0.22	26.04	23	0.3
	In dark	6726	3668, 9690	91515	64933, 160642	1.45 ± 0.21	24.70	23	0.37
T. castaneum	In light	5678	3032, 8240	59069	44322, 93034	1.62 ± 0.24	28.76	18	0.05
	In dark	4502	2308, 6568	32009	25486, 44986	1.93 ± 0.3	10.72	12	0.55
T. putrescentiae	In light	2358	1747, 2938	22014	16541, 33254	1.7 ± 0.18	18.58	18	0.42
	In dark	669	152, 1299	14290	9444, 32208	1.24 ± 0.26	11.62	18	0.87
	With food	22716	13191, 175380	805255	128460, 1.7×10^{10}	1.06 ± 0.36	55.2	28	0.0016*
A. siro	In light	751	595, 889	3802	2969, 5555	2.34 ± 0.28	18.98	23	0.7
	In dark	760	519, 968	10371	6170, 27538	1.45 ± 0.24	25.94	28	0.58
P. verrucosum	In light	8853	7465, 10149	21393	17833, 28241	4.29 ± 0.53	196.27	15	0.0001*

Limited penetration and effect through food substrates was observed. With *O. surinamensis*, *T. castaneum* and *A. siro*, no probit line could be fitted to the data because the slope was insignificant, indicating no dose response effect. With *T. putrescentiae*, although a line could be fitted through the data, there was a lot of variation between replicates with a significant difference (P<0.05) between the experimental data and the fitted line (Table 1).

3.2. Effect of UVC on progeny production

There was no significant difference in the numbers of progeny from UVC-treated females of *O. surinamensis*, *T. castaneum* and *T. putrescentiae*, compared to those from untreated females (Table 2). There was, however, a lot of variation in the numbers of progeny produced between individual replicates. With *A. siro*, there was a significant (P<0.05) reduction in the numbers of progeny produced by UVC-treated females compared to untreated females (Table 2). However, in the majority of replicates the females had died during the incubation period before any eggs had been laid.

Table 2 Mean numbers of progeny (± S.E.) produced (n=10, *n=9).

Species	Treatment time	Mean numbers of progeny
O. surinamensis	0 (Control)	106.4 ± 21.3
	2 h	83.5 ± 6.4
T. castaneum	0 (Control)	92.8 ± 20.9
	2 h	43.1 ± 22.7
T. putrescentiae	0 (Control) *	20.9 ± 7
	12 s	7.4 ± 2
A. siro	0 (Control) *	21.9 ±6
	12 s *	1.7 ± 1

3.3. Effect of UVC on P. verrucosum

Complete prevention of spore germination and spore destruction was achieved at 20,000 and 25,000 µJ cm^{-2} respectively. An ED_{95} of 21,393 µJ cm^{-2} was calculated (Table 1), however, there was a significant difference (P<0.05) in the goodness of fit between the doses and the fitted line, which may have been due to the limited number of data points, as the top two doses were 100% effective.

4. Discussion

These experiments have demonstrated that UVC is effective at reducing development in a range of storage pests. The ED values varied widely according to species with the mites more sensitive than the beetles. Photo-reactivation was only significantly demonstrated in *T. putrescentiae*. The lack of effect on development when food was present demonstrates the limited penetrative ability of UVC through substrates. In order for treatments to be fully effective, the pests must be in direct contact with the UVC for the required duration or higher doses may be needed. Anything that is likely to shield the pest from exposure, e.g., food particles, dust, debris, cracks and crevices, will affect efficacy.

There was no effect on progeny production with *O. surinamensis*, *T. castaneum* and *T. putrescentiae*. However, there was a large variation in the number of progeny produced by individual females which may have been due to no mating having taken place, variation in egg laying or death of the female during the experiment. The insects also had a tendency to run round the edge of the petri dish, which may have shielded them from the effects of the UVC. It was observed, however, that development was slower in all species following UVC treatment compared to the untreated controls. The significant effect on progeny production in *A. siro* is likely to have been due to the majority of females dying during the incubation period. It is known from the developmental experiments, that *A. siro* is the most sensitive species to UVC and, therefore, a 12-s exposure may have been too long to produce a sub-lethal effect, even though the females were active and moving freely immediately after treatment.

A practical application of UVC to a grain surface has been demonstrated by Hidaka and Kubota (2006). A thin layer of grain, inoculated with micro-organisms, was sterilized as it passed through UVC sources whilst moving along a conveyor system. The time required to obtain a 90% sterilization rate was 6.3 h for bacteria (*Bacillus* and *Pseudomonas* spp.) and 5.6 h for mould (*Aspergillus* and *Penicillium* spp.).

Grain quality was not affected by the UV irradiation. It is, however, difficult to envisage how the entire grain surface would have been treated effectively, as in our preliminary experiments we found that fungal spores appeared shielded from the direct effects of the UVC by the structure of the grain kernel.

Practical applications of UVC within a storage environment may, therefore, lie in the treatment of structural and equipment surfaces, such as conveyor systems. However, cleaning is an important consideration as the presence of food, dust and debris may affect UVC efficacy. The costs and safety implications should also be considered.

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Survival of adults and larvae of grain beetles at lethal low temperature

Eliopoulos, P.A.*#, Prasodimou, G.Z., Pouliou, A.V.
Technological Educational Institute of Larissa, Department of Plant Production, 41 110 Larissa, Greece.
Email: eliopoulos@teilar.gr

* Corresponding author
# Presenting author

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Abstract

Survival of adults and larvae of *Tribolium confusum* Jacquelin du Val., *Oryzaephilus surinamensis* (L.) and *Trogoderma granarium* (Everts) exposed to low temperature (-16ºC) was studied in the laboratory. The main effects and interactions of exposure time (0.2, 0.5, 1, 2, 4, 8, 24 and 48 h), developmental stage (larva, adult), larval age (~3, ~18 d), and adult age (~7, ~25 d), were investigated for each species (2-way ANOVA). Probit analysis was used to determine the lethal time required for 50 and 99% kill (LT_{50} and LT_{99}) of the population of each species. All experimental beetles were unacclimated and were kept at 25ºC before cold treatment. Survival differed significantly among tested species with *T. granarium* being the most cold-tolerant followed in decreasing order by *O. surinamensis* and *T. confusum*. After 4-h exposure time 100% mortality was achieved in all cases with the exception of grown larvae of *T. granarium* and *O. surinamensis,* and adults of *T. granarium* which needed 48, 8, and 24 h, respectively. Larvae were generally more cold-tolerant than adults in all species but differences were not always significant. Main effects of exposure time, developmental stage and individual's age on mortality proved to be significant for all species. Interactions between above-mentioned factors varied significantly among tested species. Our results are discussed in terms of other studies on cold tolerance in grain beetles, and analyzed on the basis of improving the efficacy of cold treatments against stored-products pests.

Keywords: *Tribolium confusum, Oryzaephilus surinamensis, Trogoderma granarium*, Survival, Low temperature

Impact of kaolin-based particle film dusts on *Callosobruchus maculatus* (F.) and *C. chinenesis* (L.) after different storage periods of treated broad bean seeds

Mahmoud, A.E.M., El-Sebai, O.A.*#, Shahen, A.A., Marzouk, A.A.
Department of Plant Protection, Faculty of Agriculture, Al-Azhar University Nasr city, Cairo, Egypt

* Corresponding author
# Presenting author

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Abstract

Adults of *Callosobruchus maculatus* (F.) and *C. chinenesis* (L.) were exposed to broad been seeds treated with kaolin-based particle film dusts (powder) at different concentrations 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025% w/w and untreated control. The effect of kaolin powder film was clearly effective in the first month of storage period of the treated seeds resulted a 100% protection of treated seeds at high concentrations from 1.0 to 0.2 w/w for both tested insects. After three months of storage of the treated seeds only the highest two concentrations 1.0 and 0.8 w/w gave a 100% protection for both tested insects. After six months of storage of the treated seeds, kaolin powder still could protect the broad bean seeds against *C. maculatus* and *C. chinensis* attacks although the efficacy of kaolin powder decreased with aging. Thus, residual effect of Kaolin powder film was reduced by prolongation of the storage period. A negative relationship was recorded between the kaolin concentration and the tested biological parameters (number of eggs laid, hatchability, developmental period, F1 adult emergence, and seeds weight loss%) for both tested insects *C. maculatus* and *C. chinensis*. The comparison of the kaolin application methods, kaolin powder was more effective than kaolin suspension which gave a better protection to the treated seeds. Broad bean seeds viability was slightly affected by kaolin powder application, the reduction of germination was most greatest at highest concentration.

Keywords: Kaolin powder, Broad been seeds, Protection, *Callosobruchus maculatus* (F.), *C. chinenesis* (F.).

1. Introduction

Pulses such as broad beans, *Vicia faba* (L.) are important sources of proteins, fats, carbohydrates, sugars, vitamin B and minerals, which is the main component in the diet of many people (Aslam et al., 2006). It is attacked by several insect pests including Pulse Beetle, *Callosobruchus chinensis* (L.) and cowpea beetle *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae,). Both insects are considered serious pests (Ahmed et al., 2003) and cause immense damage every year to legume seeds. They attack legume seeds during the warm season and are able to generate exceedingly high levels of infestation within one or two generations on the host when conditions are acceptable (Shomar, 1963). They cause substantial quantitative and qualitative losses resulting in reductions in weight, market value and viability of seeds (Seck et al., 1996; Sekou et al., 2001).

As a result of insects' ability to rapidly increase in numbers, concerns exist over their ability to overcome the effects of contact insecticides applied to the stored legumes that are infested (Lorini, 2003). The choice of insecticides for pest control in stored legumes is limited due to food safety requirements imposed by national regulatory bodies within nations (Padin et al., 2002). Therefore this has led pest control researchers to examine alternative methods including the reappraisal of inert dusts.

The use of inert dust applied alone or in combination with other control methods is considered as a strong alternative method to the use of chemicals (Lorini et al., 2003) in protecting grains in many countries (e.g. Egypt, India and Mexico). Golob (1997) categorized inert dusts into five types which can be differentiated by their chemical composition or by their level of activity. Insect mortality is induced primarily as a result of desiccation: body water loss as a consequence of cuticle disruption by removal of epicuticular waxes (Alexander et al., 1944; Wigglesworth, 1944; Lorini et al., 2003). It has been postulated that the action of these materials is not dependent on metabolic pathways and therefore resistance should not occur (Ebling, 1971). In addition, inert dusts exhibit low mammalian toxicity of these materials (e.g. insect acute oral rate LD50 >5000 mg/kg) of body weight (Majumder, et al., 1959;

and Subramanyam et al., 1994). Also, the US Food and Drug Administration recognized the inert dusts as safe for use on grains (Banks and Fields, 1995;).

Kaolin is a naturally occurring, chemically inert clay mineral. It has been used to reduce negative impacts of environmental stresses on crop plants, to suppress diseases, and to protect crops from insect pests (Glenn and Puterka, 2005; and Kahn and Damicone, 2007). Kaolin and sand have been used traditionally as grain protectants by small–scale farmers in the developing world (Krishnakumari, 1964; Verma et al., 1976). Control of a variety of common storage insect pests with kaolin is demonstrated as effective as effective in conditions of low humidity when applied as dusts or in some cases when applied as water-based slurry (Golob, 1997). Adult populations of four stored grain insects infesting paddy treated with acid-activated kaolin at 0.75% w/w. controlled stored product insect pests up to 200 days post-treatment (Permual and le Patourel, 1990). The toxicity of acid-activated kaolin to adult populations of a number of insect species was assessed and determined effective at 72 h. *C. pusillus* and *O. surinamensis* were highly susceptible to the desiccant effect, while *T. castaneum* and the *Sitophilus* spp. were relatively tolerant. The susceptibility of all species to inert dusts decreased as paddy moisture content was increased from 7.2 to 19.2%. and F_1 progeny production was also reduced (Permual and le Patourel, 1990).

Mung bean seeds treated with inert dust resulted in 100% mortality of *C. chinensis* adults, also no adverse effect on germination was recorded for up to ten months of storage (Babbu et al., 1989). Adult *Trogoderma granarium* (Everts) exposed to rice treated with kaolin resulted in 100% mortality after 4 d at concentrations of 10000 ppm and after 2 months of exposure no offspring were observed as compared with 2800 insects produced by untreated beetles (Mostafa and Moagel, 1989). C. maculatus exposed to kaolin lead to 95% mortality of females and 100% of males with 0% mortality in the control after 6 h exposure (Keita et al., 2000). Kaolin has also been effective against both *Tribolium castaneum* (Herbst) *and Tribolium confusum* Jacquelin du Val, and appears to have a potential for use in management programs to control beetles within storage facilities (Arthur and Puterka**,** 2002).

Kaolin may also be effective in controlling insects when used in combination with other components. Admixtures of isolates of entomopathogenic fungi *Beauveria bassiana* (BbGc and BbPs) with either kaolin, talc or tapioca flour (20% w/w a.i.) applied at 0.05 g/kg on long grain rice resulted in excess of 80% mortality of *Sitophilus oryzae* (L.) adults by the 7th day of exposure. A significantly higher grain weight loss was recorded with isolates mixed with tapioca flour, compared with that of kaolin or talc after 4 months of storage (Samodra and Ibrahim, 2006).

The aim of this study is to evaluate the protection performance of kaolin powder applied to broad beans against the insect pests *C. maculatus* and *C. chinensis*, and to determine if seed viability is impacted over a number of storage periods.

2. Materials and methods

2.1. Insect rearing

Callosobruchus maculatus and pulse C. chinensis stock cultures were maintained in 1-L glass jars containing *Vicia faba* and incubated in an environmental chamber at 30 ± 2 ºC and 60 ± 5% relative humidity. Both insect species were reared in the research laboratory, Department of Plant Protection, Faculty of Agriculture, Al-Azhar University Cairo, Egypt for several generations. To distinguish between the insects, morphological characteristics are used. *C. maculatus* is a more elongate species with the posterior part of the abdomen not covered by the elytra which is more definitely spotted than *C. chinensis* (Hill, 1983). Differentiations of sexes were determined by examining the elytral pattern (Southgate et al., 1957; Halstead, 1963). Females are dark colored and possess four elytral spots. In contrast, males are pale brown and less distinguished by examination of the antennae, which are pectinate only in the male (Southgate, 1958,).

2.2. Broad bean seeds

The seeds of broad bean were collected from local stores, sieved and cleaned from dusts and inert materials. Seeds were frozen at -20ºC for 48 h to kill previous infestations then kept under room conditions for 3 wks in order to reduce their moisture content to the normal rate (Huignard, 1985).

2.3. Treatment

Kaolin powder is a white nonabrasive fine-grained aluminum silicate mineral classified under, kaolinite group $(OH)_8 AL_4 Si_4 O_{10}$).

Dust was added to the beans at concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025% w/w (powder/seeds) and were prepared by mixing kaolin powder manually with broad bean for each concentration and untreated control. Twenty broad bean seeds were taken out of the 200 g of treated seed, were weighed and then exposed to five pairs of newly emerged adults (0-2 day old) of both *C. maculatus* and *C. chinensis*. Insects were placed in small transparent glass jars and were kept in 30 ± 2 ºC and 60 ± 5% r.h. covered with muslin for aeration. The trial was replicated four times for each tested concentration and storage period. The bioassays were carried out to verify the residual effect of kaolin dusts after 1, 3 and 6 months of storage on both insect species. After exposing the tested insects to treated seeds from three different storage periods (1, 3 and 6 months), determination of adult mortality, fecundity, hatchability, and F1 adult emergence were calculated. Treated and untreated broad bean weight loss was also calculated.

2.4. Biological studies and measured criteria

Parental adult mortality of the both tested insects in each tested concentration was calculated according to Abbott (1925). Fecundity was determined by counting the number of deposited eggs within each jar 7 d after removal of the adults. This time allowed the eggs to hatch and for larvae to penetrate the seeds. Hatched eggs were counted and test jars were monitored daily to determine F1 adult emergence. After adult emergence, seeds were weighed after excluding the frass and dust, and the percent weight loss was calculated using weight loss% equation (Khare and Johari, 1984) as following:

$$\text{Weight loss\%} = \frac{(\text{Initial dry weight} - \text{Final dry weight})}{\text{Initial dry weight}} \times 100$$

Reduction in fecundity, egg viability, F_1 adult emergence, and broad bean seed weight loss and seed viability of both tested insects treated with the kaolin powder were calculated according to Abbott (1925).

2.5. Kaolin application methods

An experiment was also performed to determine if there was a difference in effect of kaolin based on application of either a dust or a suspension. Dust application was performed as the method mentioned previously in the concentrations of 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025% w/w (powder/seeds). A kaolin suspension was applied by mixing the kaolin powder in distilled water to make a kaolin suspension of five concentrations; 2, 4, 6, 8 and 10% w/v (powder/water). A treatment of distilled water was applied as control. Broad beans were treated at each concentration by sub-merging 100 g of seeds for 30 sec and then aerating the beans over night for assuring complete dryness. Twenty broad bean seeds were taken from each 100 g treated seed at each concentration, were weighed and then exposed to five pairs of newly emerged adults (0-2 day old) of *C. maculatus* and *C. chinensis*. Each test was placed in small transparent glass jars and the trial was replicated four times for each concentration. Jars were kept at 30 ± 2 ºC and 60 ± 5% r.h. covered with muslin for aeration.

2.6. Seeds germination

The viability of seeds treated with kaolin was also examined. Broad bean seeds previously treated with all tested concentrations of kaolin powder were stored at room conditions and tested after initial treatment and six month intervals of storage by placing 25 seeds on a cotton pad soaked with water in Petri dishes. The experiment was replicated four times for the treated and untreated beans and germination was observed and recorded.

2.7. Statistical analysis

Percent parental adult mortality, fecundity, egg viability, adult emergence of both insect species and percent of weight loss and viability of broad bean seeds treated with the kaolin were subjected to statistical analysis by Analysis of variance (ANOVA) test and linear regression analyses using a

computer software SAS (SAS Institute, 2000). Means were determined and compared by Duncan multiple range test at 0.05% probability level (Duncan, 1955).

3. Results

3.1. Influence of kaolin after different storage periods

3.1.1. Parental adult mortality

Beans treated with concentrations from 0.8 to 1.0% w/w of kaolin powder and exposed to insects after the first month of storage showed 100% mortality after six hours of exposure period to treated seeds. Concentrations of 0.6 to 1.0% w/w resulted in 100% adult mortality with seven days of exposure period. Insects exposed to kaolin treated beans after three months of storage also had 100% mortality at 0.8 and 1.0% w/w within 2 d (Table 1). Regression analysis indicate a positive correlation between the mortality percentage of exposed insects and the concentration of kaolin powder (R= -0.74) and (R=0.69), respectively (Table 1). Results also revealed that after 6 mo of storage, seeds treated at 1.0% w/w concentration still showed a high level of adult mortality (66 and 73% compared to the untreated control 3.7 and 2.5%, for *C. maculatus and C. chinensis* respectively).

Table 1 Effect of kaolin powder on the fecundity, hatchability, life cycle, adult emergence and seeds weight loss of *Callosobruchus maculatus and C. chinensis* at different storage periods.

		Adult parents Mortality (%)		**Number of eggs laid**		**Hatchability (%)**		**Number of F1 Emerged adults**		**Weight loss (%)**	
Storage Period	**Conc. (%) w/w**	**Macu-latus**	**Chinen-sis**	**Macu-latus**	**Chine n-sis**	**Macu-latus**	**Chine n-sis**	**Macu-latus**	**Chine n-sis**	**Macu-latus**	**Chinen-sis**
Month 1	1	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.8	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.6	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.4	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.2	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.1	68	78	13	10	32	23	1.7	0.7	1.2	0.7
	0.05	46	57	38	25	44	45	6.78	5	3.6	4.7
	0.025	19	37	68	40	66	55	20	8.	9.9	8.2
	Ave.	79	84	15	9.37	17.78	15	3.6	1.7	1.9	1.7
	Control	5	5	373	283	92.4	87.14	212.5	23.02	30	23.
	R-value	0.7	0.7	-0.5	-0.5	-0.7	-0.7	-0.4	-0.5	-0.5	-0.5
	Slope	14.9	12.9	-153.7	-112.4	-69.3	-62.5	-76.9	-11.3	-13.9	-11.3
Month 2	1	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
	0.8	100	100	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0
	0.6	82	56	19	16	33	38.	8	2	4.4	2.6
	0.4	68	44	37	45	44	48	19	10	6.3	9.1
	0.2	56	33	60	58	56	57	26	14	9	13.7
	0.1	38	26	86	80	65	66	36	22.	14	16.9
	0.05	19	18	112.	115	72	71	55	48.	20	21.5
	0.025	3	8	197	190	81	78	88	78	23	25.1
	Ave.	58	48	64	63	44	45	29	22	10	11.1
	Control	2.5	2.5	325.	298	92	89	217.5	167	33	27.5
	R-value	0.9	0.97	-0.79	-0.8	-0.9	-0.9	-0.97	-0.7	-0.8	-0.9
	Slope	19.3	18.8	-250.2	-205.8	-87	-84.1	-87.5	-97.2	-25.7	-27.2
Month 3	1	66	73	50	45	50	40	9	6.8	3	4.1
	0.8	59	55	63	55	58	48	15	13	8	7.6
	0.6	49	42	83	80	68	56	24	18	12	11.5
	0.4	38.9	32	102	98	69	60	34	28	16	16.5
	0.2	32	24	130	132	67	61	55	45	21	19.4
	0.1	27	17	160	170	79	70	78	55	23	22.4
	0.05	21	10	220	243	84	81	113	90	24	24.7
	0.025	6.5	6	258	265	86	88	167	130	26	26.7
	Ave.	33	33	133	136	70	63	62	48	16	16.6
	Control	3.7	2.5	320	298	92	90	215	175	27	28.5
	R-value	0.9	0.9	-0.9	-0.9	-0.9	-0.9	-0.8	-0.8	-0.9	-0.9
	Slope	10.9	12.7	-218	-229	-35	-43.7	-158	-125.1	-23.7	-23

When data from all storage periods are averaged, the percent mortality of adult *C. maculatus and C. chinensis* increased significantly (P=0.062) as concentration of kaolin on broad beans (56.92 and 54.82% compared to the untreated 3.75 and 3.33%, Table 2).

Table 2 Effect of kaolin powder on the fecundity, hatchability, life cycle, adult emergence and seeds weight loss of *Callosobruchus. maculatus and C. chinensis.*

Concentration (%) w/w	Adult parents mortality (%)		Number of eggs laid		Hatchability (%)		Number of F1 Emerged adults		Weight loss (%)	
	Macu-latus	*Chinen-sis*	*Macu-latus*	*Chinen-sis*	*Macu-latus*	*Chinen-sis*	*Macu-latus*	*Chinen-sis*	*Macu-latus*	*Chinen-sis*
1	88.74	91.02	16.66	15.00	16.76	13.40	2.91	2.25	1.03	1.37
0.8	85.28	85.04	20.83	18.33	19.48	15.83	5.00	4.16	2.58	2.54
0.6	77.13	66.23	33.91	32.08	33.56	31.57	10.50	6.33	5.35	4.70
0.4	68.96	58.54	46.66	47.50	37.63	36.15	17.41	12.33	7.27	8.55
0.2	62.95	52.56	63.33	63.33	41.19	39.31	27.08	19.58	9.86	11.05
0.1	44.71	39.97	86.25	86.66	58.94	52.86	38.50	26.08	12.76	13.37
0.05	28.68	28.25	123.33	127.5	66.68	65.45	58.08	47.75	16.03	16.96
0.025	09.59	16.98	174.16	165.0	77.72	73.73	91.66	71.75	19.72	19.98
Control	03.75	3.33	339.16	292.5	92.17	88.66	215.00	175.0	29.91	26.33
F-value	55.40		35.59		51.13		32.79		2.88	
P=0.05	0.0001		0.001		0.0001		0.0001		0.005	

3.1.2. Fecundity

Concentrations from 0.2 to 1.0% w/w of kaolin powder was clearly effective in reducing egg production by both insect species the first month of storage as exposed adults died within a few hours. After 3 months of storage, only concentrations of 0.8 to 1.0% v/w gave a 100% protection for both tested insects (Table 1). Regression analysis indicates a negative correlation between the number of eggs laid and the concentration of kaolin (R= -0.468) and (R= -0.451), for *C. maculatus* and *C. chinensis* respectively (Table 1). Results also indicate that after six months of storage, seeds treated with 1.0% w/w concentration have a persistent effect as a seed protectant against bruchid fecundity where eggs laid averaged 50.00 and 45.00 eggs/female compared to 320.00 and 298.00 eggs/female in controls for *C maculatus and C. chinensis*, respectively.

Table 3 Residual effect of Kaolin powder on *Callosobruchus maculatus and C. chinensis* after three different storage periods of treated and untreated broad bean seeds.

Treatment	Storage period (months)	Adult parents mortality (%)		Number of Eggs laid		Hatchability (%)		Number of F1 Emerged adults		Weight loss (%)	
		Macu-latus	*Chinen-sis*	*Macu-latus*	*Chinen-sis*	*Macu-latus*	*Chine n-sis*	*Macu-latus*	*Chinen-sis*	*Macu-latus*	*Chinen-sis*
Treated	1	79.27	83.88	14.68	9.37	17.77	15.43	3.56	1.69	1.853	1.694
	3	58.33	48.07	64.12	62.96	43.85	44.83	28.90	21.68	9.707	11.152
	6	33.17	32.53	133.12	135.93	70.36	62.85	61.71	48.03	16.426	16.613
	Average	56.92	54.82	70.64	69.42	43.99	41.04	31.39	23.80	9.328	9.819
Untreated	1	5.0	5.0	372.50	282.50	92.39	87.14	212.50	23.02	30.089	23.024
	3	2.5	2.5	325.00	297.50	92.15	89.07	217.5	167.5	32.651	27.485
	6	3.75	2.5	320.00	297.50	91.97	89.77	215.00	175.00	27.004	28.503
	Average	3.75	3.33	339.16	292.50	92.17	88.66	215.00	121.84	29.914	26.337
F-value		2.83		0.30		0.90		0.30		0.00	
P-value		0.062		0.0001		0.0001		0.05		0.0001	

The impact of kaolin powder on the fecundity of both insect species regardless of concentration significantly reduces the number of eggs laid (P=0.0001) on the treated broad beans (Table 3). The reduction of mean eggs laid over the three storage periods averaged 78.0 – 76.0%, for *C maculatus and C. chinensis* respectively (Table 4).

3.1.3. Egg viability

No eggs were laid by insects exposed to beans treated with concentrations from 0.2 to 1.0% w/w of kaolin powder. Regression analysis indicates a negative correlation between the percent hatchability and

the concentration of kaolin powder (Table 1). Results also reveal that after six months of storage, seeds treated at 1.0% w/w have a persistent effect as a seed protectant against bruchid fecundity averaging 50 and 40 egg/female compared to the untreated control 92 and 90 egg/female, respectively for *C. maculatus and C. chinensis*. The viability of eggs produced by both species decreased significantly (P=0.0001) and (P=0.0005) when adults were placed on treated broad beans (44.0 and 41.0%) compared to the untreated (92.1 and 88.7%) in all periods (Table 2). Consequently, the reduction of egg viability reached to 80% and 82% in the first month of storage and was reduced about 50% after 3 and 6 months (Table 3). In general, hatchability was over 50% for both insect species (Table 4).

Table 4 Influence of kaolin powder on *Callosobruchus maculatus and C. chinensis* biological aspects after different storage periods.

Storage period (months)	Reduction % of Number of eggs Laid		Reduction % of hatchability		Reduction % of F1 emerged Adults		Reduction % of weight loss	
	Maculatus	*Chinensis*	*Maculatus*	*Chinensis*	*Maculatus*	*Chinensis*	*Maculatus*	*Chinensis*
1	96.0	96.0	80.0	82.0	98.0	92.0	93.84	92.64
3	80.0	78.0	52.0	49.0	86.0	87.0	70.27	59.42
6	58.0	54.0	23.0	29.0	71.0	72.0	39.17	41.71
Average	78.0	76.0	52.0	53.0	85.0	84.0	67.76	64.59

3.1.4. F_1 adult emergence

Overall, the number of F1 adults produced by both insect species increases as treatment concentration decreases and as storage time is increase. Survival of F_1 adults of both insect species decreased significantly (P=0.0001), in the treated broad bean beans averaging 31.39 and 23.80 adults compared to the untreated 215.00 and 121.84 for all storage periods .(Table 3). Reduction of F1 adults reached to 98% and 92% for *C. maculatus* and *C. chinensis* for the first month of storage while F1 adults were reduced from 70 to 87% for 3 and 6 months of storage (Table 4). Generally, the potential effect of kaolin reduced of F1 emergence by approximately 85.00 and 84.0% for both tested insects, respectively (Table 4).

3.1.5. Seeds weight loss

There was no weight loss of beans observed at higher application rates of kaolin and at shorter storage durations, (Table1) and regression analysis indicates a negative correlation between the weight loss and the concentration of kaolin (R= -0.52) and (R= -0.55), respectively for the insect species (Table 1). *Callosobruchus maculatus and C. chinensis* caused a significant weight loss to the broad beans regardless to the concentration of kaolin (P=0.0001). Treated broad bean were reduced by approximately 9.5% compared to the untreated where weight loss averaged 28% for the untreated beans over all storage periods (Table 3). The first month of storage showed the least bean weight loss (1.7%) for treated beans while untreated beans lost an average of up to 93%. The weight loss of treated beans for the other two storage periods (3 and 6 months) was relatively lower, ranging form 39 to 70% (Table 4). Generally, kaolin application reduced the weight loss with the average mean of 67.76 and 64.59% for the insect species, respectively (Table 4).

3.2. Kaolin application methods

Kaolin application methods were compared by exposing the two insect species to treated broad bean seeds with kaolin powder or kaolin suspension. Results in Table 5 illustrated that kaolin powder was more effective than kaolin suspension in all criteria tested. The number of eggs laid, F1 adults and seed weight loss were all greater for the dust application than the suspension application (Table 5).

Table 5 Comparison of kaolin application methods on the biological aspects of *Callosobruchus maculatus and C. chinensis*.

Tested insects	Treatment	Number of eggs laid		F1 emerged adults		Seeds weight loss (%)	
		Suspension	Powder	Suspension	Powder	Suspension	Powder
maculatus	Treated	138.25	70.64	63.85	31.39	15.34	9.32
	Untreated	340.00	345.00	230.00	235.0	24.75	26.25
chinensis	Treated	116.00	69.42	47.75	23.80	12.341	9.82
	Untreated	252.50	292.5	90.41	121.8416	15.75	26.33
P=0.05		0.022		0.008		0.014	

3.3. Seed viability

The effect of kaolin powder treatment on broad bean seeds germination is presented in Table 6. Results showed slight differences between untreated and treated beans in the germination percentage at all tested concentrations. Germination decreased in the untreated seeds from 92 for the initial time to 81.33% after six months of storage period. The general mean of the germination reduction% of the two tested storage periods regardless to the concentration averaged 2.04 and 3.02%, respectively. In conclusion, while there is a trend, kaolin application has no adverse effect on broad bean viability.

Table 6 Effect of kaolin powder on germination of treated broad bean seeds at two different storage periods.

	Initial time		After 6 months	
Conc.(%) w/w	**Germination(%)**	**Reduction (%)**	**Germination (%)**	**Reduction (%)**
1	88.66	3.63	77.00	5.32
0.8	89.33	2.90	77.00	5.32
0.6	89.00	3.26	78.33	3.69
0.4	89.33	2.90	78.66	3.28
0.2	90.33	1.81	79.00	2.87
0.1	90.66	1.46	79.66	2.05
0.05	91.66	0.37	80.33	1.23
0.025	92.00	0.00	81.00	0.41
Average	90.12	2.04	78.88	3.02
Control	92.00		81.33	
R-value	-0.90		-0.94	
Slope	-3.23		-4.04	

4. Discussion

The effectiveness of kaolin powder as a control measure against *C. maculatus and C. chinensis* is clearly apparent. At high concentrations, adult mortality, fecundity, egg viability and F1 adults were all affected by kaolin. Kaolin powder at concentrations of 8000 and 10,000 ppm provides protection for broad bean seeds for up to 6 months (Table 3). These results agreed with Strong and Sbur (1963) who reported wheat kernels were completely riddled by the end of 6 months after exposing to *Sitophilus oryzae*. While, treated wheat with inert dusts would prevent infestations for 6 months. Persistence of inert dusts has been demonstrated effective even after 3 and 6 months of storage by maintaining parental mortality and reducing progeny emergence for 4 stored grain insects (Stathers et al., 2002). Kaolin application controlled four stored grain insects up to 200 days post-treatment (Permual and le Patourel, 1990). The effectiveness of kaolin on newly emerged adults of *T. granarium* mixed with rice grains demonstrated 100% mortality after 4 d of treatment at 10000 ppm. After two months of exposure parents did not produce any offspring as compared with 2800 insects produced by the untreated beetles (Mostafa and Moagel, 1989). A significantly higher grain weight loss was recorded with isolates of *Beauveria bassiana* mixed with tapioca flour, compared with that of kaolin or talc after 4 months of storage (Samodra and Ibrahim, 2006). Prolonging storage period reduces the effectiveness of kaolin powder against the two tested insects, likely due to increasing the moisture content of the kaolin particles. This leads to changes in the physical properties that reduces the kaolin particles efficiency to disrupt the insect cuticle.

Results in Table 4 indicated that reduction of the tested biological aspects reached highest values in the first month of the storage period, then declined gradually as the storage period prolonged. Mahdi and Khalequzzaman (2006) found that the efficacy of inert dusts against insects depends greatly on the physical properties of the dust. These materials prevent insect infestations forming natural physical barrier deters insect infestation and impedes their movement, feeding and egg laying (Shadia abd El-Aziz, 2003).

Kaolin powder application showed high performance in protection of seed against bruchids than the kaolin suspension application. This may be due to the changes of physical properties of kaolin particles as a result of mixing with water. Consequently, kaolin particles get soft, and this impacts its ability to disrupt the insect cuticle. Our results show that the number of eggs laid, F1 emerged adults and seed weight loss was clearly higher in the powder application than in suspension application Kaolin powder offers unsuitable surface for laying eggs for the female than the surface treated with suspension and

reduces the number of eggs laid by approximately half. Kaolin applications are most effective in conditions of low humidity, also when applied as dusts but some retain activity even when applied as water-based slurry (Golob,1997). The efficacy of inert dusts is very sensitive to grain moisture content (Permual and le Patourel, 1990). Application of kaolin powder led to 95% mortality of *C. maculatus* females and 100% of males with 0% of mortality in the control after 6 h exposure (Keita et al., 2000). Treated mung bean with kaolin powder resulted in a 100% mortality of *C. chinensis* adults (Babbu et al., 1989).

Kaolin application had very slight adverse effect on broad bean seeds germination in the highest concentration, and no adverse effect on the low concentration of kaolin. Babbu et al. (1989) reported that mung bean mixed with kaolin powder had no any adverse effect on treated mung bean seeds germination for up to 10 months of storage period. Treated broad bean seeds with kaolin powder showed slight adverse effect seed viability for up to 6 months of storage period. Therefore, the use of kaolin inert dusts should be encouraged as stored grain protectant due to its effectiveness and being non-toxic material.

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The effect of diapause, cold acclimation and ice-nucleating bacteria on the cold-hardiness of *Plodia interpunctella*

Fields, P.G.*#[1], Timlick, B.[2]
[1] Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Rd.,Winnipeg Manitoba R3T 2M9, Canada. Email: paul.fields@agr.gc.ca
[2] Canadian Grain Commission, 844 – 303 Main St., Winnipeg, Manitoba R3C 3G8, Canada.

* Corresponding author
# Presenting author

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Abstract

Laboratory tests showed that over 50 d at 0°C, over 10 d at -5°C or 1 d at -10°C were required to control non cold-acclimated 5th instar larvae of *Plodia interpunctella* (Lepidoptera: Prylidae) (Indian meal moth). To control the most cold-hardy stage, diapausing cold-acclimated 5th instar larvae over 14 d at -10°C or 1 d at -15°C were required. A freeze-out of a single floor of a seed warehouse was carried out from 23 December 1993 until 2 January 1994 by shutting off the heat and opening the windows. The lowest temperatures achieved varied with the size and location of the seed bulk. Seed packets, that had a few grams of seed, reached -17°C, whereas, the middle of a bag stack with forty 50-kg bags of maize only reached -9°C. By the end of the 10-d freeze-out all non cold-acclimated *P. interpunctella* larvae were killed in the packets, 90% of the diapausing cold-acclimated larvae were killed in a single bag and 65% were killed middle of the stack. Mortality in the freeze-out was higher than we would have predicted from the laboratory data. The supercooling points (temperature at which freezing begins) of the *P. interpunctella* larvae range from -7°C for the non-diapausing non-acclimated to -13°C for the diapausing acclimated.

Keywords: Indian meal moth, Warehouse, Larvae, Supercooling point

1. Introduction

To survive the winter, insects in temperate areas often enter into diapause and increase their cold-hardiness before the onset of cold temperatures. The relationship between diapause, endocrine-mediated dormancy, and cold hardiness (an increase in the capacity to survive low temperatures) is unclear for most species, and it is only recently that there have detailed studies in this area (Denlinger, 1991). Denlinger (1991) describes four possible cases of the interaction between diapause and cold-hardiness: cold-hardiness without diapause, cold-hardiness coincidental but independent of diapause, cold-hardiness dependant on diapause and diapause independent of cold-hardiness (eg. summer diapause).

We believe that *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) the Indian meal moth offers a way to study this relationship, because it has an extensively studied facultative diapause (Tzanakakis, 1959; Bell, 1976; Bell et al., 1979; Kikukawa and Masaki, 1984). *Plodia interpunctella* is a cosmopolitan pest of stored grain, commodities and processed food (Rees, 2004). In comparative tests, it is one of the most cold-hardy stored-product insects (Mansbridge, 1936; Solomon and Adamson, 1955). There have been a few studies examining the cold-hardiness of *P. interpunctella* (Cline, 1971; Le Torc'h, 1977; Fields, 1992; Carrillo et al., 2006). However, there is only one study examining the relationship between diapause and cold-hardiness (Tzanakakis, 1959), but this study only used insects that had a short cold acclimation, (1 d at 5°C and 1 d at 0°C) and only used one exposure temperature (-5°C).

The use of extreme temperatures, in particular extreme low temperatures has been used to control stored product pests for many years (Fields, 1992; Mason and Strait, 1998). In western Canada, flour mills regularly use "freeze-out" techniques in their facilities during the winter to control insect pests (Worden, 1987). The effect of the insect's developmental stage and degree of acclimation, as well as the temperature, relative humidity and the duration of exposure are all variables, which will impact the success of a freeze-out (Fields, 1992).

The purpose of this study was to examine the cold-hardiness of *P. interpunctella* with and without diapause and with and without cold acclimation under a range of low temperatures, and to determine the effectiveness of freeze-outs in a seed warehouse.

2. Materials and methods

2.1. Low temperature survival in laboratory

Three times weekly, approximately 50 mg of *P. interpunctella* eggs were placed in 4-L jars with 1.5 kg of hard spring wheat and 250 g of wheat : wheat germ : honey : glycerol (ratio by weight, 40:5:1:1). The culture originated from individuals captured at a seed packaging plant in southern Manitoba, Canada. New insects captured at the seed plant were added to the culture regularly throughout these experiments.

To induce diapause, insects were reared at 25°C, 60% r.h. and 16 h light and 8 h dark for 2 wk, then placed at 20°C, 12 h light and 12 h dark for 4 wk. These conditions were shown to induce diapause in *P. interpunctella* (Kikukawa and Masaki, 1984). Corrugated cardboard discs were introduced into the jars when the wandering stage appeared. Larvae crawled into the discs and spun cocoons and entered into the prepupal stage. Discs were left for approximately 2 wk after the cessation of wandering stage to ensure that all the individuals were in diapause. Non-diapausing insects continued development and emerged as adults within two wk. To obtain non-diapausing pre-pupae, insects were held at 25°C, 60% r.h. and 16 h light and 8 h dark. Discs from the non-diapausing colony were removed for testing after the cessation of wandering. Any adults emerging from these discs were immediately counted and removed. These rearings were simultaneous, so that there was a regular supply of diapausing and non-diapausing larvae.

Discs with diapausing and non-diapausing larvae were divided into two groups; one group was subjected directly to low temperatures (non-acclimated) while the other was placed at 10°C for 4 wk (acclimated) before being placed at the low temperatures. Groups of discs from each of the four categories (acclimated diapausing, acclimated non-diapausing, non-acclimated diapausing, non-acclimated non-diapausing) were subjected to one of four temperatures (0, -5, -10, or -15°C) for a number of predetermined time periods (1 to 63 d) to ascertain the mortality due to cold injury. Sets of controls from each group were established on three occasions over the course of the experiment to determine if acclimation caused mortality before the insects were subjected to low temperatures. There were 75 to 200 insects per time-temperature-stage combination.

After the discs were removed from the low temperatures, they were placed at 25°C and 16:8 L:D photoperiod. Adults were counted and removed regularly. After adults ceased to emerge, discs were dismantled and counts were made of the dead prepupae, pupae intact (dead) and empty pupal cases.

2.2. Supercooling points

The supercooling point of *P. interpunctella* was determined for pupae and the 5th instar larvae of the four previously described stages (non-diapausing non-acclimated, non-diapausing acclimated, diapausing non-acclimated and diapausing acclimated). To determine the supercooling point for insects influenced by ice nucleators, eggs were placed on wheat treated with 0, 100 or 500 ppm of Snomax™ (Johnson Controls Inc, USA). Snomax (Fields et al., 1995) is an ice nucleating bacteria product used in artificial snowmakers. To obtain this potent source of ice nucleators, *Pseudomonas syringae* (strain 31a), a common foliar bacterium isolated from maize leaves, is grown under conditions that maximize its ice-nucleating activity. Approximately 1.5 kg of wheat was placed in 4 -L jars with Snomax. Jars were rolled for 2 min to thoroughly mix the Snomax into the wheat. Approximately 500 one-d old eggs were placed on each treatment and were incubated as described previously to induce diapause. The insects were not cold-acclimated.

To determine supercooling points insects were placed on thermocouples within a Styrofoam container. When placed into a freezer at -40°C the inner part of the container cooled at approximately 1°C /min. The container was made 2 pieces of circular Styrofoam, each 5 cm deep with a radius of 20 cm. Forty-two wells, each 2.5 cm deep with 0.75 cm radius were constructed in the Styrofoam, with thermocouples emerging through the base of each. Insects were placed into small plastic funnels placed over each thermocouple to ensure the insect came to rest against the thermocouple. After placing the container in the freezer, temperatures were recorded every second until the heat of crystallization, or supercooling point occurred.

2.3. Freeze-out in commercial seed packing plant

The seed packing plant received seeds from across North America in bags with 2 to 50 kg of seed per bag. Seed was packaged into small retail packets with a few grams of seed and sold to home owners for small gardens. The bulk-seed floor at a seed packaging plant was used as a freeze-out area for controlling *P. interpunctella* that may be present in bulk-seed bags or retail packets. This area was conducive for to a freeze-out situation for a number of reasons:

1) no water pipes to drain and risk freezing
2) windows on all walls
3) plant operations cease for the Christmas holidays. Therefore, when the plant closes and staff goes on vacation, windows in this area can be opened and the temperature can be allowed to drop.

There were of two types of *P. interpunctella* larvae tested. One set of insects were non-diapausing and non-acclimated larvae placed into seed packets and placed on the bulk-seed floor 2 d before the freeze-out. These insects represent individuals found in the heated areas of the plant. The temperatures in this part of the plant were between 15-25°C. Nineteen packets were removed on 17, 26, 31 December 1993 and 6 January 1994 or at 0, 9, 14 and 20 d after being set up on the bulk-seed floor. There were 10 insects/seed packet.

The second type of insect was composed of individuals allowed to develop on the bulk-seed floor. These insects were considered to be diapausing and acclimated prepupae at the onset of the freeze-out. The temperatures on this floor were between 8-17°C during the 4 mo proceeding the freeze-out. These insects were placed into tubes and put into various spots in the bulk-seed floor. Fifty insects were placed per 25 mL tubes containing approximately 15 g of wheat. Six tubes were placed at each of 4 locations: 1) Single bag: tubes inserted into the centre of a 50-kg bag of maize that was alone on a pallet, 2-4) Stack: tubes inserted into the middle of the top, middle and bottom bags of a stack of forty 50-kg bags of maize located on a pallet. Two tubes from each location were removed on the same dates as the seed packets.

The windows were opened on 23 December 1993 and closed on 2 January 1994. Temperatures are measured hourly by placing thermocouples at various locations and within bulk and packaged material to determine the effect of thermal mass. After removal from the freeze-out, packets and tubes were placed at 25°C and 70% r.h., and the number of emerging adults noted.

3. Results

3.1. Low temperature survival in laboratory

Regardless of the insects state, the lower the exposure temperature, the more rapidly the insects died (Fig. 1). At -15°C, all insects died within 1 d, at -10°C, all insects had over 89% mortality, after 14 d and at 0°C, it took 49 d before the non-diapausing groups had over 90% mortality. Diapausing acclimated larvae were the most cold-tolerant of all groups. Diapausing non-acclimated larvae were the next most cold-hardy. For the non-diapausing insects, there was not a great difference in acclimated and unacclimated insects at 0°C, but at -5°C and -10°C the acclimated insects were more cold hardy. However all non-diapausing insects were dead after 14 d at -5°C or -10°C.

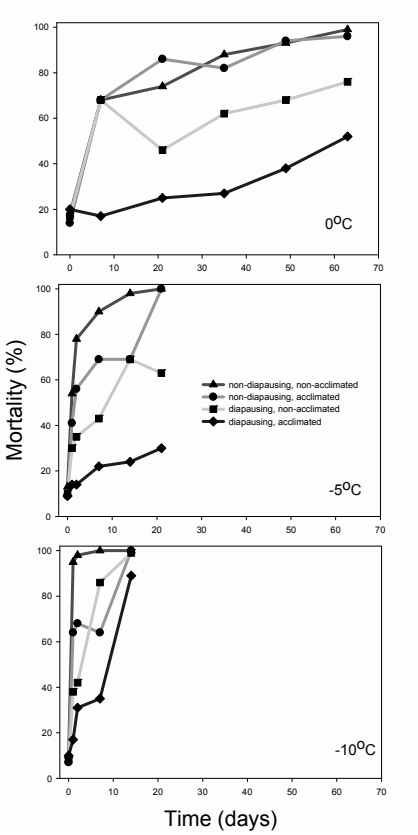

Figure 1 Mortality of 5th instar *Plodia interpunctella* larvae reared under various conditions.

3.2 Supercooling points

Pupae had the highest supercooling point at -5.0°C. The non-diapausing non-acclimated insects had a supercooling point of -7.4°C (Table 1). This explains why only 5% survived 1 d at -10°C (Fig. 1). *Plodia interpunctella* in other stages have supercooling points around -10°C or lower and had much greater survival at -10°C (Table 1, Fig. 1). Acclimation lowers the supercooling point a few degrees (-9.8°C), as does diapause (-10.7°C). The most cold hardy stage, diapausing acclimated, also had the lowest supercooling point (-13.4°C). Ice nucleating bacteria at 500 ppm significantly reduced the supercooling points, but had no effect at 100 ppm (Table 2).

Table 1 Supercooling points of pupae and 5th instar larvae of *Plodia interpunctella*, with different physiological states.

Stage	Diapuase[1]	Acclimation[2]	Snomax (ppm)	Supercooling points (+ SEM, °C)[3]	N
Pupae	ND	NA	0	-5.0 ± 0.2 a	11
Larvae	ND	NA	0	-7.4 ± 0.6 b	16
Larvae	ND	A	0	-9.8 ± 0.6 c	13
Larvae	D	NA	0	-10.7 ± 0.4 c	18
Larvae	D	A	0	-13.4 ± 0.5 d	11

1. ND = non-diapausing (25°C , 16L:8D), d = diapausing (20°C, 12L:12D); 2. NA = non-acclimacted (25°C), A = cold-acclimated (10°C for 4 weeks); 3. Means with the different letter are significantly different, Tukey's multiple range test, $P<0.05$.

Table 2 Supercooling points of 5th instar larvae *Plodia interpunctella* in different forms, (see Table 1 for footnotes).

Stage	Diapuase[1]	Acclimation[2]	Snomax (ppm)	Supercooling points (+ SEM, °C)[3]	N
Larvae	D	NA	0	-10.7 ± 0.4 a	16
Larvae	D	NA	100	-9.8 ± 0.4 a	20
Larvae	D	NA	500	-7.2 ± 0.5 b	18

3.2. Freeze-Out

The non-acclimated non-diapausing insects had 100% mortality at the end of the freeze-out. Insects held in a single bag on a pallet had 90% mortality at the end of the freeze-out. *Plodia interpunctella* larvae placed in the top bag of a stack had 80% mortality, while those placed in the middle and bottom of the stacks had the least mortality at 65 and 69% at the end of the freeze-out (Fig. 2A). These mortalities were related to the temperatures that the insects were exposed (Fig. 2B). The seed packets on the table reached -17.1°C, the single bag reached -15.2°C, the top bag of a stack reached -12.5°C, the middle bag of a stack reached -9.3°C and the bottom bag of a stack reached -10.6°C. Mortality of insects in the seed plant was greater than what we predict from the laboratory tests with diapausing acclimated insects. For example, at the end of the freeze-out the insects in the middle bag of the bag stack had 65% mortality, and were exposed to about 6 d between -5 and -10°C. In the laboratory diapausing acclimated insects held for 7 d at -5 or -10°C had only 22 to 33% mortality (Table 1).

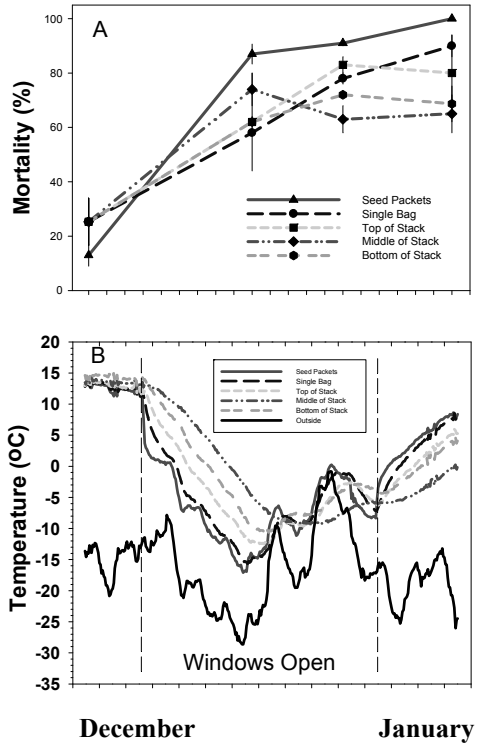

Figure 2 **A:** Mortality of 5th instar *Plodia interpunctella* larvae placed in small seed packets, at the centre of a single 50-kg bag of maize placed on a wooden pallet, or in the centre of a 50-kg bag of maize in the top, middle or bottom bag of a forty-bag stack placed on a pallet. **B**: Temperatures outside a seed warehouse in Manitoba Canada and inside the warehouse (see above). Windows were opened on 23 December 1993 and closed on 6 January 1994.

4. Discussion

Plodia interpunctella larvae increased cold-hardiness both with and without diapause. Although, as is noted by Denlinger (1991), the cold-hardiness that is associated with diapause is increased by the onset of cool temperatures. Tzanakakis (1959) showed that diapause increases the cold-hardiness of *P. interpunctella* compared to non-diapausing insects. Non-diapausing insects increased their cold-hardiness after a brief period of cold acclimation (1 d at 5°C and 1 d at 0°C), however diapausing insects did not. Our study showed that, acclimation increased the cold-hardiness of both diapausing and non-diapausing larvae. This is probably due the longer cold acclimation period in our studies compared to Tzanakakis (1959).

We obtained similar cold-induced mortality with the non-acclimated non-diapausing larvae as previous studies (Tzanakakis,1959; Le Torc'h, 1977, Carrillo et al., 2006). There was 100% mortality at -5°C after 4 d (Tzanakakis, 1959), whereas there was 78 and 90% mortality after 2 and 7 d at -5°C respectively in this study. There was 94% mortality at 2°C after 40 d (Le Torc'h, 1977), similar to our study, where we exposed insects to 0°C for 35 or 49 d and obtained 88 and 93% mortality respectively. There was 100% mortality of non-diapausing non-acclimated larvae after 16 h at -10°C (Carrillo et al., 2006) compared to 95% mortality after 24 h in our study. For field cold-acclimated larvae, Carrillo et al. (2006) found it required 13 d at -10°C for 100% mortality, compared to 89% mortality after 14 d in this study. It is more difficult to make direct comparisons for the other treatments, as our regime for diapause induction and cold acclimation was different than Tzanakakis (1959).

Carrillo and Cannon (2005) did an extensive study of the supercooling points (SCP) of *P. interpunctella*. They found that the SCP of unacclimated 5th instar larvae was -14.1°C, (-7.4°C, this study) which dropped to -22°C (-13.4°C, diapausing acclimated this study) when larvae were reared outside to enter diapause and cold acclimate. They measured pupal SCP at -22.2°C, much lower than the -5.0°C SCP we observed for pupae. In general, they found much lower SCP than we observed. They did observe some differences between strains, but it is unlikely that the large differences in the SCP between studies could be just because of different strains. Calibration errors could account for some differences. Field acclimation they subjected the insects to was likely to yield more cold-hardy individuals with lower SCP then the laboratory acclimation in our study. These insects in Carrillo and Cannon (2005) may have been subjected to a different expenditure of energy reserves and/or stresses on their metabolic pathways that regulate cryoprotection that impacts the populations success through diapause (Hahn and Denlinger, 2007)

Freeze-outs provided this company with a simple, non-chemical, inexpensive method to control insects in their seed warehouse. Not all insects were controlled in the large bag stacks with pallets of 40 bags. There are a number of options to lower the temperatures of the seed to completely control infestations; break up the bag stacks into smaller stacks that would have a smaller thermal mass, prolong the freeze out, use fans to move cold air into the warehouse, or have an area constantly exposed to outside temperatures to do freeze-outs on high-risk product throughout the winter. This particular company captured only 9 mo in pheromone traps the 5 mo after the freeze-out compared to 478 mo over the same period in the pervious year. We can not attribute the decline conclusively to the freeze-out as they implemented other control measures in parallel to the freeze-outs.

Although the freeze-out has been demonstrated in a geographical location that is blessed with particularly cold winters, this method could be used in other locations by using large freezers. In some regards, freeze-out are like fumigations in that once the building and grain warms above 20°C it can become reinfested. So monitoring for insect activity would be an important part of using freeze-out to control *P. interpunctella* populations.

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Insecticidal properties of whole meal or protein extracts of the bean seeds *Phaseolus vulgaris* L. on juvenile stages of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)

Mouhouche, F.#[1], Karbache, F.[1], Fleurat-Lessard, F.*[2]
1 Institut National Agronomique, Laboratoire de phytopharmacie, INA El-Harrach, 16200 Alger, Algéria.
2 UR INRA 1264, Mycology and Food Safety (MycSA), Qualis Research Pole INRA, 71, avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France. Email: francis.fleurat-lessard@bordeaux.inra.fr

* Corresponding author
# Presenting author

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Abstract

Callosobruchus maculatus is a pest that causes serious damage to *Cicer arietinum* (chickpea) stored seeds, but that does not develop in seeds of other legumes such as *Phaseolus vulgaris* or *Pisum sativum*. The bean seed is rich in antinutritional compounds known to inhibit the development of *C. maculatus*. In an integrated approach to protect stocks of *Cicer arietinum* against attacks of this weevil, this study had the main objective to assess the potential of using bean flours from a wild bean *Vigna caracalla*, four varieties of *P. vulgaris*, and of a crude extract from *P. vulgaris* lectins seed. The extraction method was chosen to extract lectin-like protein compounds. The biological effects of bean flour or protein extracts were observed on artificial seeds composed from *C. arietinum* flour enriched with *P. vulgaris* whole flour or extracts incorporated at different percentages. The antinutritional activity either of bean-seed whole meal or of lectin-like extracts was determined by the analysis of different biological parameters. Incorporation of bean flour mixed with chickpeas decreased fertility and fecundity of female *C. maculatus* and caused longer development times of juvenile stages. Peptide extracts of the *P. vulgaris* reduced fecundity and survival of *C. maculatus*.

Keywords: *Callosobruchus maculatus, Phaseolus vulgaris*, Lectin-like extract, Insecticidal properties, Artificial seed

1. Introduction

Large-seed legume cultivation is an important crop in Algeria (Anonymous, 2006), but these plants are exposed to many post-harvest pests, the most serious damage being caused by the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleloptera: Bruchidae). This insect is an excellent disperser, and it is capable of laying eggs in cultivated fields and in storage. Each year in tropical countries, weight losses of pulses seeds may reach 800 g kg-1 in only a few months (Ouedraogo et al., 1996). Unsubstantiated estimates claim that 30% weight loss is due to infestation of legume seeds by weevils in Africa (Rodrigues Macedo et al., 2000).

Synthetic insecticides are widely used to control and prevent infestation after the harvest. However, the use of insecticides has several disadvantages: residues on the seed, availability, costs, resistant insect populations, worker safety and consumer concerns. Therefore, there is a growing interest in finding alternatives to chemical control for legume seed weevils. Pulses have evolved a large array of antinutritional compounds to protect their seeds against insects (Janzen, 1976). Chickpea, *Cicer arietinum* (L.) (Fabaceae), have endogenous natural insecticides produced in the seed that are active against *C. maculates*, (Mouhouche and Fleurat-Lessard, 2004). There are several examples of legumes being a source of natural insecticides against other stored-product insects (Jouvensal et al., 2003; Louis et al., 2004; Taylor et al., 2004).

Sales et al. (2000) and Carlini and Grossi-de-Sá (2002) have demonstrated feeding inhibition by legume seed vicilin compounds and arcelin that may be used for the defense of legumes against bruchid beetles. From a nutritional point of view, the legume lectins are part of the human diet. Surprisingly, these highly antinutritional compounds are resistant to proteolytic degradation during their transit through the human gut (Vasconcelos and Oliveira, 2004).

Earlier work had also identified the lectins as biochemical factors in plant resistance to insects, including mainly coleopteran species. Janzen (1976) showed that larvae of *C. maculatus* are unable to attack the seeds of the common bean *Phaseolus vulgaris* (L.) (Fabaceae) seeds, because there are a number of

defensive compounds: lectins or phyto-hemagglutinins (PHA). This work focused on *C. maculatus* with a prime objective to determine the effect of insecticidal lectins present in bean seeds on *C. maculatus* developmental biology. Two specific objectives were to study the insecticidal activity of five bean flours after their incorporation into artificial chickpea seeds, and study insecticidal activity of lectin-like compounds extracted from P. vulgaris in artificial chickpea seeds.

2. Materials and methods

2.1. Insects

Callosobruchus maculatus was reared in the laboratory since 1998 at the laboratory of Zoology at the Institute National Agronomique (INA) at Algiers El Harrach, (Algeria). The food consisted in 100 g organic chickpea purchased on the market that was infested with 20 pairs of *C. maculatus*. Food and insects were held in jars in an incubator at 28± 2° C and 70± 5% r.h. in the dark.

2.2. Source of Seed

Four *P. vulgaris* varieties were tested, as well as a wild bean from India, *Vigna caracalla* (L.) (formerly Phaseolus caracalla). Four of these were supplied by the Institut Technique des Grandes Cultures of Oued Smar El Harrach. These varieties were selected for their properties of resistance to fungal diseases such as rust and anthracnose. They were introduced to Algeria in the 1990's to study their behaviour in relation to agronomical performance: variety S102 (B15V2); variety Terga (V2B2); variety Pinto (V1B2); and variety Cotender. The V. caracalla was grown and harvested at the horticultural station of INA. As a control, a *C. arietinum* a variety of Algerian commercial grade, with wrinkled external seed coat (widely consumed in Algeria, and imported from Mexico) was used.

2.3. Fecundity and longevity

Fecundity was determined as the number of eggs laid by a female during her life. Fecundity was studied in 25 pairs of adults aged 0-24 h, distributed in five replicates. Five pairs of *C. maculatus* were placed on 10 g of artificial chickpea seed in 190 mL/bottles. The number of eggs laid was counted daily using a binocular microscope. Any mortality of females and males was noted. These and other tests were run at 30±1°C and 70±5% r.h. in a dark incubator.

2.4. Adult emergence and development duration

To determine the adult emergence and development time of *C. maculatus*, 150 eggs aged 0-48 h were recovered during the study of fecundity (section 2.3). These eggs were distributed into jars, 30 eggs each containing, 15 artificial seeds with varying proportions of bean and chickpea flour or bean peptide extract and chickpea flour. There were five replicates per treatment. The duration of development was calculated as the time elapsed from the middle of the egg laying period until 50% adult emergence (Haryadi, 1994). This important parameter allowed an overall assessment of the nutritional quality of food for *C. maculatus* (ensuring the nutritional needs of juvenile stages during their active growth).

2.5. Index of Susceptibility

Index of Susceptibility (IS) was used to determine the sensitivity of artificial seeds to stored-product-insect attack (Dobie, 1974). It is based on two factors important for population dynamics: total number of emerging adults (NE) and duration of mean development (DMD). Index of Susceptibility = (Loge Yield of emerging adults)/duration of mean development.

2.6. Bean flour mixed with chickpea flour

Chickpea flour that was used as the basis of chick-pea artificial seeds was obtained from the milling of a chickpea variety imported from Turkey available on the market. Chickpeas or beans were ground using a hard seed grinder type (IKA-Werk, Germany). The seeds were ground three times to obtain a fine flour. Additionally, the particle size was homogenized by sieving flour with 0.5 mm mesh sieve to eliminate the large particles. Then, the fine flour was used to make chickpea artificial seeds enriched with various amounts of bean flour obtained from the five beans. Flour of each bean variety was added to chickpea flour in proportions of 0, 10, 20, 40, 80 or 100%. After the blending the two flours, the mixture was placed in a centrifuge mixer for 60 seconds.

To obtain a firm dough, 45 mL of water was added to 100 g of the flour mixture. The hydrated flours mixture formed a paste that was spread with a rolling pin, and cut into 1 x 1 cm squares. A spherical artificial seed from each square of paste was manually made similar in size to a chickpea seed. These artificial chickpea seeds were dried for 48 h in a dark oven set at 20°C to avoid denaturing the proteins. After drying, 20 g of seed was placed into incubation jars and insects added on the seed. The number of live and dead *C. maculatus* was counted after 3 d. There were five replicates for each treatment.

2.7. Lectin-rich bean extracts mixed with chickpea flour

The wild bean species *V. caracalla* was most toxic to inserts but due to in sufficient quantities, *P. vulgaris* variety S102, the second most toxic seed was used.The method to extract truncated lectins from beans was that described by Moreira and Perrone (1977). This method involved mixing 80 g bean flour with 800 mL distilled water. The extraction of bean flour produced 420 mL of extract in distilled water. This extract is supposed to contain lectin-like compounds which are toxic to *C. maculatus*. The pH of the solution was adjusted to 2.4 with hydrochloric acid. This solution was mixed for 4 h to obtain a homogeneous solution. After centrifugation at 2000 g-force for 20 min at 4°C, the supernatant was recovered. Toxicity tests were performed with doses of 50, 100 and 200 mL of supernatant mixed with 100 g chickpea flour; artificial seeds were made from this, and tested with insects as above.

3. Results

3.1. Fecundity

All bean varieties reduced the fecundity of *C. maculatus*. At 10% bean flour, the lowest concentration tested, fecundity dropped to below 25% of the pure chickpea control seed. At 100% bean flour, less than three eggs were laid by the five females. There were significant differences (Tukey's Multiple Range Test, $P<0.05$) between the bean flours. At 10%, *V. caracalla* and S102 had the lowest egg load, and Pinto, Cotender and Terga were not significantly different and had a higher egg load than *V. caracalla* and S102.

3.2. Adult emergence

There was high adult emergence (97%) with pure chickpea flour (Fig. 1). All five bean flours at 10% of the artificial seed significantly reduced adult emergence to 30 - 90%. There was no adult emergence at 40% *V. caracalla*, 80% S102 and Pinto, and 100% Cotender and Terga varieties. Similar trends were seen with the differences between bean flours as were observed with the fecundity. The wild bean, *V. caracalla*, S102 were significantly lower than control and dose 10% (Tukey's multiple range test, *$P<0.05$).*

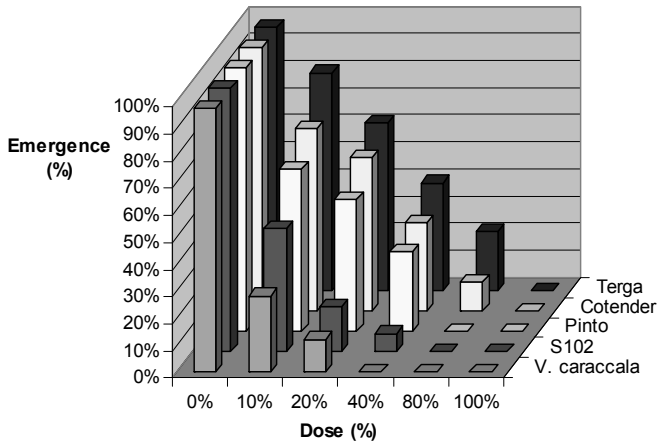

Figure 1 Emergence (%) of C. maculatus that developed on artificial chickpea seeds mixed with flour of five beans with different rates of incorporation.

3.3. Development time

There was a progressive increase in the total duration of development of *C. maculatus* from egg to adult with increasing concentrations of bean flour in the artificial see. Similar trends were seen with the differences between bean flours in development time as were observed with the fecundity and adult emergence.

Table 1 Fecundity of five *Callosobruchus maculatus* females on artificial seeds made with chickpea flour and varying amounts of five beans.

	Eggs five females (Mean + SE)				
	***V. caracalla* and *P. vulgarius* varieties**				
Bean Flour (%)	***V. caracalla***	**S102**	**Pinto**	**Cotender**	**Terga**
0	52.00 ± 0.01 a	52.04 ± 0,06 a	52.04 ± 0.06	52.04 ± 0,06	52.04 ± 0.06
10	3.60 ± 0.09 bA	4.72 ± 0.01 bA	12.56 ± 0.05 B	12.52 ± 0.03 B	13.60 ± 0.02 B
20	2.76 ± 0.05 b	4.00 ± 0.02 b	6.76 ± 0.04	8.60 ± 0.09	11.80 ± 0.02
40	1.24 ± 0.03 b	3.00 ± 0.07 b	5.20 ± 0.01	6.24 ± 0.03	8.48 ± 0.05
80	0.40 ± 0.01 b	0.72 ± 0.02 b	1.72 ± 0.04	3.52 ± 0.07	5.56 ± 0.08
100	0.36 ± 0.09 b	0.56 ± 0.07 b	0.68 ± 0.05	1.20 ± 0.06	2.24 ± 0.03

For a given bean (columns), means followed by a different small letter are significantly different, for 10% bean flour (row), means followed by a different large letter are significantly different (Tukey's multiple range test, $P<0.05$).

Table 2 The development time of *Callosobruchus maculatus* from egg to 50% adult emergence on artificial seeds made with chickpea flour and varying amounts of five beans.

	Development time (d)				
	***V. caracalla* and *P. vulgarius* varieties**				
Bean Flour (%)	***V. caracalla***	**S102**	**Pinto**	**Cotender**	**Terga**
0	29.00	29.00	29.00	29.00	29.00
10	80.40	60.93	39.03	38.59	33.25
20	145.80	86.80	47.23	41.98	38.10
40	NA	88.80	53.93	48.10	42.84
80	NA	NA	NA	45.00	47.96
100	NA	NA	NA	NA	NA

NA= no adults

3.4. Index of Susceptibility

Similar trends were seen with the Index of Susceptibility as were seen with the previous biological parameters. This is not surprising given the Index of Susceptibility is calculated from survival to adult and mean development time. Increasing proportions of bean flour caused decreases in IS (Table 3). The wild bean, *V. caracalla*, S102 were significantly lower than the other beans.

Table 3 Index of Susceptibility for *Callosobruchus maculatus* on artificial seeds made with chickpea flour and varying amounts of five beans.

	Index of Susceptibility				
Bean Flour	***V. caracalla* and *P. vulgarius* varieties**				
(%)	***V. caracalla***	**S102**	**Pinto**	**Cotender**	**Terga**
10	2.01	3.00	5.00	5.19	6.26
20	0.86	1.61	3.93	4.59	5.16
40	NA	1.12	3.04	3.51	4.15
80	NA	NA	NA	2.67	3.16
100	NA	NA	NA	NA	NA

NA= no adults; Index of Susceptibility = (Loge percentage of emerging adults)/average duration of development.

3.5. Lectin-rich bean extract mixed with chickpea flour

Given that there was insufficient *V. caracalla* and S102 was the most toxic of the *P. vulgaris* varieties, this variety was chosen for the extraction of lectin-like proteins needed for the second part of the study. Placing *C. maculatus* adults on artificial seed for 3 d caused 71, 79, and 100% mortality for 50, 100 and 200 mL of extract respectively (Fig. 2), whereas the untreated seed had 4% mortality. The lethal dose to kill 50% of the population was estimated at 35 mL of extract per 100 g of chickpea flour using probit analysis. In addition to reducing the survival, the extracts also reduced the number of eggs laid on artificial seeds. Females laid over 50 eggs on untreated seed, but only 50 mL of the extract reduce the number of eggs by almost 90% (Table 4).

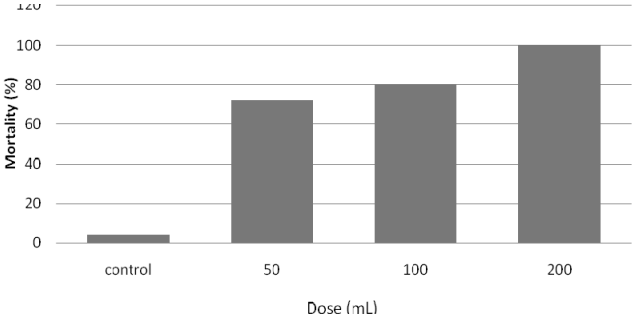

Figure 2 Effect of different doses of the water-soluble extract containing lectin-like compounds from bean seeds of S102 variety on mortality of adult Callosobruchus. maculatus.

Table 4 Effect of *P. vulagris* S102 flour extract incorporated in artificial chickpea seed on *C. maculatus* fecundity.

	Eggs/female			
Age of female (d)	**Bean flour extract with lectins (mL)**			
	0	**50**	**100**	**200**
2	16.80	4.08	1.64	1.04
4	14.60	2.12	0.96	0.08
6	9.60	0.00	0.00	0.00
8	6.56	0.00	0.00	0.00
10	2.88	0.00	0.00	0.00
12	1.04	0.00	0.00	0.00
14	0.20	0.00	0.00	0.00
16	0.00	0.00	0.00	0.00
Total eggs/female	51.7 ± 6.6	6.2 ± 1.5	2.6 ± 0.6	1.1 ± 0.4

4. Discussion

Our results show that the wild bean *V. caracalla* and *P. vulgaris* variety S102 were potent inhibitors for egg laying of *C. maculatus* when incorporated at 10% into artificial chickpea seeds. *C. maculatus* prefer to lay on smooth varieties (Lepesme, 1944). However, our results showed a much lower rate of eggs laid by this pest species on artificial chickpea with a smooth surface when these seeds are enriched with a small amount of bean flour or bean flour water-soluble protein extract. This means that the texture of the seed coat is not the only factor influencing the egg laying behavior of female *C. maculatus*. This work suggests behavior is also related to the biochemical composition of seeds perceived by females, which refused to lay eggs and which were rapidly killed after a few days in contact with “toxic” artificial chickpea seeds.

The high percentage of eggs failing to hatch on bean flour enriched chickpea artificial seeds showed that insecticidal potential of certain bean varieties may be related to the presence of hydrophilic protein such as lectins that could prevent egg hatching and larval development of *C. maculates* as previously observed

by Janzen et al. (1976), Gatehouse et al. (1995), Gatehouse and Gatehouse (1998), Okeola et al. (2002), and Boleti et al. (2007). These authors found that high rates of lectins in certain species of legume such as Dolichos lablab (L.) and Rhycocea saucia prevent the development of *C. maculatus*. However, according to Goossens et al. (2000), with lower incorporation level of an extract containing glycoproteins isolated from bean at about 5% in weight, did not reduce fertility or development time of *C. maculatus*. The limit of the content of these insecticidal compounds in chickpea could be estimated between 5 and 10 % to expect a control of *C. maculatus* and the reduction of damage on stored chickpeas.

Our results were in agreement with those obtained in earlier studies by other authors: Hamelryck et al. (1996), Louis (2004), Brinda et al., (2004) and Zambre et al., (2005) have already reported the resistance of bean seeds of Phaseolus toward *C. maculatus*. Among resistant varieties, we found variety referenced G02771, and two other species of Phaseolus Genus: *P. calcaratus* (L.) and *P. lathyroides* (L.).We deduced that the extracts toxicity was probably related to the presence of "reserve glycoproteins" (Hamelryck et al., 1996) whose role is to protect bean seeds against attack by non-adapted bruchid species to antinutritional compounds present in bean seeds, such as the chickpea weevil *C. maculatus*.

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Heat treatment: A viable methyl bromide alternative for managing stored-product insects in food-processing facilities

Hulasare, R.*#[1], Subramanyam, B.[2], Fields, P.G.[3], Abdelghany, A.Y.[3,4]
1 Temp-Air Inc., Burnsville, MN 55337, U.S.A. Email: rhulasare@temp-air.com
[2] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, U.S.A.
[3] Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba R3T 2M9, Canada
[4] Economic Entomology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt

* Corresponding author
# Presenting author

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Abstract

Heat treatment involves raising and maintaining temperatures of grain storage structures, warehouses, and food-processing facilities between 50 to 60°C to manage stored-product insect species. The duration of heat treatment is application-specific and may vary from 6 h for an empty storage facility to 24 h for an entire food-processing facility. Laboratory and commercial trials with high temperatures during the last decade, especially with forced air gas heaters, have resulted in a wealth of information on (1) understanding responses of insect species and life stages to heat, (2) heat distribution within a treated area, and (3) techniques necessary for gauging effectiveness of commercial heat treatments. Insect responses vary with the temperature, among species, and within a species among life stages. Air movement and strategic placement of fans are important for eliminating cool spots (<50°C) and for uniformly heating a treated area. Insect bioassays and monitoring insect populations before and after a heat treatment are important to understand the degree and duration of insect suppression obtained in commercial facilities. Heat treatments are safe, effective, and a viable tool for the organic and non-organic sector. Research in both laboratory and food-processing facilities has shown heat treatments to be a viable alternative to methyl bromide fumigation.

Keywords: Heat, Forced air, Flour mills, Methyl bromide alternative

1. Introduction

Heat treatment, a 100-year old technique (Dean, 1911), involves raising the temperature of a room, equipment, or an entire facility to 50 to 60°C to kill insects, primarily stored-product insects (Heaps, 1994; Mahroof et al., 2003a,b; Roesli et al., 2003; Beckett et al., 2007). The duration of the heat treatment depends on the site being treated. Whole facility heat treatments typically last 24-36 h. There is renewed interest in exploring heat treatments as an alternative to methyl bromide, a structural fumigant that has been phased out in the United States, Canada and Europe, except for certain critical uses, because of its adverse effects on stratospheric ozone levels (Makhijani and Gurney, 1995).

Electric heaters, forced air gas heaters (Figure 1), or steam heaters (Figure 2) can be used to conduct a heat treatment. With the forced air gas heaters the building is placed under positive pressure during a heat treatment, and the entire air within the building is exchanged four to six times per hour. The number of air exchanges when using electric and steam heaters may be one or two per hour. The forced air also allows heat to reach gaps in the building and equipment much better than electric or steam heaters. The forced air gas heaters can use natural gas or propane as fuel. Since these heaters have an open flame they are placed outside a facility, and nylon ducts (Figure 1) are placed within the facility to introduce heated air. Hot air has a tendency to stratify horizontally and vertically within a facility. Therefore, several fans should be placed on different floors of a facility to redistribute heat and to uniformly heat a facility. Fan placement is an art, and during heat treatments, fans should be moved to eliminate cool spots-areas where the temperature is less than 50°C. In addition to food-processing facilities, heat treatment can also be used in empty storage structures (bins, silos), warehouses, feed mills, and bakeries. It is an environmentally benign method for managing insects.

Figure 1 Propane-fired heater (Temp-Air) heater (source of heat for studies in Figure 3 and 4). The door was sealed with plywood, and a flexible fabric duct delivered heated air through a hole cut in the plywood,

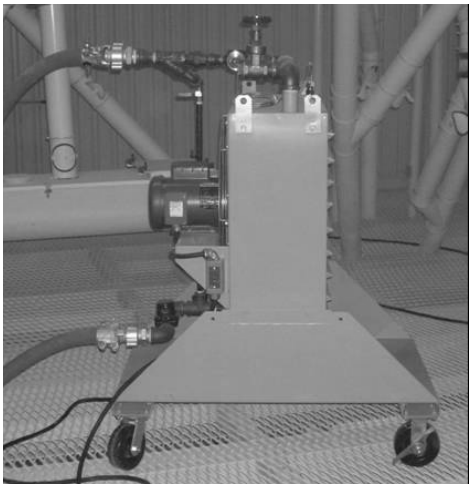

Figure 2 Portable steam heater (Armstrong International Inc.) used in heat treatment (Fields, 2007).

2. Heat treatment of commodities versus structures

Heat has also been used to disinfest perishable and dry, durable food products. High temperature treatments are used for disinfestations of dried fruits and nuts, perishable commodities (fruits) (Hansen and Sharp, 1998), and grains (Beckett and Morton, 2003). Facility heat treatments are distinctly different from heat treatment of fresh fruits, nuts, or grains. In facility heat treatments, heaters are used to slowly heat the ambient air. A long heat treatment period is necessary for the heat to penetrate wall voids and equipment to kill insects harbouring in them. A typical heat treatment may last 24-36 h (Mahroof et al., 2003a; Roesli et al., 2003). In heat treatments of fresh commodities, nuts, dried fruits, or grains, high temperatures of 60-85^{o}C are used for short time periods (in min). Typical heating rates during heat treatment of perishable commodities, nuts, dried fruits, and grains range from 1-15^{o}C per minute, whereas during facility heat treatments heating rates should generally be around 3-5^{o}C per hour for effective disinfestation. However, in both cases the products or the facility subjected to high temperatures are allowed to cool to ambient temperature, and this may take several hours. During heat treatments, it is important to remove all food products and packaging materials (bags) from the facility. Equipment should be opened and thoroughly cleaned of any food product where possible. It is important during heat treatments of products to ensure that the quality is not affected. Similarly, in the case of structural heat treatments, it is important to ensure that there is no damage to the equipment, uninfested materials stored within the facility, and the structure.

3. Issues to consider before a heat treatment

Dosland et al. (2006) gave detailed step by step procedures for conducting and evaluating a facility heat treatment. One important aspect of conducting an effective heat treatment involves calculating how much heat energy is required after accounting for heat losses due to exposed surfaces, equipment, and infiltration. Research at Kansas State University and discussions with heat service providers showed that the amount of heat energy should range from 0.074-0.102 kW per cubic meter of the facility per h, and during a 2009 heat treatment of a flour mill at Kansas State University, the heat energy used was as high as 0.16 kW per m^3 per h. An indirect method of determining whether or not adequate heat energy is being used is by observing how quickly ambient temperatures reached 50°C. In proper heat treatments, the time to reach 50°C should usually take about 8-10 h, and depending on the time of year and the leakiness of a structure, this time can take as long as 15 h.

4. Characterizing temperature profiles

A typical temperature profile during heat treatment is shown in Figure 3. From the temperature data, the following information should be extracted: the time taken to reach 50°C, the time temperatures were maintained above 50°C and the maximum temperature (Mahroof et al., 2003a; Roesli et al., 2003). The time to reach 50°C is important to determine the heating rate, which is calculated as the difference between 50°C and the ambient temperature at the start of the heat treatment divided by the time to 50°C. This rate should be between 3 and 5°C per hour in properly conducted heat treatments for effective disinfestation. Temperatures should be held at least for several hours above 50°C to kill insects. The maximum temperature should not exceed 60°C to prevent any structural damage or damage to equipment. Information broken down in this fashion can be related to insect mortality if live insects confined in cards or vials are used to gauge the effectiveness of a heat treatment.

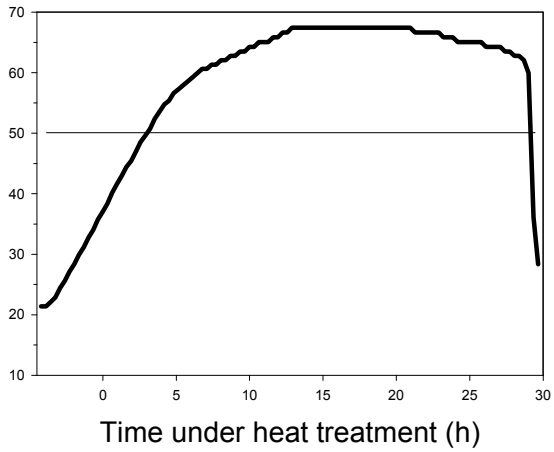

Figure 3 Temperature during a heat treatment (Fig. 4) in a flour mill using propane-fired heaters (Fig. 1).

5. Effects of heat on insects

Lethality in insects at high temperatures depends on both the temperature and time of exposure (Denlinger and Yocum, 1999; Evans and Dermott, 1981; Fields, 1992; Mahroof et al., 2003b). Temperature and exposure time to achieve a certain percentage of insect kill are inversely related. At high temperatures insect cuticular wax becomes compromised allowing loss of water. This affects water balance in insects, leading to death by desiccation as well (Hepburn, 1985). High temperature exposure denatures proteins, affects hemolymph ionic balance and pH, and adversely affects enzyme activity (Denlinger and Yocum, 1999; Neven, 2000).

High temperatures that do not kill insects can adversely affect the insect's reproduction. Recently, Mahroof et al. (2005a) have shown that when pupae and adults of the red flour beetle, *Tribolium castaneum* (Herbst) were exposed to 50°C for 39 and 60 min, respectively, the surviving adults from

these insects showed significant reduction in oviposition, egg-to-adult survival rate, and progeny production.

6. Heat tolerance in insects

Insects when exposed to high temperatures may produce heat shock proteins (HSPs). Generally, these proteins protect the cells by preventing aggregation or improper folding of proteins (Currie and Tufts, 1997). There are several families of HSPs largely classified by molecular weight. HSP 70 is one of the most highly conserved heat shock proteins with the largest specific activity and thus may be easy to detect (Currie and Tufts, 1997). The heat tolerance in young larvae of *T. castaneum* was due to increased expression of HSP 70 (Mahroof et al., 2004; 2005b). Time, and temperature-dependent expression of HSP 70 showed that the increased heat tolerance in young larvae lasted only as long as 8 h at 40°C or 30 min at 46°C (Mahroof et al., 2004) The HSP 70 may not confer tolerance to *T. castaneum* at temperatures of 50-60°C typically used during heat treatments.

The stage that is heat tolerant varies with the species (Table 1), especially at temperatures between 50 and 60°C. Mahroof et al. (2003b) and Boina and Subramanyam (2004) have shown that the heat tolerance of a stage varies with the temperature, and tolerance to heat at temperatures of 50-60°C is therefore more important than at temperatures below 50°C. All of these studies were based on laboratory studies at fixed temperatures. Heat tolerance of life stages of a species has not been determined during commercial heat treatments, and experiments should be designed to confirm laboratory findings with field data.

Table 1 Time for 99% mortality of heat tolerant stages of four stored-product insect species at constant temperatures between 50 and 60°C.

Species	Stage	Temp. (°C)	LT_{99} (95% CL) (min)	Reference
T. castaneum	Young larvae	50	433 (365-572)	Mahroof et al. (2003a)
		54	82 (60-208)	
		58	38 (29-76)	
		60	24 (20-33)	
T. confusum	Old larvae	50	90 (82-102)	Boina & Subramanyam(2004)
		54	56 (49-67)	
		58	38 (30-71)	
		60	24 (20-33)	
P. interpunctella	Old larvaea	50	34 (29-43)	Mahroof & Subramanyam (2006)
		52	34 (26-67)	
L. serricorne	Eggsb	50	190 (170-220)	Chun Yu (2008)
		54	39 (36-43)	
S. paniceum	Young larvae	50	234 (176-387)[c]	Abdelghany et al. (2010)d
		55	10.8 (6.6-13.8)	
		60	4.8 (4.2-4.8)	

[a]Fifth instars; [b]Time-mortality relationships were based on egg hatchability data; [c]These values are LT_{90s} (95% CL); [d]Abdelghany A.Y., Awadalla S.S., Abdel-Baky, N.F., EL-Syrafi H.A., Fields, P.G., 2010 (unpublished data).

6.1. Flour mill treatment with six insects; methods

The following insects were used in tests of efficacy of heat treatments in flour mill: *Stegobium paniceum* (L.), *Lasioderma serricorne* (F.), *Cryptolestes ferrugineus* (Stephens), *T. castaneum*, *Tribolium confusum* Jacquelin du Val and *Tenebrio molitor* (L.). All insects were adults, except for *T. molitor* which was exposed as late instar larvae and *S. paniceum* which exposed as young larvae (5-d old) and as adults. Insects were reared at 30°C, 60% r.h., except *T. molitor* which was reared at room temperature.

Field data was collected in Western Canada at a medium-sized mill on 23-24 October, 2008. The various species were prepared at the Cereal Research Center, Agriculture & Agri-Food Canada, Winnipeg, Canada. Ten grams of wheat flour mixed with brewers yeast (95:5 by weight) was placed into a vial (29 mm diameter with 50 mm high) and 50 insects of a given species were placed into the vial with sealed

with a screened lid. There were three days between placing the insects in the vials and the exposure to heat in the mill. During shipment, the insects experienced the temperatures between 20 to 30^{0}C. Seven vials, one for each species, were grouped tightly around a HOBO data logger and twelve sets of seven vials were placed on the floor of the mill in a ring with about 20 cm diameter at two locations in the mill. At the middle of the ring, there was one set of vials for measuring flour temperatures, with the same amount flour and yeast, but without insects. The temperatures inside these vials were measured by introducing T-type thermocouples which were connected with HOBO data logger (Onset Computer Corporation, Bourne, MA). The tip of a thermocouple was located at the flour center inside the vial.

The mill was heat treated from 9:15 am 23 October 2008 until 10:00 am 24 October 2008. During that period, groups of the vials were taken out of the mill, one set at a time when the flour temperatures reached approximately; 32, 35, 37, 40, 42, 45, 47, 50, 52, 55 and 60°C. The vials were shipped back to the laboratory and held at 30°C, 70% r.h. until the emergence of adults for *S. paniceum* and *T. molitor* larvae. The survival of adults was assessed upon arrival at the laboratory.

6.2. Flour mill treatment with six insects; results

Lasioderma serricorne in location 1A and *L. serricorne* and *S. paniceum* larvae in location 1B all had greater than 20% mortality at temperatures below 35^{o}C, therefore the mortality was adjusted by using Abbott's equation (Abbott, 1925). Although there were large differences in response of insects to heat, there was significant deviation from the probit model making it impossible to calculate the lethal time for 50% of the population (LT_{50}) using probit analysis. We estimated LT_{50} graphically (Figure 4). In general, *C. ferrugineus* was the most heat tolerant, *T. molitor* larvae was the least heat tolerant with the other species falling between these two extremes.

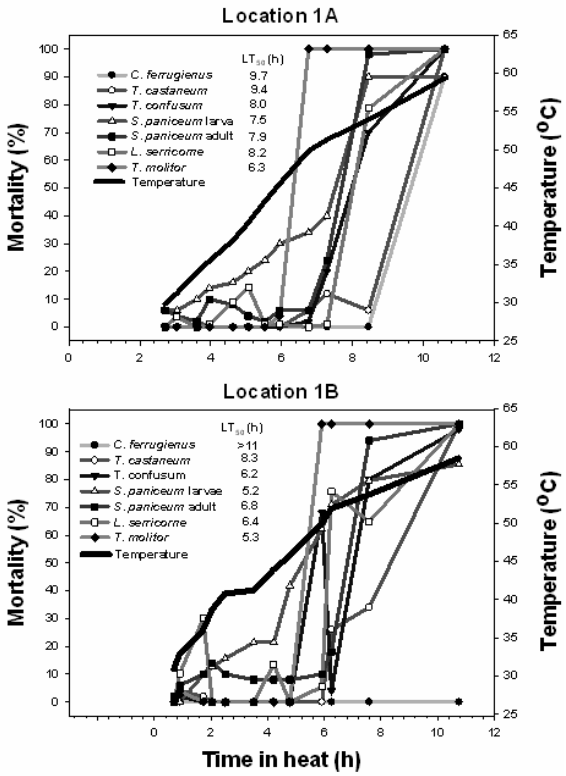

Figure 4 The lethal time to kill 50% of the population (LT_{50} estimated graphically), mortality of stored-product beetles and temperature during a heat treatment in a flour mill at two different locations in a flour mill.

7. Gauging heat treatment effectiveness

To gauge heat treatment effectiveness, it is important to identify critical areas in the facility. These areas are usually places where insects can hide and breed or places where temperatures can not penetrate or reach at least 50°C. Such places are usually identified through inspections. Temperature sensors should be placed in these areas to measure temperatures. Cards with insects such as those marketed by Alteca (www.alteca.com) or insects in vials with food should be placed in critical areas and examined during or after a heat treatment to determine effectiveness against insects. Insects in the cards are usually without food and therefore these stressed or starved insects tend to succumb quickly to a heat treatment, and may falsely indicate that the treatment was effective, when in fact it was not. For example, in a pet food facility, the mortality of adults of *T. castaneum* in cards and in bioassay vials with flour (3 g) were compared. These results showed that during the 23-h-heat treatment, all insects in the cards died at 15 h, whereas in the vials at the end of the heat treatment, the mortality of adults was below 20%. The use of live insects to gauge heat treatment effectiveness provides valuable information, but in some facilities bringing live insects may be prohibited. Resident insect populations within a facility should be monitored before and after a heat treatment. At least thirty-five traps should be used inside the facility and five outside the facility. In some facilities such as flour mills, it is possible to sample tailings to determine insect load. These observations should occur every week and should be resumed soon after a heat treatment. The trapping or visual observations of products/tailings following a heat treatment should be done at least on a daily basis for the first week and should continue weekly for at least 8-16 wk. These data provide valuable information on the degree and duration of control obtained after a heat treatment intervention. Table 2 shows mean trap captures of *T. castaneum* adults before and after a heat treatment of a pasta facility. These data show that a single treatment's effectiveness lasted close to two months, because the facility managers use sanitation and exclusion tactics. Similar heat treatments in Canada has shown that heat treatments can be effective for over 5 mon (Fields, 2007) The doors and windows should be tightly closed to prevent insects from outside coming into a facility. Insects can be brought into a facility on raw materials, and care must be taken to inspect all materials to ensure that they are insect-free. Inspection, sanitation and exclusion practices can help extend the degree and duration of insect suppression obtained with a heat treatment.

Table 2 Captures of *T. castaneum* adults in pitfall traps before and after a heat treatment of a pasta facility in 2006.

Date	**Mean number of adults/trap/week**[a]		
	Press room	**Flour room**	**Outside**
May 30	0.46	0.40	0.50
June 14	0.20	0.42	0.65
June 28	0.32	0.65	0
July 4	**Heat treatment**[b]		
July 11	0 (100%)c	0.09 (86%)c	0
July 25	0.03	0.10	0.38
August 8	0	0.05	0.50
August 23	0.01	0.05	0.20

Source: Bh. Subramanyam (unpublished data); [a]The number of traps in the press room, flour room, and outside was 35, 10, and 5, respectively; [b]Traps were replaced immediately after the heat treatment was done; [c]Percentage reduction in trap catch, based on catch just prior to the heat treatment.

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Suppression of the population of *Lasioderma serricorne* in stored tobacco by relocation of warehouses to cooler areas

Imai, T.*#
Leaf Tobacco Research Center, Japan Tobacco Inc.; Oyama, Tochigi 323-0808, Japan.
Email:toshihiro.imai@jt.com

* Corresponding author
# Presenting author

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Abstract

To suppress populations of cigarette beetles, *Lasioderma serricorne,* during winter, domestic tobacco warehouses in Japan have been relocated since 2004. Warehouses located in warmer tobacco-cultivated areas were closed, and new ones were constructed in places where the daily mean temperature is below 5ºC for over 70 d per year, a value chosen based on lethal low temperature conditions determined under constant laboratory and fluctuating field situations. In 2008, with relocations almost complete, the total number of beetles captured in pheromone traps in all warehouses had decreased by 98% compared to the 2001-2002 levels. Domestic tobacco production and the number of warehouses were reduced by 40% during the time.

Keywords: *Lasioderma serricorne*; Cigarette beetle; Tobacco warehouse; Low temperature; Winter survival

1. Introduction

The cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae), is a cosmopolitan pest that is found throughout tropical, subtropical and warm-temperate parts of the world. It is the most problematic stored-tobacco pest in Japan. Pesticide application to stored tobacco and tobacco products is restricted, where phosphine fumigation is the only available method for disinfestation of warehouses. Nevertheless, phosphine-resistant beetles have continued to spread, and current fumigation techniques are becoming less effective (Rajendran and Narasimhan, 1994; Zettler and Keever, 1994). In addition to the emerging problem of resistance, public concern has been growing regarding the potential health and environmental hazards of pesticides. Consequently, non-chemical control methods have become increasingly sought as future pest-management strategies. Storage facilities that cool the air to 4ºC have been designed to eliminate this pest in stored tobacco (Childs et al., 1983; Beard et al., 1983; Beard et al., 1986); however, the cost of such facilities has prevented their construction.

In Japan, *L. serricorne* is prevalent in southwestern areas, but is not commonly found in northern areas. This fact suggests that the cigarette beetle is not fully adapted to temperate climates in Japan, and that the relocation of warehouses to cooler areas may reduce damage during storage without the need for cooling facilities. To identify the temperature conditions necessary to achieve suppression of the beetle population in storage facilities, beetle mortality was examined at constant low temperatures or under fluctuating field conditions. The conditions for beetle eradication were determined to be 11 wks at a constant temperature of 5ºC or 9-10 wks below 7ºC plus 1-2 wks below 6ºC under a natural winter situation (Imai and Harada, 2006; Imai and Tsuchiya, 2007). Based on these results, tobacco warehouses in Japan have been relocated from warmer tobacco-cultivated areas to cooler areas where the daily mean temperature is below 5ºC for over 70 d per year. The present paper describes the results of a supplementary laboratory test to verify the winter extinction conditions and the effect of warehouse relocation on the population of *L. serricorne*.

2. Materials and methods

2.1. Evaluation of mortality at constant low temperatures

Laboratory tests were performed as described in Imai and Harada (2006). Acclimated fourth (final)-instar larvae pre-exposed to 15ºC for one month were exposed to 7.5ºC and 10ºC for 4, 8, 12, 16, 20 and 24 wks, and their viability was checked. The 50% and 99% lethal times (LT_{50} and LT_{99}) were calculated

with the PriProbit (ver. 1.63) computer program developed by Sakuma (1998), which was downloaded from http://bru.gmprc.ksu.edu/sci/throne/.

2.2. Contour mapping of 5ºC/70-day lines

The mean of the daily temperature from 1971 to 2000 at 255 meteorological data points over central and western areas of Japan were taken from the Japan Meteorological Agency website:

(http://www.data.jma.go.jp/obd/stats/etrn/index.php).

Contour analysis was done for the annual number of days with a mean temperature below 5ºC using the Surfer 8.0 computer program (Golden Software Inc., Golden, CO, USA).

2.3. Monitoring data in tobacco warehouses

The appearance of adult *L. serricorne* in tobacco warehouses was monitored by JT Logistics Co. Ltd. using commercial pheromone traps (SERRICO, Fuji Flavor Co. Ltd., Tokyo, Japan). The traps were placed at a density of 1 trap per 200 m^2 in every warehouse. The annual monitoring data for the years 2001, 2002, 2007 and 2008 were supplied by JT Logistics Co. Ltd.

3. Results and discussion

3.1. Winter extinction conditions for L. serricorne

Table 1 shows the lethal exposure times at constant temperatures of 0, 5, 7.5 and 10ºC for the acclimated larvae of *L. serricorne*. These results coincide with the mortality data in tobacco hogsheads: the larvae in hogsheads were eradicated in 12, 20, and 32 wks at constant temperatures of 4.4, 7.2, and 8.9ºC, respectively (Childs et al., 1968). Almost three months were required to eradicate the acclimated larvae at 5ºC and half a year was required at 7.5ºC. Because the latter condition is not achievable in natural situations in temperate areas, the critical temperature × duration for this pest to overwinter in temperate areas should be around 5ºC × three months. An actual winter extinction under fluctuating natural situations occurred in milder conditions than those expected from laboratory data at constant temperatures; 9-10 wks below 7ºC plus 1-2 wks below 6ºC (Imai and Tsuchiya, 2007). During that experiment, test insects were exposed to the atmospheres of warehouses for five months (from mid-November to mid-April). Under practical warehouse conditions, exposure to sublethal low temperatures before and after deep winter accelerates the lethal effect of low temperatures.

Table 1 Lethal exposure time for acclimated larvae[a] of *Lasioderma serricorne* at constant low temperatures.

Temperature, ºC	LT_{50} (fiducial limits), day	LT_{99} (fiducial limits), day
0[b]	21 (19–25)	47 (43–55)
5[b]	22 (18–25)	78 (60–117)
7.5	43 (32–53)	188 (113–150)
10	64 (50–77)	226 (180–358)

a Larvae were exposed to 15ºC for one month before exposure to each low temperature;
b Data were reproduced from Imai and Harada (2006).

3.2. The effect of warehouse relocation to cooler areas on the population of L. serricorne

Based on the laboratory and field experimental data on lethal low-temperature conditions, tobacco warehouses in Japan have been relocated since 2004. In 2001 and 2002, approximately 60,000 t of tobacco were produced domestically and stored in 22 warehouses located near the cultivated areas; 10 of the warehouses were in Kyushu, the southwestern island of Japan (Fig. 1a). During that time, more than 1,000 beetles were captured annually, and phosphine fumigation was applied twice per year, in May and August. Since that time, domestic tobacco warehouses in warmer areas have been closed, and new ones have been constructed in places where the daily mean temperature is below 5ºC for over 70 d per year (Fig. 1b). Regular fumigation has not been applied since 2005. The total number of adult *L. serricorne* captured in monitoring traps in all warehouses in 2008 decreased by 98% compared with those in 2001 and 2002, whereas domestic tobacco production and the number of warehouses have dropped by 40% during the time (Table 2). The trap catch is a good index of pest population, although it is affected by temperature condition. The flight behavior and trap catch of *L. serricorne* are suppressed below 20ºC (unpublished data). The mean temperatures exceeded 20ºC at least three months (mid-June to the end of

September) in every location that the warehouses were located, and the reduction in number of catches per trap should reflect population decrease. Even though pests accompanying tobacco from processing plants can not be prevented from occasional entering these warehouses, the results indicate that the overall population has remained low almost all year.

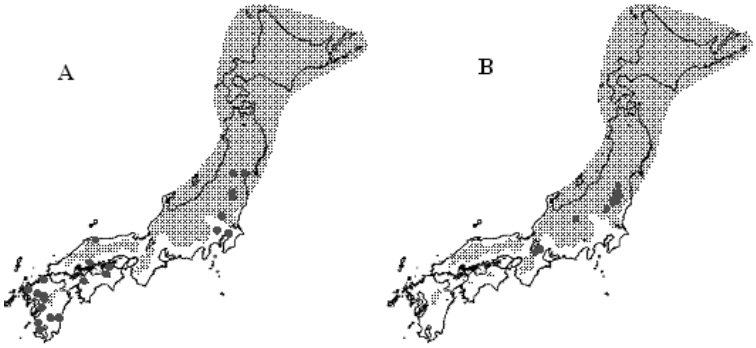

Figure 1 The distribution of domestic tobacco warehouses in Japan (A, 2002; B, 2008). The circles show the locations of domestic tobacco warehouses and the gray area shows the area experiencing mean temperatures below 5ºC for at least 70 days.

Table 2 Annual domestic tobacco production, the number of warehouses and total number of *Lasioderma serricorne* adults captured by pheromone traps in all domestic-tobacco warehouses in Japan.

Year	Leaf production, t	No. warehouses	Total *L. serricorne* caught	*L. serricorne* caught/trap/year
2001	60,565	22	1,916	1.53
2002	58,174	22	1,676	1.34
2007	37,803	15	203	0.35
2008	38,484	12	37	0.07

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The control of the drugstore beetle, *Stegobium paniceum* (Coleoptera: Anobiidae) with high and low temperatures

Abdelghany, A.Y.#[1, 2], Awadalla, S.S.[2], Abdel-Baky, N.F.[2], EL-Syrafi, H.A.[2], Fields, P.G.*[1]
[1] Cereal Research Centre, Agriculture & Agri-Food Canada, 195 Dafoe Road, Winnipeg, Manitoba, R3T 2M9, Canada. Email: paul.fields@agr.gc.ca
[2] Economic Entomology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt

* Corresponding author
# Presenting author

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Abstract

Botanicals; dried plants, roots, stems, leaves, seeds and flowers, have been used from the dawn of history as drugs or spices (Craker, 2007). Botanicals have been used in the health care system to improve blood circulation, reduce chronic fever and cure chronic constipation (Golob et al., 1999; Samy et al., 2008). The drugstore beetle, *Stegobium paniceum* (L.) (Coleoptera: Anobiidae), is a pest of stored medicinal and aromatic plants and one of the most common insects found in botanical warehouses (Abdelghany et al., 2010). Generally, mortality of each stage increased with an increase of temperature and exposure time. Heat tolerance for different stages from highest to lowest was; young larvae, old larvae, eggs, adult and pupae. The mortality after 7 h, at 42°C for young larvae, old larvae, eggs, adult and pupae respectively was; 16 ± 5, 31 ± 6, 48 ± 3, 63 ± 8 and 86 ± 2% (mean ± SEM). Similarly, the lethal time for 90% mortality (LT_{90}) at 42°C was; too low to estimate, 773, 144, 12 and 11 h. The LT_{90} value for young larvae at 42, 45, 50, 55 and 60°C was 25, 20, 3.9, 0.18 and 0.08 h respectively. The cold tolerance of different stages at 0°C from highest to lowest was adult, old larva, young larva, pupa, and egg. The LT_{90} at 0°C was 298, 153, 151, 89 and 53 h, respectively. The LT_{90} value for adults at 5, -5, -10 and -15°C was 792, 58, 2 and 0.8 h, respectively. The supercooling point of adults, young larvae, old larvae and pupae was -15.2 ± 2°C, -9.0 ± 0.8°C, -6.5 ± 0.5°C, and -4.0 ± 1.4°C respectively. Heat treatments that control young larvae should control all other stages of *S. paniceum*. Cold treatments that control adults should control all other stages of *S. paniceum*. Dried plants stored at 5°C for 45 days or 42°C for 30 h and then kept below 18°C throughout the rest of the year, should remain pest-free without any chemical control. The full paper was submitted to Entomologia Experimentalis et Applicata.

Keywords: Anobiidae, Heat, Cold, Supercooling point, Tolerance, Storage, Warehouse

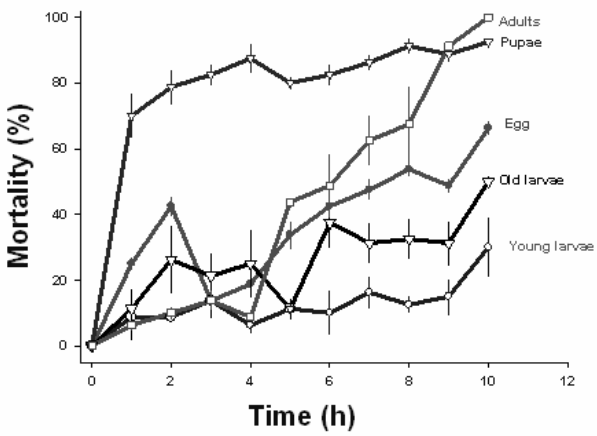

Figure 1 Mortality of various stages of the *Stegobium paniceum* at 42°C.

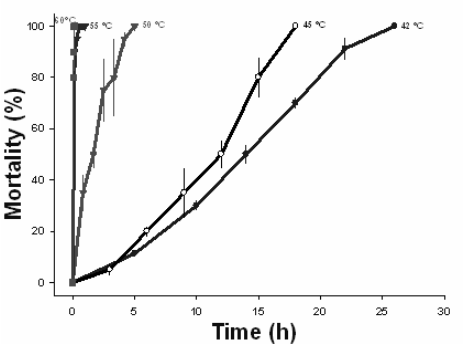

Figure 2 Mortality of *Stegobium paniceum* young larvae at five constant temperatures.

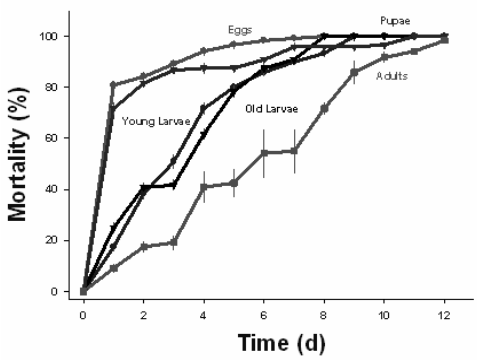

Figure 3 Mortality of various stages of the *Stegobium paniceum* at 0°C.

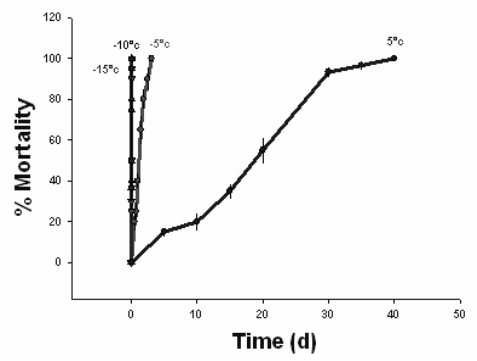

Figure 4 Mortality of *Stegobium paniceum* adults at low constant temperatures.

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Egg removal device for the management of three stored product pests

Jayaprakash, S.A.*#, Mohan, S., Ramaraju, K.
Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore – 641 003, India.
Email: entojayaprakash@yahoo.com

* Corresponding author
# Presenting author

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Abstract

Investigations were carried out to assess the efficiency of pulse beetle egg removal device in the removal of eggs of *Tribolium castaneum* and *Rhyzopertha dominica* from infested sorghum, wheat, maize and paddy grains and the eggs of *Lasioderma serricorne* from infested coriander. The efficiency of the device or the impact of rotation was assessed based on the number of offspring adults emerged and percentage reduction in adult emergence compared to untreated controls. Rotation of the grains for three consecutive days for 15 min/day gave the highest reduction in the emergence of offspring adults. Reductions in emergence of *T. castaneum* and *R. dominica* were found to be 54 and 57% in sorghum; 69 and 69% in wheat; and 71 and 76% in maize, respectively. There was a 77% reduction in *L. serricorne* on coriander seed, and a similar level for *R. dominica* on paddy.

Keywords: Pulse beetle egg removal device, Tribolium castaneum, Rhyzopertha dominica, Lasioderma serricorne

1. Introduction

The stored grains are attacked by more than a dozen of stored grain insect pests (Simwat and Chahal, 1982). They assume greater importance as they start their damage in the field itself (Mohan and Subba Rao, 2000). Generally stored-product insects fly from nearby farms, farm store houses or farmer storehouses and start laying eggs on the maturing grains. So eggs are the basic root in causing damage to the grains during storage. Synthetic insecticides, residual and fumigants, are widely used to control insects in stored grain. However, there are number of reasons people are seeking alternatives to chemical insecticides; concerns over worker and consumer safety, the development of insecticide-resistant populations and problems with the environmental damage, methyl bromide as an ozone depletor is an example. Thus there is an interest in mechanical control methods, like removal of eggs from the grains before storing. Physical or mechanical methods like rotation, tumbling and impact of infested grains are an effective method of control for stored-product insect populations (Bailey, 1962; Joffe, 1963; Joffe and Clarke, 1963; Bailey, 1969; Loschiavo, 1978; Ungsunantwiwat and Mills, 1979; Quentin et al., 1991; Plarre and Reichmuth, 2000). Until now, only limited information was available in using the mechanical mode for controlling the egg stage of insects. Hence, in the goal of this study was, to assess the performance of the pulse beetle egg removal device in removing the eggs of red flour beetle, *Tribolium castaneum* (Herbst), the lesser grain borer, *Rhyzopertha dominica* (F.) and the cigarette beetle, *Laisoderma serricorne* (F.) from various food grains.

2. Materials and methods

2.1. Insects

The test insects used for the various experimental studies were, the *T. castaneum*, *R. dominica* and *L. serricorne*. They were mass reared in plastic containers in the laboratory. Sorghum grains infested with *T. castaneum* were collected from Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India, and were cultured on whole wheat flour at 30°C and 70% r.h. (White, 1982). Sorghum grains infested with *R. dominica* were collected from Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India, and were reared in the laboratory at 28°C and 70% r.h. on organic whole wheat kernels (Mohan et al., 2007).Coriander, turmeric powder and chili powder which were infested with *L. serricorne* were collected from local markets for initiating culture. During storage, whole coriander is infested by cigarette beetle causing considerable damage and deteriorates the quality (Agrawal and Srivastava, 1984). The insects were reared at 27 ± 1°C and 60 ± 5% r.h. with a 12 h

photoperiod on a diet of whole wheat flour (10 parts), white cornmeal (10 parts), and brewers' yeast (1.5 parts) (Arbogast et al., 2003).

2.2. Pulse beetle egg removal device

Egg removal device for pulse beetle (Mohan, 2005) was used to assess the efficiency of its egg removal against other important stored-product insects namely, *T. castaneum*, *R. dominica* and *L. serricorne.* The device comprises of an outer container enclosing an inner perforated container (Fig. 1). The outer container (18.5 cm high and 21 cm diameter) was made of aluminum and the inner perforated container made up of galvanized iron sheet with a diameter of 15 cm. The outer container and inner perforated container (3 mm perforations) were arranged in such a manner that a gap of 3 cm exists between them. The containers were provided with a lid at the top, the lid having an opening at its centre. A rotatable rod is provided with smooth brushes of length 4.5 cm fixed equispaced (Fig. 2). The sides of brushes touch the inner walls of the inner perforated container. The rotatable rods are fixed to the bottom of the inner container and pass through the opening, connecting the lid at the top. The other end of the outer container is provided with a transparent container to collect the insects which fall down from the inner perforated container.

Figure 1 Outer view of pulse beetle egg removal device .

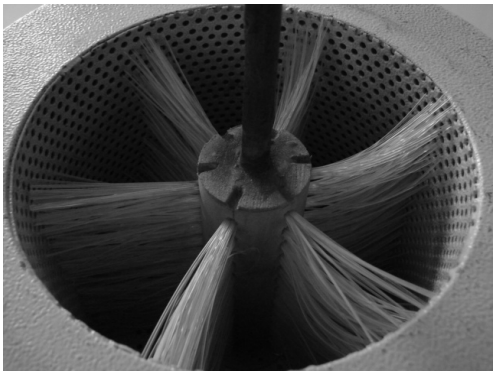

Figure 2 Inner view of pulse beetle egg removal device.

2.3. Efficiency of egg removal device

Sorghum and maize grains were obtained from the Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India and the paddy grains were procured from Paddy Breeding Station, TNAU, Coimbatore, Tamil Nadu, India, whereas, wheat grains were obtained from Horticultural Research Station, Ooty, Tamil Nadu, India. The grains were sieved to remove dust and insects and then kept at -18°C in 1 kg batches sealed in polythene bags for 10 d to destroy any prior infestation by insects (Shazali and Smith, 1986).

Unsexed adults of *T. castaneum* and *R. dominica* were released inside the grains of sorghum, wheat, maize or paddy (*T. castaneum* was not used for paddy) at the rate of 100 adults into 1 kg of grain. The grains were placed in plastic containers, covered with cloth. The grains were kept as such for 7 days for ovipositon at room temperature (26 – 27°C). After 7 d, adult insects were sieved from the grain (1 kg) and the grains were placed in the inner perforated container of the egg removal device (Fig. 2). Circular rotation involving clockwise and anticlockwise movements in an alternate manner were done for 15 min. once a day. There were 3 treatment levels; seeds were rotated for 1, 2 or 3 consecutive days.

The grains were taken out and kept undisturbed for a period of 40 days to allow for the development and emergence of offspring adults. The offspring adults were removed three times between 40 and 60 d and the all adults from these three sievings were totaled to give the number of offspring adults for each replicate.

Coriander were obtained from local groceries and were sieved to make it free from dust and insect stages if any and it was disinfested as above by freezing. Likewise, for assessing the performance of the device in the removal of eggs of *L. serricorne* from coriander, the same methodology stated earlier was followed.

2.4. Statistical analysis

The data pertaining to the observations in the laboratory were transformed using square root transformation and then analyzed in a Completely Randomized Design (CRD). The mean values of the experiments were separated using Duncan's Multiple Range Test (Gomez and Gomez, 1984).

3. Results

There were very similar results for all insects on all grains. A single rotation for 15 min on day one reduced populations from 16 to 37% compared to controls, 2 days of treatment reduce populations from 37 to 67% and 3 days of treatment reduced populations the greatest amount, with declines of 54 to 77%. There was a regular decrease in the population with each progressive day of treatment, with populations decreasing on average 15 ± 3% per day. Mechanical damage observed was very meager in the test grains when they were subjected to rotational impact.

Table 1 Impact of egg removal device on the egg stage by way of assessing the emergence of *Tribolium castaneum* and *Rhyzopertha dominica* adults from infested sorghum and wheat grains.

	Sorghum				**Wheat**			
	T. castaneum		***R. dominica***		***T. castaneum***		***R. dominica***	
Days treated	**Adults emerged Mean ± SE**	**Reduction (%)**	**Adults emerged Mean ± SE**	**Reduction (%)**	**Adults emerged Mean ± SE**	**Reduction (%)**	**Adults emerged Mean ± SE**	**Reduction (%)**
0	671 ± 4.1 d		537 ± 12.3 d		705 ± 5.5 d		612 ± 6.7 d	
1	508 ± 6.0 c	24	441 ± 8.3 c	17	587 ± 10.3 c	16	437 ± 7.2 c	28
2	419 ± 4.2 b	37	304 ± 5.6 b	43	420 ± 5.4 b	40	316 ± 11.3 b	48
3	306 ± 8.2 a	54	230 ± 8.6 a	57	218 ± 4.4 a	69	187 ± 5.3 a	69
CD (P = 0.05)	0.4471		0.6998		0.4654		0.6587	
CV%	1.54		2.72		1.61		2.55	

Means followed by the same letter are not significantly different, Duncan's Multiple Range Test ($P < 0.05$), n=5.

Table 2 Impact of egg removal device on the egg stage by way of assessing the emergence of *Tribolium castaneum* and *Rhyzopertha dominica* adults from infested maize and paddy grains.

	Maize				Paddy	
	T. castaneum		*R. dominica*		*R. dominica*	
Days treated	**Adults emerged Mean ± SE**	**Reduction (%)**	**Adults emerged Mean ± SE**	**Reduction (%)**	**Adults emerged Mean ± SE**	**Reduction (%)**
0	593 ± 9.0 d		517 ± 7.3 d		687 ± 6.8 d	
1	498 ± 13.6 c	16	432 ± 9.8 c	16	502 ± 10.3 c	26
2	364 ± 7.6 b	38	302 ± 6.9 b	41	364 ± 13.4 b	47
3	169 ± 5.4 a	71	123 ± 5.0 a	76	161 ± 13.1 a	76
CD (P = 0.05)	0.6859		0.6284		1.0205	
CV%	2.60		2.60		3.79	

Means followed by the same letter are not significantly different, Duncan's Multiple Range Test ($P < 0.05$), n=5.

Table 3 Impact of egg removal device on the egg stage by way of assessing the emergence of *Lasioderma serricorne* adults from infested coriander seeds.

Days Treated	**Adults emerged Mean ± SE**	**Reduction (%)**
0	116± 10.3 c	
1	72 ± 2.6 b	37
2	38± 2.2 a	67
3	26 ± 3.1 a	77
CD (P = 0.05)	1.2498	
CV%	8.86	

Means followed by the same letter are not significantly different, Duncan's Multiple Range Test ($P < 0.05$), n=5.

4. Discussion

Brushing infested seed had a dramatic reduction in insect populations, with control being over 70% after three consecutive days of treatments. Generally, the females of *L. serricorne* and *T. castaneum* oviposit directly on the surface of grains, and *R. dominica* laying eggs both inside and outside the kernels (Ashworth, 1993; Rees, 2004). Also young larvae of the insects are free living, starting on the outside of the seed, before finding cracks in the grain to establish themselves. This is contrast to the *Sitophilus* spp. that lay their eggs in the grain and larvae complete their life cycle inside the kernel. Brushing the seed could control populations by removing or destroying eggs or young larvae.

The regular bean tumbling dramatically lowered the bean weevil *Acanthoscelides obtectus* (Say) populations by approximately 97% in kidney bean (*Phaseolus vulgaris* L.) (Quentin et al., 1991). The mortality of rusty grain beetle *Cryptolestes ferrugineus* (Stephens) adults generally increased with increasing number of drops in wheat (Loschiavo, 1978). Joffe and Clarke (1963) working in elevators, showed that the type, timing and frequency of disturbance played a significant role in determining the extent of damage to *Sitophilus oryzae* (L.). They reported that the daily disturbance of maize grains resulted in a higher percentage of control of *S. oryzae*

Future experiments could examine if more frequent rotations or longer duration of rotations would affect mortality. Other experiments could examine if complete control can be obtained by rotating the grain for every day for 4, 5, 6 or 7 days. In this study both eggs and first instar larvae were be present. Experiments with a more well defined age structure would determine if there are differences in susceptibly between, eggs, first instar larvae, late instar larvae, pupae and adults to this type of control.

Acknowledgements

We thank Paul Fields, Agriculture and Agri-Food Canada for encouragement and support.

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Development and comparison of two models to predict survival rates of young larvae of *Stegobium paniceum* (L.) (Coleoptera: Anobiidae) under heat treated temperatures

Jian, F.[1], Jayas, D.S.*#[1], Fields, P.G.[2], Abdelghany, A.Y.[2,3]
[1] Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB, R3T 5V6 Canada.
Email: digvir_jayas@umanitoba.ca
2 Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Road, Winnipeg, MB, R3T 2M9 Canada.
3 Department of Economic Entomology, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt.

* Corresponding author
# Presenting author

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Abstract

Predicting mortality or survival rate of insects at heat treatment temperatures is critical for commercial heat treatment of food and storage facilities. Two models were developed to predict the survival rate of young larvae of drugstore beetle, *Stegobium paniceum* (L.) (Coleoptera: Anobiidae) under steady state or transient temperature condition: modified fundamental kinetic model and modified complementary log-log transformation model. Published young larvae mortalities and raw data of the temperature history, determined at different heat treatment temperatures, were used to develop these models. The models were verified and compared by using the field data collected in a medium sized mill. Both of the developed models overestimated the insect mortality in the mill when temperature was >53°C and underestimated insect mortality when temperature was <50°C. The lowest mean of the absolute difference between the predicted and measured insect mortality in the mill was 16.7±1.1% which was generated by using the modified complementary log-log transformation model. The possible reason of this divergence from the model is discussed.

Keywords: *Stegobium paniceum* (L.), Survival, Heat treatments, Modeling.

1. Introduction

Managing stored-product insects by heating food processing and storage facilities to lethal temperatures is an old and effective technology (Dean, 1913). There is renewed interest in using this old technology (Beckett et al., 1998; Mahroof et al., 2003) due to the desire to develop alternatives to the current systems of grain preservation and pest control. A heat treatment consists of heating all or part of a facility to 50–60°C and maintaining the temperature for 24–36 h (Mahroof et al., 2003; Tang et al., 2007). This method is used in facilities constructed with a wide variety of materials, brick, concrete, stone, and wood with metal sheathing, but structural damage sometimes might be a risk (Dowdy and Fields, 2002; Beckett et al., 2007). Although modern building construction can tolerate temperatures in excess of 50°C, some of the equipment in food processing and storage facilities cannot. If the temperature requirements necessary for effective control could be reduced, costs can be reduced. Therefore, predicting survival rate of insects under heat treatments would make this alternative to methyl bromide more widely used.

To systematically develop and assess heat treatments, models have been developed to predict survival insects under heat treatment temperatures. Models in the literature describing the thermal death kinetic data of insects range from fundamental kinetic models to semi- or purely- empirical models (Wang et al., 2007). The common characteristic of these published models was that it used one mathematical equation to describe the relationship between insect mortality and time at a given temperature. This assumption is usually developed based on the laboratory observation under constant temperatures. Therefore, it is usually scaled back to predict insect mortality at constant temperatures. Some developed models, such as fundamental kinetic model and complementary log-log transformation, could not be used to calculate insect mortality at transient temperature condition. Based on the logarithmic model, Boina et al., (2008) developed a dynamic model and this model could be used to predict insect mortality under transient temperature.

The fundamental kinetic model (Wang et al., 2002a, b; Gazit et al., 2004; Johnson et al., 2003 and 2004; Hallman et al., 2005) and complementary log-log transformation model (Jones et al., 1995; Thomas and Mangan, 1997; Waddel et al., 1997) have been successfully used to predict insect mortality at constant

temperature. It is not known if its basic assumption could be used to develop a model to predict insect mortality under transient temperatures.

Selection of the appropriate model depends on research preferences, intended use, target insects, and temperature range (Wang et al., 2007). These multiple choices make the model selection and using part art and part science. An ideal mathematical model for the prediction of insect mortality under heat treatment should be developed based on experimental data and be used in heat treatment with certain accuracy. It should be usable for different insects under different treatment conditions. Model comparison of the developed models by using the same laboratory and field data might be an effective tool to find the ideal and robust model.

Stegobium paniceum (L.) (Coleoptera: Anobiidae) is one of the most common insects found in botanicals warehouses (Awadallah et al., 1990; Arbogast et al., 2002; Abdelghany et al., 2009a, b). It has been recorded from wide range of food, but its distribution is more temperate than tropical (Lefkovitch and Currie, 1967). Young larvae are more heat tolerant than the other stages (Abdelghany et al., 2009b). Therefore, heat treatments that results in a 100% mortality of young larvae should control all other stages of *S. paniceum*.

The aim of this study was to: 1) develop the following two models (by using the same laboratory data) which could predict the mortality of young larvae of *S. paniceum* under steady state or transient temperature conditions: modified fundamental kinetic model, and modified complementary log-log transformation; 2) validate and verify the developed models by using the same field data; and 3) compare the developed models.

2. Data used in the development and verification of the models

During model development and model calibration, the raw and published data related to the young larvae of *S. paniceum* published by Abdelghany et al., (2009b) were used, except specified otherwise. In the laboratory, the time-mortality relationships of the young larvae were determined at constant temperatures of 42, 45, 50, 55 and 60^0C and at different treatment times. The temperature rose to the target temperature within 30 min and once the target temperature was obtained it remained stable.

To verify the developed models, comparison between the predicted and measured insect mortalities was conducted. The measured insect mortality included the data collected in both the laboratory and field. The laboratory data were the same as that used in the model developments and calibrations. Field data was collected in Western Canada at a medium sized mill from Oct 23 and 24, 2008.

3. Data collection in mill

The young larvae were prepared at the room temperature in the laboratory of Cereal Research Center, Agriculture and Agri-Food Canada, Winnipeg (Abdelghany et al., 2009b). Wheat flour (10 g at about 12% moisture content, wet basis) mixed with brewer yeast (95:5 by weight) was loaded into vials (3 cm diameter, 5 cm high). Vials were covered with 600-µm wire mesh after the 50 young larvae were introduced at the top of the flour. The vials with insects were shipped to the mill and there were three days between the insect introduction and the beginning of the heat treatment. During shipment, the larvae experienced the temperatures from 20 to 30°C.

Seven vials, each containing a different species (other species presented in Hulasare et al., 2010), were grouped together around a Hobo data logger (Onset Computer Corp. Bourne, MA). There were 13 sets of vials placed in a 20 cm diameter circle on the concrete floor. At the middle of the ring, there was one set of vials with the same amount flour and yeast, but without insects. The temperatures inside these vials at the middle of the ring were measured by introducing T-type thermocouples connected with Hobo data logger, with temperatures recorded every 1 min. The thermocouples were located at the center of the flour inside each vial. During heating treatment, the floor temperature was also measured with hand held thermocouples (52 Thermometer, John Fluke MFG) taped to the floor and readings taken every 30 min. The thermocouples were calibrated within ±0.1°C using a mercury thermometer.

The mill was heat treated from 9:15 am to the next day 10:00 am. The temperatures took 9 h to rise maximum temperature of 58°C at the floor. The heating rate was about 3.8°C/h. During that period, groups of the vials were taken out of the mill when the vial temperatures were approximately 40, 42, 45,

47, 50, 52, 55, and 60°C. The heat-treated young larvae were shipped back and held in the laboratory at 30°C, 70% r.h.. until the emergence of adults. The criterion for survival was emergence to adults.

Insect mortality was calculated by using Abbott's equation (Abbott, 1925) and control was at 40°C. Four replications were conducted in the heat treated mill. However, three replicates had a higher than 42% control mortality (possible reason being the long distance of shipment, mortality during sample preparation, or problems with the initial rearing). Therefore, only one replicate with control mortality at 20% was used to verify the model.

The temperature inside the vials was higher than the temperature on the floor. The relationship between these two temperatures in the temperature range from 40 to 60°C was:

$$T_{floor} = -6.8 + 1.1T_{vial} \qquad R^2 = 0.96 \qquad (1)$$

where, T_{floor} and T_{vial} are the temperatures on the floor and inside the vial, respectively.

It was assumed that some young larvae might be at the bottom of the vials due to larvae seeking cooler temperatures during the heat treatment in the mill. Some insects at the bottom of the vials might experience different temperatures as those insects at the top or middle of the vial. Therefore, both the vial and calculated floor temperatures were used to predict the insect mortality.

4. Development of the models

4.1. Modified fundamental kinetic model (FKM)

The method used in the development of the fundamental kinetic model was the same as described by Wang et al., (2007). It was found: 1) the 0 –order model (Eq. 2) was the best fitted equation with an average determination coefficient (R^2) = 0.85 and minimum = 0.72 (which was at 50°C); 2) K value in the 0-order model followed the Arrhenius relationship (Eq. 3); and 3) C value in the 0-order model was close to 1.0.

$$S = -Kt_c + C \qquad (2)$$

$$log_{10}^{K} = log_{10}^{K_{ref}} - \frac{E_a}{R}\left(\frac{1}{T_{t_c}} - \frac{1}{T_{ref}}\right) \qquad (3)$$

where; S is the insect survival rate at a constant temperature and in t_c (min) is the treatment time; C is a constant at each constant temperature; K is the rate constant (min^{-1}) at a constant temperature; K_{ref} is the reaction rate constant at the reference temperature T_{ref} =314.35K (41.2°C) (Table 1); T_{t_c} is the temperature as a function of chronological time (t_c); E_a is the activation energy (J/mol) (Table 1); and R is the universal gas constant (8.314 J/mol.K).

Table 1 Value of the parameters in Eq. 3.

Parameter	Mean±SE	P
$log_{10}^{K_{ref}}$	-3.5042±0.2063	0.0004
E_a	136483.3825±17015.8166	0.0040

The C values could be calculated by using the following regressed Lorenzian equation:

$$C = 1.1475 - \frac{0.3057}{1+\left(\frac{T_{t_C}-52.6263}{5.7746}\right)^2} \qquad R^2 = 0.93 \qquad (4)$$

The Eq. 2 could not be used to calculate the insect survival rate at transient temperatures. Therefore, the following assumption was made: 1) C = 1; and 2) K was only influenced by temperature. The reason for the first assumption was: 1) C values remain constant at around 1.0 for several studied insects (Stumbo, 1973; Gazit et al., 2004; Wang et al., 2007); and 2) the calculated average C value was ≈ 0.9940 when Eq. 4 and filed temperature data was used. Eq. 2 was re-written as:

$$1 - S = Kt_c \qquad (5)$$

Eq. 5 was written in a differential form as following:

$$1 - \frac{dN_{t_c}}{N_0} = K(T_{t_c})dt_c \quad (6)$$

where; dN_{t_c} is the insect survival in a small time period of t_c; N_0 is the initial number of insects; $K(T_{t_c})$ is K as show in Eq. 2, 3 and 5 in the small time period of t_c and it is function of T_{t_c}; and dt_c is the small time period of t_c and in differential form.After integration of both sides of Eq. 6 and conversion of the mathematical equation to a numerical equation, Eq. 6 becomes:

$$M = \sum_{0}^{t_c} K\left(T_{t_c}\right)\Delta t_c \quad (7)$$

where; M is the accumulated mortality from time 0 to t_c; and Δt_c is the incremental exposure time. At a constant temperature, Eq. 7 could be reduced to Eq. 5. During the model verification by using the field data, $K(T_{t_c})$ value was calculated by using Eq. 3; the T_{t_c} was the temperature at each time period; and $\Delta t_c = 1$ min.

4.2. Modified complementary log-log transformation model (CLLT)

The relationship between insect mortality and time at a given temperature was assumed as (Jones et al., 1995; Thomas and Mangan, 1997; Waddell et al., 1997):

$$M = 1 - e^{-e^{Kt_c + C}} \quad (8)$$

where; K and C are constant at each constant temperature. Eq. 8 yielded the following equation after taking logarithms for two times:

$$Ln(-Ln(1 - M)) = -Kt_c + C \quad (9)$$

It was found Eq. 8 fitted the insect mortality data with an average R^2 =0.93±0.04 and minimum = 0.80 (Table 2). Therefore, Eq. 8 was used to find the K and C value at each constant temperature by regression. To predict the K and C values at other temperatures (at which insect mortality was not determined in the laboratory), the found K and C values were used to conduct non-linear regression with K or C value as the dependent variable and temperature as an independent variable. The following were the best equations found (Table 2):

Table 2 K and C value at different constant temperatures

Temperature (°C)	K		C		
	Eq.8 [a]	Eq.10 [b]	Eq.8 [a]	Eq.11 [b]	R^2, Eq. 8[c]
42	0.0029 ± 0.0002	0.0023	-2.8375 ± 0.1570	2.8375	0.98
45	0.0041 ± 0.0005	0.0046	-3.1936 ± 0.3367	3.1936	0.93
50	0.0127 ± 0.0025	0.0126	-1.7407 ± 0.3273	1.7407	0.80
55	0.8932 ± 1.7613	0.8857	-8.0975 ± 7.6124	8.1026	0.99
60	0.8783 ± 0.1762	0.8857	-3.1196 ± 0.7161	2.7877	0.96

[a] The K or C value (mean ± SE) regressed by using Eq. 8. All the p <0.0001. [b] The K or C value calculated by using Eq. 10 and 11, respectively.[c] R^2 value regressed by using Eq. 8.

$$\begin{cases} k = \dfrac{0.7765}{1 + e^{-\frac{T_{t_c} - 47.4806}{4.3844}}} & 41.2℃ < T_{t_c} < 50℃ \quad R^2 = 0.98 \\ k = 0.0035 + \dfrac{0.8822}{1 + e^{-\frac{T_{t_c} - 48.8874}{0.1126}}} & T_{t_c} \geq 50℃ \quad R^2 = 0.99 \end{cases} \tag{10}$$

$$\begin{cases} C = 3.2538e^{-0.5\left(\frac{T_{t_c} - 44.2930}{4.6604}\right)^2} & 41.2℃ < T_{t_c} < 50℃ \quad R^2 = 0.98 \\ C = 2.7877 + 76.096e^{-0.5\left(\frac{T_{t_c} - 54.4018}{0.2912}\right)^2} & T_{t_c} \geq 50℃ \quad R^2 = 0.96 \end{cases} \tag{11}$$

To calculate the insect mortality at transient temperature, the following assumption was made: 1) C and K were only influenced by temperature; 2) Eq. 9 could be written in a differential form as follows:

$$Ln\left(-Ln\left(\frac{dN_{t_c}}{N_0}\right)\right) = -K\left(T_{t_c}\right)dt_c + C\left(T_{t_c}\right) \tag{12}$$

Where; $C\left(T_{t_c}\right)$ is C as show in Eq. 8, 9 and 11 in the small time period of t_c and is a function of T_{t_c}. After integration and mathematical conversion, Eq. 12 yields:

$$Ln\left(-Ln\left(\frac{dN_{t_c}}{N_0}\right)\right) = C\left(T_{t_c}\right) - \sum_0^{t_c} K\left(T_{t_c}\right)\Delta t_c \tag{13}$$

$$\sum_0^{t_c} M = 1 - e^{-e^{C\left(T_{t_c}\right) + \sum_0^{t_c} K\left(T_{t_c}\right)\Delta t_c}} \tag{14}$$

At a constant temperature, Eq. 14 could be reduced to Eq. 9.

During the model verification by using the field data, the value of $K\left(T_{t_c}\right)$ and $C\left(T_{t_c}\right)$ were calculated by using Eq. 10 and 11, respectively; the T_{t_c} was the temperature at each time period; and $\Delta t_c = 1$ min.

5. Results and discussion

The modified fundamental kinetic model could predict the insect mortality at constant temperature of 50 and 55°C (Fig. 1 and 2). It underestimated insect mortality at 42 and 60°C (Fig. 1, and 2), and overestimated insect mortality at 45°C (Fig. 1). The modified complementary log-log transformation model could predict insect mortality at constant temperature of 45, 50, 55, and 60°C (Fig. 1 and 2). It underestimated insect mortality at 42°C (Fig. 1).

To compare the performance of the developed models at each constant temperature, the absolute difference between the insect mortalities measured and the predicted was calculated. The mean of the absolute difference calculated from the modified fundamental model was 18.6 ± 1.8% with a maximum 65%. The mean of the absolute difference calculated from the modified complementary log-log transformation model was 9.4 ± 1.0% with a maximum 53.8%. The paired t-test was conducted between the absolute differences of two developed models with t= 5.7916 and p<0.0001. The prediction accuracy of the modified complementary log-log transformation model was significantly higher than that of the modified fundamental model.

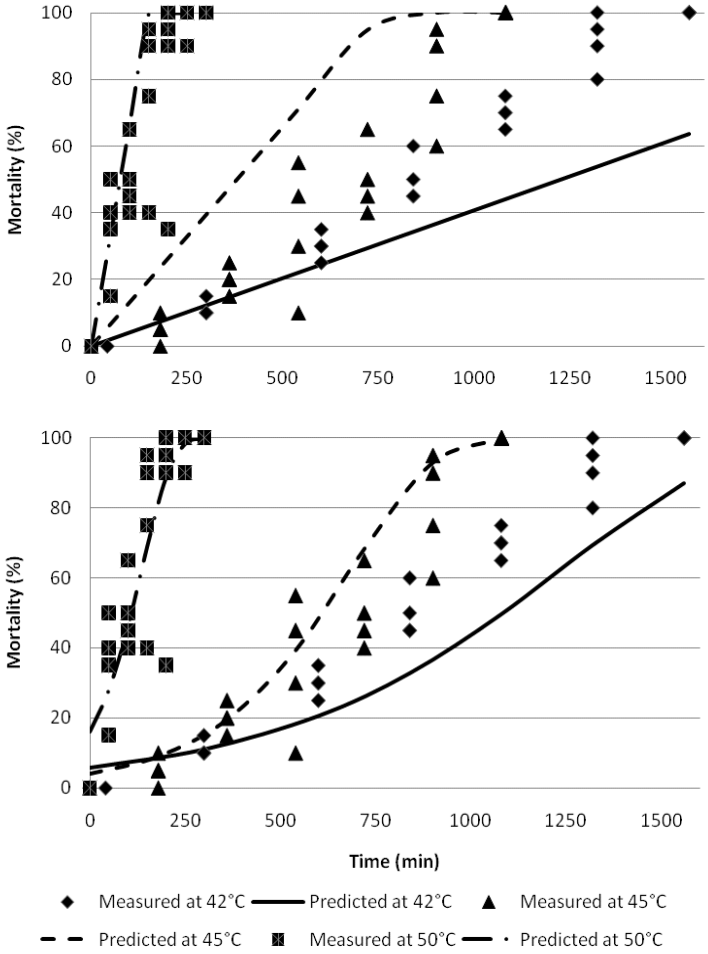

Figure 1 Insect mortality measured and predicted by using the following models at target temperature 42, 45, and 50°C: modified fundamental kinetic model (Top) and modified complementary Log-Log transformation model (Bottom).

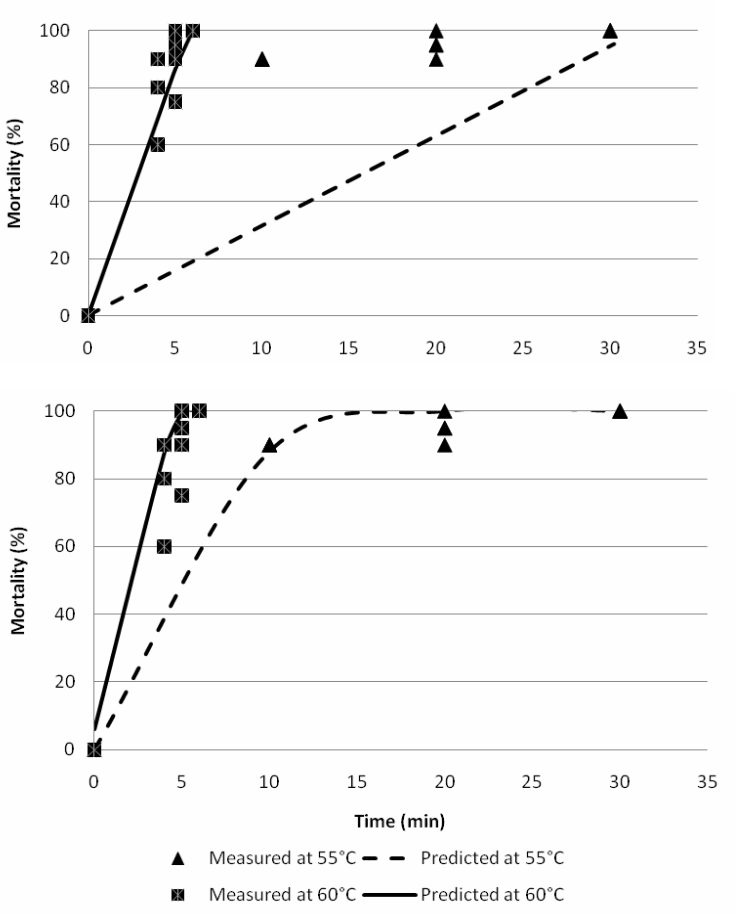

Figure 2 Insect mortality measured and predicted by using the following models at target temperature 55, and 60°C: modified fundamental kinetic model (Top) and modified complementary Log-Log transformation model (Bottom).

Both of the developed two models overestimated the insect mortality in the mill when temperature >53°C and underestimated insect mortality when temperature <50°C (Fig. 3). Using floor temperature increased the prediction accuracy (Fig. 3). This indicated that the insects in the vial might migrate to the bottom of the vial during heat treatment in the mil. The statistic result of the absolute difference between the predicted and measured insect mortality in the mill showed that (Table 3): when the floor temperature (T_{floor}) was used, the CLLT model had a better prediction; while the FKM had a better prediction when T_{vial} was used. However, both models had the same prediction trend (Fig. 3) and there was no significant different (SAS, 2008) between the predictions (Table 4), even though the modified complementary log-log transformation model had a better performance to predict insect mortality at each tested constant temperature.

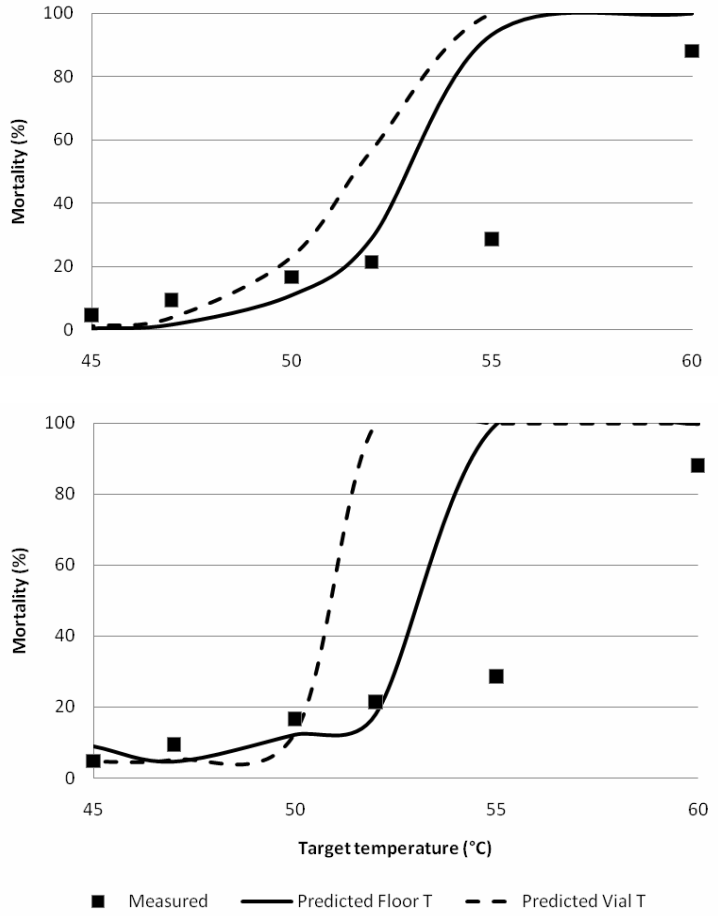

Figure 3 Insect mortality measured and predicted by using the following models in the heat treated mill: modified fundamental kinetic model (Top) and modified complementary Log-Log transformation model (Bottom). In the graph, 'Predicted Floor T' means the predicted insect mortality based on the calculated floor temperature; and 'Predicted Vial T' means the predicted insect mortality based on the measured vial temperature.

Table 3 Statistic of the absolute difference between the predicted and measured insect mortality in the mill

Statistic of the absolute difference	Predicted by using T_{floor}		Predicted by using T_{vial}	
	FKM	CLLT	FKM	CLLT
Mean ± SE	17.0 ± 9.6	16.7 ± 11.0	22.4 ± 10.9	28.4 ± 14.8
Maximum	64.7	71.4	71.4	78.6
Minimum	4.4	3.4	3.6	0.26

Table 4 Results of the paired t test between the absolute differences between measured and predicted insect mortality calculated from developed models by using the tested constant temperature, floor temperature in the mill, and the vial temperature

	t	P
Constant temperature	5.7916	<0.0001
T_{floor}	0.2068	0.8443
T_{vial}	-0.8148	0.4522

Both of the developed models: 1) underestimated the insect mortality determined at constant temperature of 42°C and when temperature <50°C in the mill; and 2) overestimated the insect mortality when temperature >53°C in the mill. These facts indicated that these two developed models worked better when treatment temperature was about 50°C. The reason for the overestimation or underestimation at other temperatures might be that these two models did not count the following factors: 1) heating rates (Beckett et al., 1998); 2) death effect during heating period (the time from room temperature to lethal temperature); 3) individual death time distribution; 4) different physiological deaths during heat treatment; and 4) enzyme complexes causing insect death. These un-accounted factors play important role in the insect death time during heat treatment (Tang et al., 2007). For example, the temperatures rose to 60°C within 15 min at laboratory condition or 10 min at 55°C will result 90% mortality of young larva of *S. paniceum* (Abdelghany et al., 2009b). Therefore, these two models could be used to predict mortality of insects when subjected to steady-state isothermal heating, and might have limitation when used at transient and non-isothermal conditions.

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Radio frequency treatments for insect disinfestation of dried legumes

Johnson, J.A.*#[1], Wang, S.[2], Tang, J.[2]
[1] San Joaquin Valley Agricultural Sciences Center, 9611 South Riverbend Avenue, Parlier, CA 93648-9757, USA, Email: judy.johnson@ars.usda.gov
[2] Department of Biological Systems Engineering, Washington State University, 213 L.J. Smith Hall, Pullman, WA 99164-6120, USA

* Corresponding author
# Presenting author

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Abstract

Dried legumes (chickpeas, green peas or lentils) are valuable export commodities in the US Pacific Northwest. A major problem in the marketing of these products is infestation by insect pests. Typically, chemical fumigants are used to disinfest product, but regulatory issues, insect resistance, environmental concerns and the increase of the organic market have forced the industry to explore non-chemical alternatives. One possible alternative is the use of radio frequency (RF) energy to rapidly heat product to insecticidal levels. To determine the potential of RF treatments to control insect pests in dried pulse products, the heat tolerance of the cowpea weevil (*Callosobruchus maculatus* F.) was evaluated and compared to the tolerance of previously studied insects, and the dielectric properties of both the insect and the products were compared. The most heat tolerant stage of the weevil was found to be the pupal stage, with adults being the most susceptible. Cowpea weevil pupae were fairly heat tolerant; to obtain rapid mortality (exposure <10 min) temperatures of 56-58°C were needed. At frequencies commonly used by industry for RF heating, dielectric loss factors for both adult and larval cowpea weevil was higher than those for legumes, suggesting that cowpea weevils would heat at a faster rate than the product. Previous studies showed that suitable heating uniformity during RF treatments was obtained through the addition of hot air (60°C) and conveyor belt movement. These studies showed that chickpeas, green peas and lentils were able to tolerate RF treatments of 60°C for 10 min without adverse effects on quality. The results suggest that practical large scale RF treatments to disinfest pulses may be possible.

Keywords: Heat treatments, Dried pulses, Cowpea weevil, Radio frequency, Disinfestations

1. Introduction

Chickpea (*Cicer arietinum* L.), green pea (*Pisum sativum* L.) and lentil (*Lens culinaris* Medikus) are three important rotational legumes in the western United States. Infestation by postharvest insect pests can be a major problem in the processing and marketing of dried legumes. Of particular economic importance is the cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), a serious internal pest of several legume crops. Another pest of concern is the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), a common pest of many stored products. These insects reduce the quality of products through direct feeding damage and contamination of the product with exuviae, webbing and feces. Legumes infested with cowpea weevils and other internal feeders are not often easily detected by external inspection. Regulatory agencies and importers in many countries have therefore established phytosanitary protocols, often including postharvest disinfestation treatments.

Historically, the legume industry has relied on chemical fumigation (methyl bromide or phosphine) for postharvest insect control. In 2004, India imposed a non-tariff barrier requiring all imported legumes to be fumigated with methyl bromide and certified free of bruchids (USADPLC, 2007). However, most phytosanitary uses of methyl bromide within the U.S. were phased out in 2005 by the U.S. Environmental Protection Agency under the Montreal Protocol (UNEP, 2006). In addition, methyl bromide fumigation is less practical at treatment temperatures <5°C, with lower treatment temperatures requiring higher doses or extended exposure times. Legume processing plants and warehouses in the interior northern states of the U.S. have night temperatures below 5°C for more than 6 months each year (USADPLC, 2007). Therefore, there is a need to develop a practical alternative to methyl bromide for control of insect pests in legumes. Any alternative must also have a minimum impact on product quality and environment.

Heat treatment methods using hot air have been investigated extensively as non-chemical alternatives for disinfesting stored commodities. However, it is difficult to accomplish disinfestation using conventional hot air heating methods without causing deleterious effects to product quality (Armstrong, 1994), and the slow rate of heat transfer due to a high resistance of conduction within bulk materials results in extended treatment times (Evans et al., 1983). Low heating rates also may increase the thermotolerance of the targeted insects (Beckett and Morton, 2003) by causing the induction of heat shock proteins in insects (Yin et al., 2006).

Radio frequency (RF) energy offers the possibility of rapidly increasing temperatures within bulk materials. RF energy directly interacts with commodities containing polar molecules and charged ions to generate heat volumetrically, significantly reducing treatment times when compared to conventional heating methods. Many studies have explored the possibility of using RF energy to disinfest insect pests (Hallman and Sharp, 1994; Nelson, 1996). More recent studies demonstrated the potential of RF treatments for industrial disinfestation of in-shell walnuts with acceptable product quality (Wang et al., 2007a, b). The demonstrated ability of RF treatments to disinfest low moisture products suggests this method for potential applications in dried legumes.

The most important considerations in developing heat treatments using RF energy are the thermotolerance of targeted insects and treated products, and the heating uniformity of the product. In RF treatments, heating uniformity is largely a function of the dielectric properties of the product and the design of the treatment. This paper presents information on the thermotolerance of target insects and the dielectric properties of products and target insects. The treatment effect on product quality and heating uniformity of proposed RF treatments will also be discussed.

2. Materials and methods

2.1. Insect thermotolerance

The relative heat tolerance of cowpea weevil stages was determined using a computer-controlled heat block system designed by Washington State University (Ikediala et al., 2000; Wang et al., 2002). Test insects were from a culture of cowpea weevils maintained at the San Joaquin Valley Agricultural Research Center, Parlier CA, on black-eyed peas, *Vigna unguiculata* (L.), at 28 ± 0.5°C and a photoperiod of 14:10 (L:D) h. Cowpea weevils were treated in mung beans, *Vigna radiata* (L.) Wilczek, a small legume that fit within the heat block and provided relatively rapid heat transfer. Adult cowpea weevils were allowed to oviposit on clean mung beans for 24 h. Infested mung beans were held under rearing conditions and treated 2, 7, 15, and 21 d after infestation when developing cowpea weevils were in the egg, young larval, old larval and pupal stage, respectively. All stages were treated at 50°C for 50, 100 and 150 min and 54°C for 7, 14, and 21 min. A second series of tests treated old larvae and pupae at 54°C for 12, 16 and 20 min, and 56°C for 5, 8 and 11 min. All infested mung beans were held for adult emergence at rearing conditions. Emerging adults were counted and treatment survival estimated by comparing emergence from treated beans with emergence from untreated controls. About 100 adult weevils in flat nylon screen bags were also treated at the above temperature/time combinations. Adult mortality was evaluated 24 h after treatment. When adults proved to be much less tolerant than the immature stages, adults were also treated at a series of less extreme temperature-time combinations. For all experiments each treatment was replicated three times.

Preliminary estimates for the thermal tolerance of cowpea weevil were made for both the most tolerant immature cowpea weevil stage (as determined above) and cowpea weevil adults. Thermal death time (TDT) curves based on the observed minimum exposure time that resulted in 100% mortality were compared with those previously determined for Indianmeal moth, red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) in Johnson et al. (2003), Johnson et al. (2004), and Wang et al. (2002), respectively.

2.2. Dielectric property measurements

Immature stages (late larvae and pupae) were dissected from black-eyed peas, while adult weevils were collected from laboratory cultures. Both immature stages and adults were killed and stored at -20°C until they could be shipped to Washington State University for measurement. Dielectric measurements of cowpea weevils were made using the open-ended coaxial probe technique with an impedence analyzer

(model 4291B, Hewlett-Packard, Santa Clara, CA, USA) over a frequency range of 10-1800 MHz (Wang et al., 2003). Measurements of the dielectric constant (ε ') and loss factor (ε ") were made at 20, 30, 40, 50 and 60°C. Insect and legume moisture contents were determined on wet basis in aluminium moisture dishes in a vacuum oven (ADP-31, Yamato Scientific America Inc., Santa Clara, CA, USA) at 130 ºC for one hour (AOAC, 2002).The dielectric properties were compared to similar values obtained for Indianmeal moth (Wang et al., 2003). Of the four legumes previously studied (chickpea, green pea, lentil and soybean) in Guo et al. (2010), lentils were chosen as a representative legume for comparing dielectric properties.

3. Results

3.1. Insect thermotolerance

The survival of cowpea weevil eggs, young larvae, old larvae and pupae exposed to 50 and 54°C by the heat block method is given in Figure 1. Eggs and young larvae were consistently the least tolerant to the treatment in the heat block, while old larvae and pupae were the most tolerant. Complete mortality occurred in all adult cowpea weevil exposed to the temperature-time treatments in Figure 1.

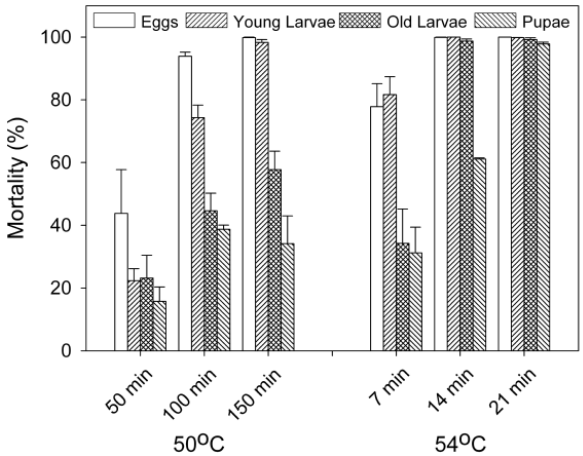

Figure 1 Mortality of immature stages of the cowpea weevil at 50°C for 50, 100 and 150 min, and 54°C for 7, 14 and 21 min.

The results of further tests with old larvae and pupae at 54 and 56°C are given in Figure 2. Mortality of old larvae was consistently higher than that for pupae. Based on these results, pupae were selected as the most tolerant cowpea weevil stage for more detailed thermal death studies. Preliminary results from these studies, given in Figure 3, show that cowpea weevil pupae are quite temperature tolerant when compared to navel orangeworm, Indianmeal moth and red flour beetle. Figure 3 also shows that cowpea weevil adults, the least tolerant of the cowpea weevil stages using the heat block method, were also more tolerant than all the other insects previously tested.

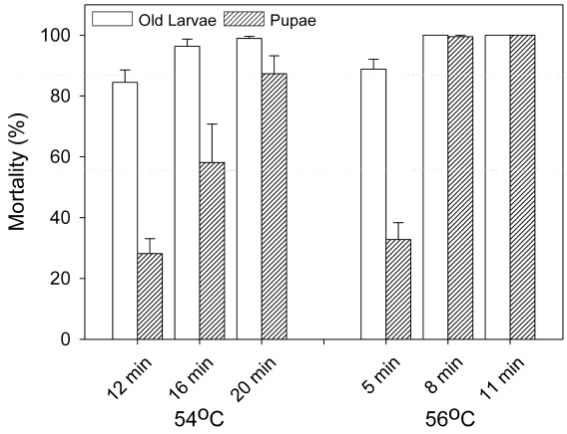

Figure 2 Mortality of cowpea weevil older larvae and pupae at 54°C for 12, 16 and 20 min, and 56°C for 5, 8 and 11 min.

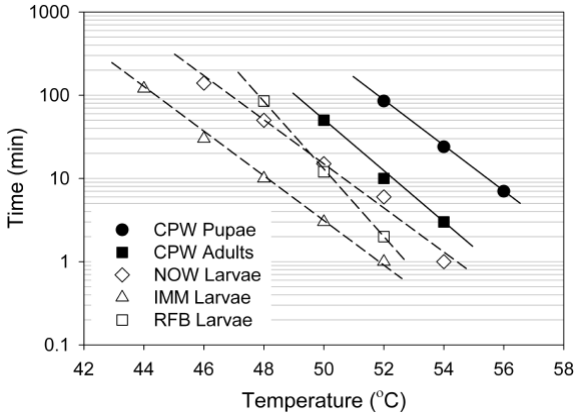

Figure 3 Thermal death time curves for cowpea weevil pupae and adults, navel orangeworm larvae, Indianmeal moth larvae and red flour beetle larvae.

3.2. Dielectric property measurements

Figure 4 shows dielectric constant (ε′) and dielectric loss (ε″) for cowpea weevil immature stages, cowpea weevil adults, Indianmeal moth larvae and lentil (8% m.c.) as a function of frequency at 20°C. The ε′ and ε″ for both cowpea weevil immature stages (larvae and pupae) and adults were comparable to those found for Indianmeal moth larvae (Wang et al., 2003). Both ε′ and ε″ for all insect stages were higher than those for lentil due to the higher moisture (70.8%) of insects. This was consistent for all temperatures tested. Figure 5 shows the effect of temperature on dielectric loss for cowpea weevil immature stages and lentil at two different moisture contents (8 and 22%). The difference between the cowpea weevils and the product increased with increasing temperature. These results suggest that both cowpea weevils and Indianmeal moth would heat at a faster rate than the product.

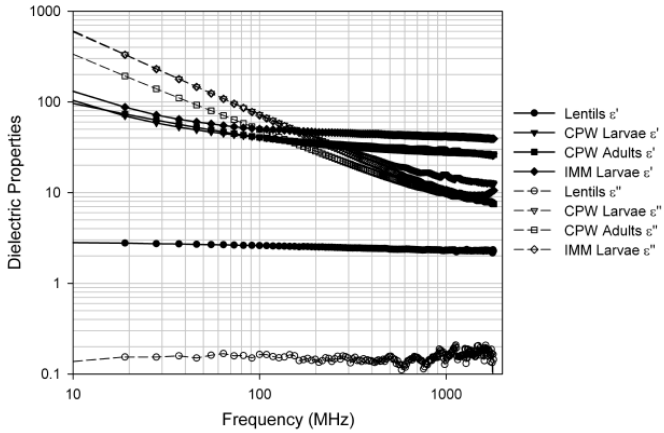

Figure 4 Frequency dependence of the dielectric constant (ε′) and loss (ε″) for cowpea weevil immature stages (large larvae and pupae), adults, Indianmeal moth larvae and lentil (8% m.c.) at 20°C.

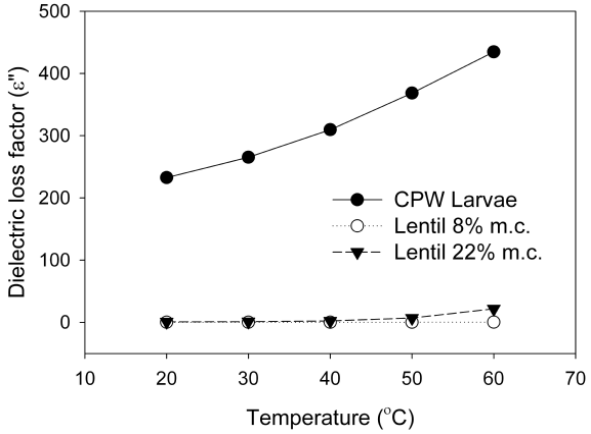

Figure 5 Temperature dependence of dielectric loss (ε″) at 27 MHz for cowpea weevil immature stages (large larvae and pupae) and lentil at 8 and 22% m.c.

4. Discussion

The cowpea weevil is the first internal seed feeding insect to be tested using the heat block system designed by Washington State University. Because of the carefully controlled and rapid heating rates obtained with the heating block system, it has proven a valuable tool in determining thermal death kinetics for several target insect pests in previous radio frequency feasibility studies (Wang et al., 2002; Johnson et al., 2003, 2004). However, there are some difficulties inherent in using this method on an internal seed feeder. Dissecting the necessary number of test insects without causing extreme control mortality was difficult, so test insects were treated within small mung bean to minimize the effect of insulation by the bean. Because test insects were treated in bean, it was difficult to be certain of the exact number of insects and stage being treated, resulting in sometimes variable results.

The current study indicates that the pupal stage is the most heat tolerant stage of the cowpea weevil. However, care must be taken in applying this information. Eggs and adults were the least tolerant stages tested, and both these are external stages. It is difficult to compare these stages directly with the internal larval and pupal stages, due to the insulation of the bean. The heating rate experienced by internal stages is much slower than that experienced by the external stages, but so is the cooling rate, making the estimation of the actual accumulative temperature effect very difficult. Because of this uncertainty, it is

recommended that studies to confirm the relative heat tolerance of cowpea weevil stages under RF heating be conducted before large-scale confirmatory studies using industrial RF units are attempted.

Regardless of the relative tolerance of the internal stages, it is clear that the cowpea weevil is heat tolerant when compared to other species, as the least tolerant cowpea weevil stage (adult) was more tolerant than any of the other species tested with the heat block. Protocols for RF treatments will likely need to raise products to 56°C or higher for more than 7 min to obtain adequate control of cowpea weevil, and any such treatment should also control Indianmeal moth.

The comparison of the dielectric properties of the target insects with those of the products indicates that the target insects should heat faster than the product when treated with RF energy. Theoretical differential heating has been reported for several other products (Wang et al., 2003) and was demonstrated experimentally in almond (Wang and Tang, 2007). The occurrence of differential heating improves the likelihood of developing a successful RF treatment protocol, resulting in increased insect mortality at relatively low product temperatures that cause no harm to the product.

Guo et al. (2010) calculated penetration depths of various legumes from their dielectric properties, noting that penetration decreased as frequency increased. It was determined that the penetration depths in 27 MHz industrial RF systems should provide sufficient throughput of product to be practical. Wang et al. (2010) suggested an RF treatment protocol for legumes using 27 MHz RF to rapidly heat product in a 10-cm-deep bed to 60°C, then holding the product at 60°C for 10 min through the application of hot forced air. Afterwards, the product would be reduced to a 1-cm-deep bed to allow for rapid cooling through forced ambient air. In this treatment, the heating uniformity of the product was maximized by the addition of 60°C forced hot air and movement along a conveyor belt. Mixing of the product between successive RF treatments did not improve heating uniformity, as had been found with RF treatments of inshell walnut (Wang et al., 2007a). The results of the current study indicate that this treatment should be sufficient to control cowpea weevil and Indianmeal moth, but additional tests are needed to confirm the treatment protocol.

The effect of the proposed treatment protocol on product quality was also examined by Wang et al. (2010). No significant differences in weight loss, moisture content, color or germination of chickpea, green pea or lentil were observed between RF treatments and unheated controls. RF treatments, therefore, should provide a practical, effective and environmentally friendly method for disinfestation of postharvest legumes while maintaining product quality.

❖ This paper represents the results of research only. Mention of a proprietary product or trade name does not constitute a recommendation or endorsement by the US Department of Agriculture.

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Effectiveness of flameless catalytic infrared radiation against life stages of three stored-product insect species in stored wheat

Khamis, M.[1], Subramanyam, B.*#[1], Dogan, H.[1], Flinn, P.W.[2], Gwirtz, J.A.[1]
[1] Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA.
Email: sbhadrir@k-state.edu
[2] USDA-ARS, Center for Grain and Animal Health Research, Manhattan, KS 66502, USA.

* Corresponding author
# Presenting author

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Abstract

A bench top flameless catalytic infrared emitter was evaluated in the laboratory to disinfest wheat containing different life stages (ages) of the lesser grain borer, *Rhyzopertha dominica*; rice weevil, *Sitophilus oryzae*; and red flour beetle, *Tribolium castaneum*. The emitter produces infrared in the 3 to 7 um range. A non-contact infrared thermometer obtained real-time grain temperatures during exposures of uninfested and infested wheat containing various life stages of the three insect species. The grain temperatures attained were influenced by wheat quantity, distance from the emitter, and exposure time, which in turn influenced effectiveness against various life stages of the three species. In general, higher grain temperatures were attained in 113.5 g of wheat as opposed to 227.0 g, at 8.0 cm from the emitter surface rather than at 12.7 cm, and during a 60-sec exposure compared to a 45-sec exposure. Logistic regression indicated the probability of death of various life stages of *R. dominica*, *S. oryzae*, and *T. castaneum* was temperature-dependent. About 99 to 100% mortality of all life stages of the three species occurred when the mean wheat temperatures were in the range of 108 to 114°C. The promising results show flameless catalytic infrared technology to be a viable option for disinfestation of stored wheat, provided such high temperatures do not affect grain quality.

Keywords: Infrared radiation, Stored-product insects, Non-chemical method, Efficacy assessment

1. Introduction

Stored-product insects in grain have been traditionally managed by chemical methods (Martin et al., 1997). Reliance on chemicals has led to several stored-product insects developing resistance to traditionally used pesticides (Subramanyam and Hagstrum, 1996). In addition, new government policies on chemical residues in food and consumer's demand for safer, healthier food necessitate exploring nonchemical alternatives for managing insects associated with stored commodities.

Infrared radiation technology, a nonchemical alternative, has been used to disinfest both soft wheat grain and paddy rice (Tilton and Schroeder, 1963; Cogburn et al., 1971; Kirkpatrick et al., 1972; Kirkpatrick, 1975; Tilton et al., 1983). A 40-sec exposure resulted in 99.6% mortality of adults of 12 stored-product insects in wheat (Kirkpatrick et al., 1972). The infrared generators used in these evaluations had an open flame with temperatures >926°C (Kirkpatrick and Cagle, 1978). Such high temperatures and open flames are not suitable for use in grain-handling facilities because of explosion hazards. In addition, in previous studies, grain temperatures were measured after exposure to infrared radiation, which resulted in underestimating actual temperatures attained by the grain. Flameless catalytic infrared radiation is a new and green technology developed by Catalytic Drying Technologies, LLC., in Independence, KS, USA. (www.catalyticdrying.com). The flameless infrared radiation is emitted when propane combusts in presence of a platinum catalyst generating temperatures around 400ºC (Gabel et al., 2006; Pan et al., 2008). The 3 to 7 µm infrared energy is readily absorbed by water molecules, resulting in increased temperatures. Since stored-product insects have higher moisture content (~60%) than grain (11-13%), the former will receive a lethal dose of infrared energy. This green technology may be a viable alternative to traditionally used grain protectants and the fumigant phosphine. The objectives of this research were to examine factors influencing effectiveness of flameless catalytic infrared energy against various life stages (ages) of three economically important insect species associated with stored wheat.

2. Materials and methods

2.1. Insect rearing

The lesser grain borer, *Rhyzopertha dominica* (F.)(Coleoptera: Bostrichidae) and rice weevil, *Sitophilus oryzae* (L.)(Coleoptera: Curculionidae) were reared on organic whole wheat (Heartland Mills, Marienthal, KS, USA). The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), was reared on 95% whole wheat flour plus 5% (by wt) of Brewer's yeast diet. All cultures were reared at 28ºC, 65% r.h., and 14:10 (L:D) h photoperiod in laboratory growth chambers.

2.2. Grain infestation and infrared treatment

Three factors that influence infrared radiation intensity, grain quantity (113.5 and 227.0 g), distance from the emitter (8.0 and 12.7 cm), and exposure time (45 and 60 s) were investigated. Only organic hard red winter wheat was used in biological tests reported in this paper. One hundred unsexed adults of *R. dominica*, or *S. oryzae* from cultures were added to each jar (113.5 or 227.0 g). After 3 d of infestation, the adults were removed by sifting the contents, and all grain contents including eggs of *R. dominica* that were laid outside of kernels were collected and placed back in the jar. The jars with 3-d-old eggs represented age 0 for the two species. Jars held in the growth chamber at 28ºC and 65% r.h. for 7, 14, 21 and 24 d represented larvae in different developmental stages, and jars held for 28 d represented pupal and adults within kernels only for *R. dominica*. To expose adults of *R. dominica* to infrared radiation, 100 adults collected from culture jars were directly added to wheat. For *S. oryzae*, 7, 14, and 21 d infested samples represented larval stages while 24 d samples represented pupal stages, and 28 d sample represented adults within kernels and a few that emerged from kernels. Jars held 2 wks past 28 d had adults of *S. oryzae* and these were directly exposed to infrared radiation. The development of internal stages for both species was verified by radiographic analysis using the Faxitron X-ray device (Model 43855A; Faxitron X-Ray Corporation**,** Lincolnshire, IL, USA).

In the case of *T. castaneum*, 100 unsexed 2-wk-old adults from cultures were introduced into several jars each containing 100 g of flour plus yeast diet. The procedures for extracting eggs, young larvae, old larvae, pupae, and adults of *T. castaneum*, for use in tests, were similar to those described by Mahroof et al. (2003). Eggs were collected within 24 h of infestation (day 0), and larvae in various developmental stages were collected from these infested jars on days 7, 14, and 21; insects collected on day 24 presented the pupal stage. Adults (2-wk-old) needed for exposures were directly collected from the jars. For exposures, 100 individuals of all stages were used, except for the egg stage where 50 were used.

The bench top infrared emitter model, donated by Catalytic Drying Technologies LLC., has a circular heating surface of 613.36 cm^2, and is fueled by a 473-ml container of propane (Ozark Trail Propane Fuel, Bentonville, AR, USA) at 28.0-cm water column pressure. A steel pan of 27.94-cm diameter and 3.8-cm deep with a steel handle (43-cm long) was used to expose infested wheat in a single layer to infrared radiation. Temperature of exposed wheat was measured continuously at the center of the pan using a non-contact infrared thermometer (Raynger MX4 Model 4TP78 Raytek®, Santa Cruz, CA, USA) placed 75 cm away from the emitter. The thermometer was connected via an USB port to a laptop computer to record "real-time" grain temperatures every second (LabVIEW (National Instruments Corporation, Austin, TX, USA).

2.3. Assessment of insect mortality

Wheat exposed to infrared radiation was placed back in jars and held at 28ºC and 65% r.h. Adult mortality was determined after a 24 h holding time. Mortality of immature stages was based on number of adults that emerged from those stages. These values were compared with adult emergence in untreated wheat samples that were infested similarly.

2.4. Experimental design and data analysis

The experiment was run as a completely randomized design. The mean temperature attained every second was plotted as a function of time for 113.5 and 227.0 g of wheat exposed for 45 and 60 s at 8.0 and 12.7 cm from the infrared emitter. The temperature profile for each replicate was also averaged over time for use in logistic regression (see below).

The number of adults that emerged from untreated wheat and those exposed to infrared in the various treatment combinations was recorded. The main effect of insect age, wheat quantity, distance from

emitter, and exposure time, and their two-way interactions on the probability of death were determined using logistic regression at P= 0.05 (SAS Institute, 2002). Odds ratios from logistic regression were used to show differences in susceptibility (odds of dying) of various life stages exposed to infrared. Differences in susceptibility of various life stages was also shown by plotting probability of death as a function of mean temperature attained averaged over wheat quantity, distance from the emitter, and exposure time.

3. Results and discussion

A comparison of temperature profiles across various ages showed that for any given quantity of grain, distance from emitter and exposure time, the profiles were essentially similar. The temperatures attained were generally greater when wheat was exposed for longer time periods at same grain quantity and distance from the emitter. Figure 1 is a representative temperature profile obtained for wheat exposed to infrared radiation in 113.5 and 227.0 g of wheat at distances of 8.0 and 12.7 cm from the emitter for 45 and 60 s. The time-dependent temperature profile was highest for 113.5 g of wheat exposed for 45 or 60 s at a distance of 8.0 cm from the emitter followed by 113.5 g of wheat exposed at 12.7 cm than in 227.0 g samples. Additional information about temperature profiles and statistical comparisons are not provided here because of space limitations, but these are explained in detail in a thesis (Khamis, 2009).

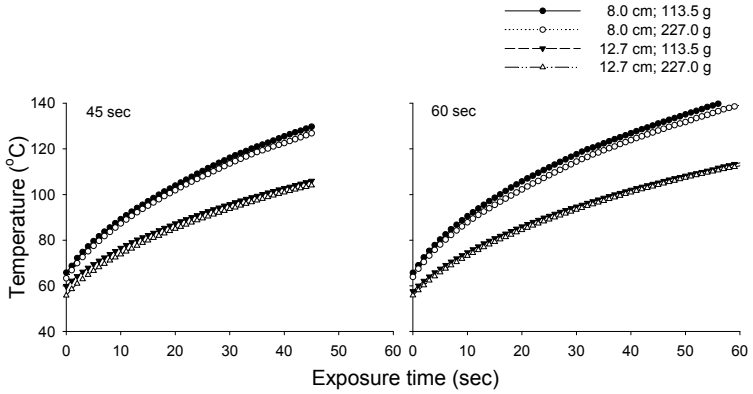

Figure 1 A generalized time-dependent temperature profile attained with different quantities of wheat exposed at 8.0 and 12.7 cm from the emitter surface for 45 or 60 sec.

The temperatures we observed were twice as high as those reported by other researchers (Tilton and Schroeder, 1963; Kirkpatrick, 1975; Kirkpatrick and Cagle, 1978; Tilton et al., 1983; Pan et al., 2008) in various stored commodities, including wheat. Unlike our tests, previous researchers measured grain temperature after infrared exposures rather than in real time.

About 212 to 581 *R. dominica* and 216 to 403 *S. oryzae* adults emerged from uninfested wheat samples. Consistently more adults of both species emerged in 227.0 g of wheat compared with 113.5 g of wheat, irrespective of insect age. It is hard to explain why more *R. dominica* adults emerged from 227.0 g than 113.5 g of wheat, besides the fact that there were twice as many kernels in 227.0 g of wheat. Toews et al. (2000) reported on adult progeny production when 100 g of each of eight United States wheat cultivars were infested with 50 unsexed *R. dominica* adults for 7 d. In their study, they carried out three separate experiments and progeny production was determined at 27 and 34ºC at 70% r.h. They found large differences in progeny production, which varied from a low of 123 to a high of 940 adults. Campbell (2002) reported that *S. oryzae* laid more eggs in kernels that were = 20 mg. The consistently greater progeny production of *S. oryzae* in 227.0 g of wheat compared with 113.5 g could be due to the availability of more kernels that weighed = 20 mg, or it could just be natural variability in the number of eggs laid by the mixed age adults used in our study.

Logistic regression analysis showed that the probability of death of all three species was influenced significantly ($P < 0.05$) by insect age, wheat quantity, distance from the emitter, and to a lesser extent on exposure time (Table 1). Increasing the grain quantity or increasing the emitter distance resulted in lower

probability of insect death (Figures 2-4). In the case of *R. dominica*, irrespective of insect age, the best treatment appeared to be 113.5 g of wheat exposed for 60 s at a distance of 8.0 cm from the emitter, because in these treatments mean temperatures attained 108 to 114ºC, and the probability of death ranged from 0.99 to 1.00 (99.0 to 100.0% mortality). Complete mortality of all insect ages of *S. oryzae* was achieved in 113.5 g of grain, exposed for 60 s, at 8.0 cm from the emitter surface resulting in grain temperatures of 108 to 112ºC. The variation in probability of death of various life stages of *T. castaneum* was evident at mean grain temperatures below 105ºC. Across grain quantities, exposure times, and distance from emitter, all life stages of this species were killed when the mean grain temperatures attained were between 108 and 111ºC.

Table 1 Logistic regression statistics showing the influence of main and interactive effects on probability of death for three insect species exposed to infrared radiation.

Effect	*R. dominica*			*S. oryzae*		*T. castaneum*		
	df	χ^2	$P>\chi^2$	χ^2	$P>\chi^2$	df	χ^2	$P>\chi^2$
Insect age	6	642.6	<0.0001	1404.5	<0.0001	5	26.7	<0.0001
Grain quantity	1	323.1	<0.0001	18.1	<0.0001	1	67.9	<0.0001
Emitter distance	1	342.7	<0.0001	111.6	<0.0001	1	51.3	<0.0001
Exposure time	1	223.8	<0.0001	2.5	0.1111*	1	97.7	<0.0001
Age × Quantity	6	154.7	<0.0001	89.6	<0.0001	5	34.8	<0.0001
Age × Distance	6	281.5	<0.0001	144.4	<0.0001	5	18.1	<0.0001
Age × Time	6	565.6	<0.0001	182.2	<0.0001	5	44.3	<0.0001
Distance × Quantity	1	82.8	<0.0001	12.0	<0.0005	1	8.4	<0.0001
Quantity × Time	1	47.1	<0.0001	---**	-----	-----	-----	-----
Distance × Time	1	84.0	<0.0001	47.1	<0.0001	1	13.3	<0.0001

*Not significant ($P > 0.05$). This is the only non-significant value. **The model could not be fit to the data.

Odds ratios showed differences in susceptibility among life stages to infrared radiation, and these susceptibility trends varied by species. For example, with *R. dominica*, the odds ratio for 21-d-old insects was 0.94 when compared with the adults (1), which were the next least susceptible stage to infrared. Odds ratios for the other stages in increasing order were eggs (1.45), 24-d-old insects (2.35), 14-d-old insects (2.41), and 7-d-old insects (3.80). With *S. oryzae*, eggs were the least susceptible stage (odds ratio, 0.02), followed by adults within kernels (28-d-old; 0.14), pupae (24-d-old, 0.18), young-to-old larvae (7- to 21-d-old; range, 0.49-0.73), and 42-d-old adults (1). Both insect mortality (probability of death data) and odds ratios for *T. castaneum* showed that pupae were the least susceptible stage (odds ratio, 0.52), followed by eggs (0.66), adults (1), old larvae (21-d-old, 1.46), mid-to-old larvae (14-d-old, 1.78), and young larvae (7-d-old, 2.86).

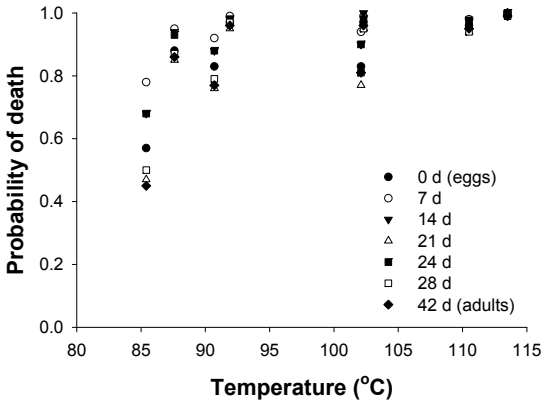

Figure 2 Probability of death of different ages of *R. dominica* as a function of mean wheat temperature.

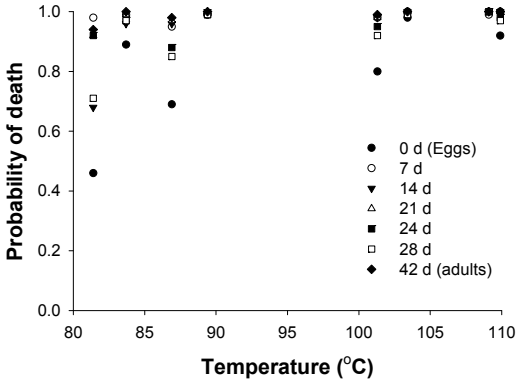

Figure 3 Probability of death of different ages of *S. oryzae* as a function of mean wheat temperature.

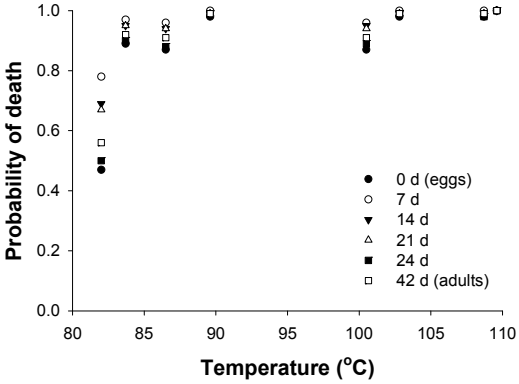

Figure 4 Probability of death of different ages of *T. castaneum* as a function of mean wheat temperature.

The variation in susceptibility among life stages and species may be related to the adverse effects of infrared on the insect's physiological processes. Except for the egg and adult stages, all other stages of *R. dominica* and all life stages of *S. oryzae* are spent within kernels. The location of the insect within the kernel may also influence its susceptibility to heat. In the case of adults of all species and larvae of *T. castaneum*, their ability to move away from areas that are hotter to seek cooler areas may make them less susceptible to heat. We found eggs to be relatively more tolerant to infrared radiation. Kirkpatrick (1975) reported only 8% percent mortality of eggs and first instars when exposed to infrared radiation. Some of the eggs could have escaped infrared treatment, perhaps by being shielded by kernels.

In summary, to completely control all life stages of the three species, the best infrared treatment appeared to be 113.5 g of wheat exposed for 60 s at a distance of 8.0 cm from the emitter; in these treatments mean temperatures attained 108 to 114°C. These results show that flameless catalytic infrared radiation technology may be valuable tool for disinfesting stored grain. Quality tests with infrared treatments reported here revealed no adverse effects on the physical, chemical rheological, and end-use qualities of wheat (Khamis, 2009).

❖ Mention of trade or proprietary names in this publication does not imply an endorsement by Kansas State University or the USDA.

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Laboratory evaluation of diatomaceous earth against main stored product insects

Kostyukovsky, M.*#[1], Trostanetsky, A.[1], Menasherov, M.[1], Yasinov, G.[1], Hazan, T.[2]
[1] The Volcani Center, Dept. of Food Science, P.O.Box 6, Bet Dagan, 50250, Israel.
Email: inspect@volcani.agri.gov.il
[2] Hazan Pest Control Ltd, P.O. Box 1147 Acre Old City, 24501, Israel

*Corresponding author
Presenting author

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Abstract

The sensitivity of the main external and internal stored product insect pests to the commercial formulation of Detia Degesch Diatomaceous Earth – DDDE - Inerto (DE) was studied in laboratory experiments. The tested insects were adults of internal feeders *Sitophilus oryzae Rhyzopertha dominica* and external feeders *Oryzaephilus surinamensis*, *Tribolium castaneum*, and larvae (third instar) of *T. castaneum*. The DE was applied to wheat grain of 12% moisture content at concentrations of 0.5, 1.0, 2.0 and 4.0 g/kg of grain. The treated and untreated (control) grain were kept at 28˚C and 65 ± 5% r.h. The numbers of dead and survived insects were counted two, three and four weeks after treatment. The number of adult progeny was counted nine weeks after treatment. At a concentration of 0.5 g/kg, mortality of *S. oryzae* and *O. surinamensis* after three weeks of exposure to DE were 92 and 86%, respectively. In contrast, mortality of *T. castaneum* and *R. dominica* adults was 3 and 37%, respectively. Progeny production of *O. surinamensis* and *T. castaneum* at a concentration of 2 g/kg was negligible, since only few individuals were recorded nine weeks after treatment, in comparison with the high progeny production in the control grain. The progeny of *S. oryzae* was also reduced. In contrast, for *R. dominica* was reduced only twice, in comparison with the control. In the case of *T. castaneum* larvae, at a concentration of 2 g/kg, after 4 weeks of exposure, 37% of the larvae emerged to adults, compared with 95% in control. Nine weeks after treatment, the number of F_1 adults was 100% suppressed. DE efficacy was similar at 4 g/kg. Based on the findings of the present study, the efficacy of the tested DE was influenced by DE concentration, insect species, developmental stage and exposure interval to the treated commodity.

Keywords: Diatomaceous earth, Stored product insects, Wheat grain

1. Introduction

The use of contact insecticides as grain protectants against stored product insect pests is a common and effective treatment in Israel and worldwide. However, the demands for residue-free food and environmental safety, as well as development of resistant insect pest populations to residual insecticides, have led to attempts to find non-toxic to human and environmentally friendly alternative protectants. Diatomaceous earth (DE) is known as one of the most promising alternatives to traditional residual insecticides (Athanassiou et al., 2003; 2004; 2007; 2008; Athanassiou and Korunic, 2007; Vayias and Stephou, 2009). DE is a non-toxic, safe, natural origin material with a unique, non-chemical mode of action against insects which die through desiccation (Korunic, 1998; Subramanyam and Roesli, 2000). Today, DE is in wide use for various products and processes, from toothpaste to cigars, plastics to paprika, filter media in swimming pools to home fish tanks, as well as insect and parasite control in animals and grain. The efficacy of commercial formulations of DE has been proved for a number of insect species on various stored grains. However, the DE efficacy often varies with the formulation, treated commodity and other factors (Desmarchelier and Dines, 1987; Subramanyam et al., 1994; Subramanyam and Roesli, 2000; Athanassiou et al., 2003; Vayias and Athanassiou, 2004; Athanassiou and Kavallieratos, 2005; Athanassiou et al., 2003; 2004; 2007; 2008; Kavallieratos et al., 2005).

In the current study, the laboratory evaluation of the sensitivity of the main external and internal stored product insect pests to commercial formulation of DE Detia Degesch Diatomaceous Earth – DDDE-Inerto (Detia Degesch GmbH) was conducted.

2. Materials and methods

The tested insects were adults of internal feeders, rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), and external feeders, saw-toothed grain beetle *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), as well as larvae (third instar) of *T. castaneum*. All tested insects were from laboratory-reared cultures. The DDDE was applied to wheat grain at 12% moisture content. Wheat grain lots of 3 kg were treated with DE at concentrations of 0.5, 1.0, 2.0 and 4.0 g/kg of grain. Each treatment was repeated three times (replicates). The treated and untreated (control) infestation-free grain were kept at 28˚C and 65 ± 5% r.h. Samples, of 500 g each, of treated or untreated grain were placed into glass jars of 1 lt capacity. Twenty individuals of each tested species were separately inserted into each jar, which was covered with paper to allow sufficient aeration. All jars were placed in incubators at 28˚C. The number of dead and survived insect was counted after two, three and four weeks of exposure to DE. The number of adult progeny was counted nine weeks after treatment.

3. Results

After exposure of two weeks to DE at a concentration of 0.5 g/kg, mortality of *O. surinamensis and S. oryzae* adults were 67 and 82%, respectively. In contrast, mortality of *T. castaneum* and *R. dominica* adults at the same concentration were 2 and 23%, respectively. At a concentration of 2 g/kg, adult mortality of above-mention species was 96, 93, 11 and 67%, respectively. At 4 g/kg, all (100%) adults of *O. surinamensis and S. oryzae* were dead. For *T. castaneum* and *R. dominica* adults, mortality reached 52 and 75%, respectively. Prolonged exposure to DE resulted in increased mortality of the tested species in all concentrations (Table 1).

Table 1 The efficacy of the DDDE - Inerto against adults of major stored product insects.

		Mortality (%)			
Concentration (g/kg)	**Exposure time (d)**	***Sitophilus oryzae***	***Rhyzopertha dominica***	***Tribolium castaneum***	***Oryzaephilus surinamensis***
	14	2	0	0	3
	21	2	11	2	8
0	30	8	13	2	19
	14	82	23	2	67
	21	92	37	3	86
0.5	30	94	47	5	88
	14	96	61	13	92
	21	100	77	59	97
1	30		90	82	100
	14	93	67	11	96
	21	100	84	72	10
2	30		86	96	100
	14	100	75	52	100
	21		90	96	
4	30		96	98	

Progeny production of the external feeders *O. surinamensis* and *T. castaneum*, at a concentration of 2 g/kg, was almost totally suppressed, given that only few individuals were recorded in the treated substrate, nine weeks after treatment. Similarly, progeny production of *S. oryzae* was also notably reduced. In contrast, progeny of *R. dominica* was decreased only two times in comparison with progeny production in the control grain. At a concentration of 2 g/kg, after exposure of 4 weeks to DE, 37% of the larvae of *T. castaneum* have emerged to the adult stage, compared with 95% in the control. Nine weeks after treatment, no F1 adults were found in the treated substrate. Similar results were also recorded in the case of 4 g/kg of grain. Twenty individuals of each tested species were separately inserted into each glass jar of 1 L capacity, filled with 500 g of wheat grain. The data is average from three replicates.

4. Discussion

The results of the current experiment indicate that DE concentration, insect species (external or internal feeder), developmental stage and exposure time to the treated commodity influenced the efficacy of tested DE. Among adults, *S. oryzae* and *O. surinamensis* were found to be the most susceptible to DE, regardless of the dose rate. After two and three weeks of exposure to DE, even at the lowest concentration of 0.5 g/kg, mortality of *S. oryzae* was 82 and 92%, respectively. In contrast, *T. castaneum* adults were much more tolerant to DE, given that, at 0.5 g/kg, mortality of *T. castaneum* did not exceed 3%. At the same concentration, mortality of *R. dominica* reached 37%, which was considerably lower than that for *S. oryzae* or *O. surinamensis.* For *T. castansum* and *R. dominica* mortality was high only in the case of the highest dose rate tested (4 g/kg). Hence, longer exposures and dose rates are needed for the control of these species at the adult stage. Our findings are consistent with results obtained by other researches (Korunic, 1998; Fields and Korunic, 2000; Arthur, 2001; 2002; Vayias and Athanassiou, 2004, Arnaud et al., 2005; Athanassiou et al., 2007). Based on the available literature, *Tribolium* spp. are considered the most tolerant to DEs stored-grain beetle species, at the adult stage (Fields and Korunic, 2000; Vayias and Athanassiou, 2004). However, larvae of *T. castaneum,* were very susceptible to DE. Even in cases where larvae survived and reached the adult stage, no progeny was produced. Therefore, it is expected that, despite the fact that adults are tolerant to DEs, susceptibility of larvae may slowly control *T. castaneum* populations. For the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae), Vayias and Athanassiou (2004) also indicated that DE was much more effective against larvae than against adults. From the internal feeders, *S. oryzae* was by far more susceptible than *R. dominica*. This could be attributed to the fact that *R. dominica* adults were more slow-acting, which may reduces the overall contact with the DE particles (Fields and Korunic, 2000). It is known, that, in general, mobile species, such as the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae), are more susceptible to DE than less mobile species (Rigaux et al., 2001; Vardeman et al., 2007). The current results confirm the findings from previous studies, about the rank of stored-product insect species according to their susceptibility to DEs (Korunic, 1998, Fields and Korunic, 2000, Subramanyam and Roesli, 2000; Athanassiou et al., 2004; Vayias and Athanassiou, 2004; Athanassiou and Kavallieratos, 2005; Kavallieratos et al., 2005).

In conclusion, the findings of this work indicate that DE is effective against stored-grain pests, at the dose rate of 1 g/kg or higher. A longer exposure may alleviate the need for increased doses in order to control species that are less susceptible to DEs. It is well established that DE does not react much with the environment, which makes DEs ideal candidates for long-term protection (Korunic, 1998; Subramanyam and Roesli, 2000; Vayias et al., 2006).

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Analysis of monoterpenoids in inclusion complexes with β-cyclodextrin and study on ratio effect in these microcapsules

López, M.D.*#, Pascual-Villalobos, M.J.
Instituto Murciano de Investigación Agrario y Alimentario (IMIDA) C/ Mayor s/n, La Alberca, 30150, Murcia, Spain. Email address: mdolores.lopez29@carm.es

* Corresponding author
# Presenting author

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Abstract

In recent studies, the insecticide activity against some stored products pests of monoterpenoids, such as linalool, *S*-carvone, camphor, geraniol, γ-terpinene and fenchone, and phenylpropanoids, like *E*-anethole and estragole, has been proved. Currently, applications of these volatile compounds are complicated due to their chemical and physical properties. This is one of the major problems for their use as insecticides; therefore, microencapsulation could be the solution to problems of stability, evaporation and release. Microencapsulation of these chemicals was carried out with β-cyclodextrin using a chemical precipitation method at four different ratios (β-cyclodextrin: monoterpenoids), 1.33:1, 3.33:1, 4.66:1 and 6.66:1 (w/w) in order to determine the ratio effect. This study establishes that encapsulation at the ratio of 3.33:1 to linalool and γ-terpinene was higher, whereas *S*-carvone, camphor, *E*-anethole, geraniol, estragole and fenchone showed the greatest encapsulation when the ratio was 6.66:1. Furthermore, the efficiency of encapsulation was estimated by measuring the content of the compounds in the powder by gas chromatography. The maximum inclusion efficiency of β-cyclodextrin was reached by camphor (52%) followed by geraniol (34%) using 10 g of β-cyclodextrin and linalool (31%) using 5 g of this matrix. The present study indicates that natural products such as monoterpenoids or phenylpropanoids could be microencapsulated in an efficient way using an appropriate amount of β-cyclodextrin.

Keywords: Microencapsulation, β-cyclodextrin, Camphor, Geraniol and Linalool

1. Introduction

Essential oils and others phytochemicals have been studied as insecticides to control pests lately due to their particular properties. These natural products show toxic (Don-Pedro, 1996; Clemente et al., 2003), repellent (Pascual-Villalobos and Ballesta-Acosta, 2003), antifeedant and ovicidal effect in insect pests (Regnault-Roger and Hamraoui, 1994; Alvarez-Castellanos et al., 2001). Some monoterpenoids and phenylpropanoids, obtained directly from plant secondary metabolism, have insecticidal activity against stored product pests (Lee et al., 2003; García et al., 2005).

Recently, López et al. (2008) demonstrated that some monoterpenoids such as linalool, *S*-carvone, camphor, geraniol, γ-terpinene and fenchone and phenylpropanoids like *E*-anethole and estragole, could be alternatives to synthetic insecticides against some stored-product pests such as *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) or *Cryptolestes pusillus* (Schönherr) (Coleoptera: Cucujidae). However, applications of these volatiles have turned out to be particularly complicated due to their chemical and physical properties (low stability, high evaporation and release).

Microencapsulation allows immobilization, protection, release and functionalisation of active ingredients. The use of microcapsules in food (Versic, 1988) or pesticides (Fuyama et al., 1984) are generally one of the main applications in the industry since the main purpose of microencapsulation is to entrap sensitive ingredients, such as volatile and flavours into solid carriers to increase their protection, reduce evaporation, promote easier handling, and control their release during storage and applications (Baranauskiené et al., 2007).

Starch matrix is one of the natural materials that are receiving considerable attention because of its great abundance, ease of recovery from plant sources, low cost and its ready conversion chemically, physically and biologically into a broad spectrum of low molecular weight polymers (Doane, 1993). Starch and starch-based ingredients (modified starches, maltodextrins, *β*-cyclodextrins) are widely used to retain and protect volatile compounds (Madene et al., 2006). Cyclodextrins (*α, β* and *γ*) are cyclic oligosaccharides consisting of six, seven and eight glucose units, respectively.

The binding of volatile compounds to starch has been classified into two types. On the one hand, the flavour compound surrounded by the amylase helix through hydrophobic bonding is known as an inclusion complex. On the other hand, polar interactions have been determined which involve hydrogen bonds between the hydroxyl groups of starch and aroma compounds (Arvisenet et al., 2002; Boutboul et al., 2002).

The aim of this study is to investigate if 6 monoterpenoids (linalool, camphor, geraniol, *S*-carvone, *γ*-terpinene, and fenchone) and 2 phenylpropanoids (*E*-anethole and estragole) could be microencapsulated in an efficient way using different ratios of *β*-cyclodextrin to monoterpenoids during the complexation process.

2. Materials and methods

2.1. Chemicals

Six monoterpenoids, (-)-linalool (97%), camphor (96%), *γ*-terpinene (98%), geraniol (99%), *S*-carvone (98%), fenchone (98%) and 2 phenylpropanoids, *E*-anethole (99%) and estragole (98%) were used as guest molecules and were obtained from ACROS Organics BUBA/SPRL. To carry out the complexation process, the matrix chosen was *β*-cyclodextrin (98%) which was purchased from Sigma-Aldrich.

2.2. Preparation of microcapsules

A chemical precipitation method based on Reineccius (1989) was used to prepare monoterpenoid or phenylpropanoid-*β*-cyclodextrin complexes. Different amounts of *β*-cyclodextrin (2, 5, 7 and 10 g) were dissolved in 500 mLof an ethanol to water (1:2) mixture and were maintained at 50ºC on a stirring hot plate. 1.5 g of monoterpenoid or phenylpropanoid were dissolved in ethanol (10% w/v) and were slowly added to the *β*-cyclodextrin solution with continuous stirring. After this addition, the heating was stopped and was maintained at room temperature and stirred for 4 h. This solution was maintained at 4ºC overnight and the precipitated *β*-cyclodextrin-monoterpenoid or phenylpropanoid complex was recovered by filtration and was dried in a oven at 50ºC for 24 h. Finally, the powder was allowed to air-dry at 25ºC for one day in order to reach its equilibrium moisture content. The powder was observed by optical microscopy (40x) to check the microcapsules formation. This process was carried out for each isolated monoterpenoid and phenylpropanoid. Four starting ratios of *β*-cyclodextrin to core material (monoterpenoids and phenylpropanoids) were used: 1.33:1, 3.33:1, 4.66:1 and 6.66:1. Each ratio was replicated twice; both results (first and second replication) were very similar.All samples were stored at 25ºC in airtight vials.

2.3. Total monoterpenoids and phenylpropanoids extraction

To determine the total amount of volatile compounds in the powder an extraction method was used. 0.5 g of powder (for each monoterpenoid, each phenylpropanoid and each ratios studied) were mixed with distilled water (8 mL) and hexane (4 mL) in glass vials (15 mL) and were sealed.

This solution was heated and stirred in a hot plate at 75ºC for 20 min. The organic phase containing the volatile compounds was decanted, and the aqueous phase was exhaustively extracted with hexane 3 times (4 x 4 mL). These 4 phases were combined. For each treatment the hexane was removed by using a nitrogen stream. Finally the concentrated extracts were transferred to insert vials and stored at 4ºC until required for GC/MS analysis.

2.4. Efficiency of encapsulation

The identification of each monoterpenoid or phenylpropanoid was accomplished by comparing the mass spectra and retention times of compounds with standards. Quantitative analysis of the volatiles extracted from the powder was carried out using GC-MS and internal standards for each monoterpenoid and phenylpropanoid in different range of concentrations. The quantitative analysis of each compound was carried out using a model 5890 Series II equipped with a DB-Waxetr 30 m x 0.32 mm capillary column coated with a polyethylene glycol film (1 μm thick). The chromatographic conditions were as follows: an Agilent model 5972 inert mass spectrometry (MS) detector (Agilent, Palo Alto, CA). The initial oven temperature was held at 60ºC for 1 min. Afterwards, it was increased by 3ºC min^{-1} to 225ºC, injector at 250ºC, column head pressure at 8.00 psi, helium carrier gas, flow rate 2.6 mL min^{-1}, splitless with 2 μL of sample injected. Content of monoterpenoids and phenylpropanoids was calculated according to the area of the chromatographic peaks. A linear regression model was computed and was obtained using standard dilution techniques to quantify components.

2.5. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS. There were four ratios of *β*-cyclodextrin to monoterpenoids. Differences among treatments were determined by Duncan's multiple test at the 5% level ($P<0.05$).

3. Results

For the great majority of components studied, we observed that the higher the ratio of β-cyclodextrin, the greater the encapsulation (Table 1), although there were some exceptions, such as linalool at 3.33:1 ratio (0.4680 g) and *γ*-terpinene at the same ratio (0.0195 g), but in the latter case there was no statistically significant difference. The rest of monoterpenoids and phenylpropanoids showed clearly the highest values for the ratio 6.66:1 (eg. S-carvone: 0.1678 g, camphor: 0.8105 g, E-anethole: 0.4791 g or geraniol: 0.5311 g).

Table 1 Amount of natural insecticides, monoterpenoid or phenylpropanoid, microencapsulated at different *β*-cyclodextrin to monoterpenoid or phenylpropanoid ratios.

Efficiency of encapsulation	Ratio[1]	*β*-cyclodextrin[2] (g)	Natural insecticiede (g)	Natural insecticides recovered from 1.5 g microencapsulated formulation[3]							
				Camphor	Geraniol	Linalool	*E*-Anethole	S-Carvone	Estragole	Fenchone	Terpinene
Insecticide	1.33:1	2	1.5	0.0088 a	0.0171 a	0.0021 a	0.0000 a	0.0136 a	0.0000 a	0.0006 a	0.0004 a
Encapsulated	3.33:1	5	1.5	0.5001 c	0.1862 b	0.4680 c	0.0941 a	0.1135 b	0.1051 c	0.0130 a	0.0195 b
(g)	4.66:1	7	1.5	0.2036 b	0.0424 ab	0.0162 a	0.0325 a	0.0140 a	0.0172 b	0.0037 a	0.0043 a
	6.66:1	10	1.5	0.8105 d	0.5311 c	0.2363 b	0.4791 b	0.1678 c	0.1410 d	0.0515 b	0.0162 b
Insecticide	1.33:1	2	1.5	0.6	1.2	0.1	0.0	0.8	0.0	0.1	0.1
Encapsulated	3.33:1	5	1.5	33.4	12.4	31.3	6.3	7.6	7.0	0.9	1.3
(%)[4]	4.66:1	7	1.5	13.5	2.7	1.1	2.1	0.9	1.1	0.2	0.3
	6.66:1	10	1.5	52.3	34.2	15.2	30.9	10.8	9.1	3.3	1.0
Vapour pressures (mmHg at 20 °C)				--	0.20	0.17	0.05	0.40	0.11	--	0.70
Boiling Point (°C)				204	229	198	235	229	215	--	174

[1]Ratio *β*-cyclodextrin : monoterpenoid or phenylpropanoid. [2]Dry weight basis when 1.5 g of monoterpenoid or phenylpropanoid is added. [3]Treatments having the same letter are not significantly different, Duncan's multiple range test, $P<0.05$, (column comparison), n=2. [4]Insecticides varied from 1.4927 to 1.6337 g.

At the ratio 1.33:1 (2 g of β-cyclodextrin), all monoterpenoids studied (S-carvone, linalool, camphor, γ-terpinene, fenchone and geraniol) exhibited the lowest values of encapsulation or were not encapsulated at all in the case of phenylpropanoids (estragole and E-anethole). This indicated that more amount of matrix was necessary to improve or even achieve the encapsulation.

On the other hand we also observed that adding more matrix (β-cyclodextrin) did not always cause more encapsulation. Microencapsulation values for ratio 4.66:1 (7 g of β-cyclodextrin), were less than 5 g of β-cyclodextrin (3.33:1 ratio). Even though, in some cases, statistical comparison indicated there was no significant difference ($P>0.05$) between 3.33:1 and 4.66:1 ratios (eg. fenchona, E-anethole and geraniol).

Efficiency of encapsulation depends on monoterpenoids and phenylpropanoids studied and the different amount of matrix in dry weight basis used (Table 1).

The percentage of encapsulation for each compound, working with 2 g of β-cyclodextrin, is lower than when we assayed other amounts of β-cyclodextrin (5, 7 and 10 g), indicating that with the lesser amount of matrix, there was less encapsulation and consequently less recovery.

The data from 5 and 10 g of *β*-cyclodextrin pointed out that some monoterpenoids such as linalool (31.3% to 5 g of *β*-cyclodextrin), camphor (33.4% and 52.3% to 5 and 10 g of *β*-cyclodextrin, respectively). *E*-anethole (30.9% to 10 g of *β*-cyclodextrin) and geraniol (34.3% to 10 g of matrix) had reached a remarkable recovery.

We also conclude that some monoterpenoids are difficult to encapsulate using our methodology: low values for γ-terpinene (recovery: 0.1, 1.3, 0.3 and 1.0% to 2, 5, 7 and 10 g of β-cyclodextrin, respectively) and fenchone (recovery: 0.1, 0.9, 0.2 and 3.3% to 2, 5, 7 and 10 g of matrix, respectively) were obtained (Table 1). We should take into account that other aspects may contribute to the low recovery of these chemicals such as operational loss, evaporation and so on.

4. Discussion

The β-cyclodextrin inclusion complexes with terpenoids have been reported to be effective in preventing oxidation, retaining volatile substances, in masking undesired tastes and odours, and in solubilising water-insoluble substances (Donze and Coleman, 1993). In fact, in recent works the behaviour as modulated release, of volatile compounds within starch matrixes which are precursors of dextrins (Yilmaz et al., 2002; Yilmaz et al., 2004) have been examined. Other authors (Bertolini et al., 2001) have studied the stability of some monoterpenes encapsulated (β-pinene, citral, limonene, β-myrcene and linalool) with gum Arabic as a matrix, indicating that oxidative processes occurred for some core materials and concluded that gum Arabic was not efficient as a wall material.

In our study we have established how different ratios of β-cyclodextrin to monoterpenoids or phenylpropanoids can determine the encapsulated amount of guest molecule. As a result, the major ratio (6.66:1) presents the higher values, although occasionally lesser ratios (3.33:1) gave better results.

Bhandari et al. (1998), investigated the characteristics, including the profile of flavour volatiles, of the complex as affected by the ratio of lemon oil to β-cyclodextrin used during the complexation process. They found that a lemon oil powder could be successfully produced by a microencapsulation technique using β-cyclodextrin: lemon oil with a ratio to 0.4:1 treatment, which was not the highest ratio. In our assay the same occurs with monoterpenoids linalool and γ-terpinene. However, we cannot completely compare our results since we have just worked with isolated compounds instead of essential oils.

The highest rates of encapsulation were between 30 and 50% for camphor, E-anethole, geraniol and linalool. The remaining compounds only had rates of incorporation between 1 and 11%. These rates of incorporation are in agreement with Adamiec and Kalemba (2006) who analyzed two essential oils (elemi and peppermint) using maltrodextrin as carrier. Kim et al. (2006) accomplished a percentage of efficiency of encapsulation of isoflavone from 50 to 90%, although they assayed other coating materials, polyglycerol monostearate and triacylglycerol.

Our results show that a microencapsulation of monoterpenoids and phenylpropanoids using β-cyclodextrin, is possible, but optimum ratios have to be established for each compound and encapsulation method. Besides relating these results to our previous studies about insecticidal activity of monoterpenoids and phenylpropanoids against stored product pests, we have moved forward with regard to the use of these more appropriate and cleaner natural products in integrated pest management. Although these formulations also need to be studied in depth and assayed for toxicity to other stored product insects, with regards to the efficacy and duration of activity.

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Environmentally friendly technologies to maintain stored paddy rice quality

Lazzari, F.A.*#, Lazzari, S.M.N., Lazzari, F.N.
Department of Zoology, Universidade Federal do Paraná, Caixa Postal 19020. 81531-980 Curitiba, Paraná, Brazil.
Email: flaviolazzari@gmail.com

* Corresponding author
# Presenting author

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Abstract

Exports of processed rice have been increasing every year, as well as legislative restrictions and consumer demand for certified chemical free rice, pressing the rice processing industry to new challenges. The objective of this work was the implementation of a Rice Quality Certification Program. The package includes the association of a rigorous sanitation program and safe environmentally friendly control measures. It was accomplished in a large paddy rice facility with 40 silos during the 2008/09 rice crop. Silo sanitation was done by washing with pressurized water the conveyor belt structure above the silo roof, around the externals walls and thorough aspiration of the aeration system. During silo filling, the lower and top portions of the rice grain were treated with a mixture of diatomaceous earth (DE) and powder deltamethrin. Artificial chilling was applied as soon as the top layer of the grain mass was leveled by insufflating cool air (6 to 8ºC) with a large cooling machine through the aeration system. The grain mass temperature stabilized at about 12-14ºC, and kept this range of temperature for about 60 d. As the temperature of the grain mass increased, mainly on the top layer, aeration was performed with natural air from the cold fronts during the winter months. After 8 months on storage the rice was free of external insects, as proved by the grain sampling just before processing. For the 2009/10 crop season, the rice quality program will be repeated on 60 silos with few adjustments.

Keywords: Chilled aeration, Inert dust, Physical insect control, Rice quality maintenance.

1. Introduction

Processed rice exports have been increasing every year challenging the rice processing industry to adapt to new legislative restrictions and to attend the demand from the consumers for certified chemical free rice. Among the many problems in grain storage, pest infestation demands constant attention and efficient control measures, especially in large scale storage facilities under tropical/subtropical conditions (Trematerra, 2004; Lazzari et al., 2006; Lazzari et al., 2007; Ceruti et al., 2008; Vayas, 2009; Barbosa and Adler, 2007).

The control of insect and mite pests in stored rice using fumigation and residual insecticides is still the most common practice in Brazil; however, these methods may not be the most cost effective. Also, the residues of active ingredients can be risky for people and domestic animals, cause environmental contamination, and result in resistance of insect populations (Subramanyam and Hagstrum, 1995; Collins et al., 2000; Fields, 2006).

Post-harvest integrated pest management (IPM) focuses greatly on structure modifications, sanitation of the facilities and target pest control. Clean and sanitized structures are less likely to be favorable to pest establishment (Subramanyam et al., 2005).

The combination of physical and chemical methods has been used worldwide for insect control, including applications of diatomaceous earth (DE) (Fields, 1992; 2000; Athanassiou, 2007). Deltamethrin is a pyrethroid of low mammalian toxicity and can be used as dust or liquid in combination with DE (Ceruti and Lazzari, 2005). Chilling aeration is another physical method that presents several advantages mainly during the warmer periods of the year in warm climates (Maier and Navarro, 2002; Navarro, 2007; Adler, 2007). Evaluation with infrared radiation from flameless catalytic heater shows potential for killing insects inside rice kernels (Subramanyam, 2007). Therefore, there is a need for a diverse set of integrated measures including natural and environmentally friendly procedures to guarantee grain rice quality for the industrial processes. The main objective of the present study is to establish and apply a technological package or Rice Quality Program, as we call it, comprising a series of coordinated actions and measures in order to achieve an insecticide-free rice certification by 2011.

2. Materials and methods

This technological package is being carried out in a large rice receiving, drying, storage and processing facility (Figure 1) in the county of São Borja, State of Rio Grande do Sul, Brazil (28º 39' 38'' S and 56º 00' 16'' W; 96 m above sea level). For this large scale procedure, 40 silos with capacity for 3500 tons each of paddy rice from the crop season of 2008/09 were treated as follow.

Figure 1 Receiving, drying, storing and processing rice facility in São Borja, State of Rio Grande do Sul, Brazil, where the Rice Quality Program is being applied, since April 2008.

Silos were thoroughly cleaned from the roof to bottom, inside and outside, by washing, with pressurized water, the conveyor belt structure above the silo roof to remove starch dust, broken kernels, fines and clumps of grain. Also; application of water under pressure was done to clean external walls. The next step was spraying the external walls with DE plus deltamethrin (Figure 2 and 3). In each silo, the bottom and top layers, of about 60 tons of rice, were treated with a combination of 300 ppm diatomaceous earth (DE) (commercial name KEEPDRY) and 30 ppm of powder deltamethrin 2% a.i. (commercial name K-obiol 2 P) per t of rice. The treatment was applied as the grain was being transported to the silo by a transportation system including a bucked elevator, a screw, and a conveyor belt. After the bottom portion of the silo was filled with about 60 tons of treated grain it was leveled and the silo filled with untreated grain until close to the top. The top layer that consisted of about 60 tons of treated rice was then leveled.

Figure 2 Buildings and silo sanitation. Prior grain filling silos were washed with pressurized water and a mixture of diatomaceous earth + deltamethrin was applied on the outside walls of buildings and silos.

Figure 3 Application of diatomaceous earth + deltamethrin on the rice transported to the silos. Silos floor and internal walls were treated as the treated rice mass dropped from 25 m high.

After the silos were completely filled, artificially chilled air was insufflated throughout the aeration system with airflow at 30.000 m3/h (Figure 4). The temperature of the air insufflated into the grain mass was between 5-8ºC and 65-70% r.h. The grain mass reached a temperature range between 11 and 12ºC after 86 h of continuous applications of chilled air. Natural air was used to maintain the rice mass cool by using the cold fronts available from May through August mainly during the night. Monitoring of the grain mass temperature was performed with thermocouples installed in each silo.

Figure 4 Artificial chilling equipment used to insufflate chilled air through the aeration system of the silos.

Food-baited cage traps were placed on several spots near the silos in order to monitor the resident and migrant insect populations. The top layer of the rice mass was checked periodically and superficial grain samples were taken for insect detection. Before the milling of the rice from each treated silo, screenings from the cleaning machine were evaluated for live insect detection. The package also includes periodical training in order to instruct and change the attitude of the personnel involved with monitoring, control application and in charge of the rice stocks.

3. Results

Trappings revealed that only 15% of the insects captured in the cage traps were directly associated with grains and about 76% were sap beetles, Carpophilus spp. Among the insects collected were the Coleoptera: *Sitophilus oryzae* (L.) (Curculionidae), *S. zeamais* Motschulsky, *Oryzaephilus surinamensis* (L.) (Silvanidae), *Rhyzopertha dominica* (F.) (Bostrichidae) and *Tribolium castaneum* (Herbst) (Tenebrionidae).

From February to May 2009 all the silos and the lots of rice received were prepared for long term storage. After 8 months of rice storage, 29 silos were processed and none of them showed any external infestation of stored grain insects. The evaluation was done by checking the screenings of the cleaning machines during the expedition of rice to the mills. Only a residual population of R. dominica was found outside of a silo, but it was promptly controlled with DE + deltamethrin. The source of this infestation was not found.

Table 1 and Figure 5 show the temperature conditions outside and inside of a silo. Temperatures were taken with the thermocouples inside the grain mass at different levels of every 1.5 m: ambient air temperature, temperature inside the silo near the surface under the metallic sheath, temperature at 0.5 m above the grain mass, chilled air temperature and temperature at 0.3 m below the grain. The ambient air temperature from March 19th to 31st varied from 26 to 36ºC. The average temperature in March (end of summer in the region) was 34ºC. Temperatures inside the silo and just beneath the metallic ceiling of the silo ranged from 26 to 68ºC. The temperature at 0.5 m above the grain mass ranged from 17.7 to 42.6ºC, reaching the lowest point of 16.8ºC at 4 d after chilled air application. However, at the 5th day the temperature increased to 36.8ºC, due to the effect of head space heat trapped inside the silo. Figure 5 shows the temperature fluctuation during the period of this preliminary test. Chilled air was insufflated for about 4 d bringing temperatures down at all points inside the silo, despite the ambient temperature. Grain mass temperature was kept below 20oC for about 10 d without further chilled air insufflation; however at the upper layers and at the ceiling temperatures started increasing again following external ambient temperature.

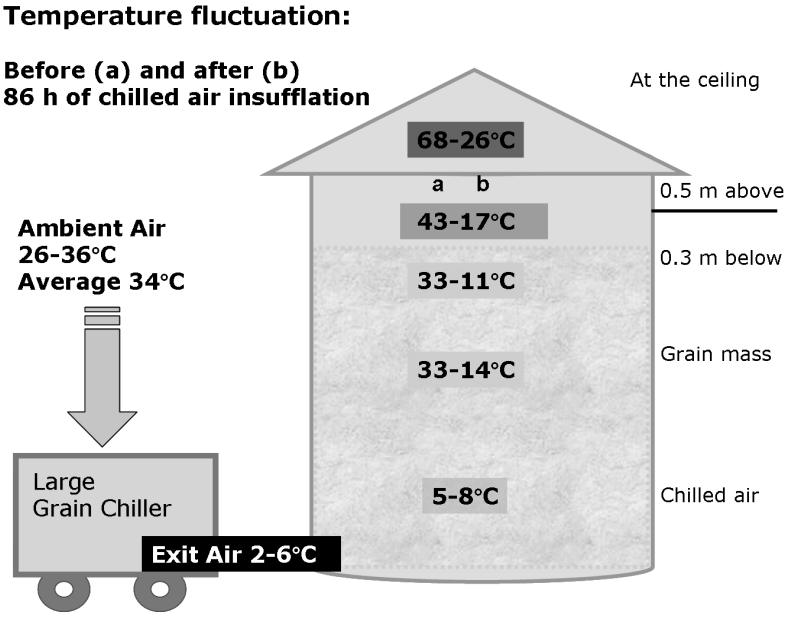

Figure 5 Scheme of the silo showing the application of artificial cold air and temperature profile at different points inside the rice mass before and after 86 h of chilling.

Table 1 Ambient air temperature, temperature of chilled air entering the grain mass, average temperature of the grain mass, temperature at 0.3 m below the grain surface, temperature at 0.5 m above the grain mass, temperature inside the silo near the metallic sheath, from 30 points of temperatures during March 2009 in metallic silo with paddy rice submitted to artificial chilling in São Borja, RS, Brazil.

Dates in March 2009	Temperature (ºC)					
	Air ambient	Entering chilled air	Grain mass (average)	0.3 m inside grain	0.5 m above grain	Under metallic sheath
19	29.6	7.7	32.9	33	31.9	68
20	34	8.4	20.7	21.9	24.2	55
21	33.3	6.8	14.8	19.1	21.7	51
22	35	6.3	14.8	12.1	17.7	48
23	33.6	4.9	14.3	10.9	16.8	45.3
24	32.5	-	14.1	11.2	36.7	50.6
25	32	-	14.3	12.5	38	51.5
26	25.5	-	13.5	17.8	28.7	26
27	30.8	-	14.4	22	40.8	57.3
28	33.7	-	14.8	26.8	40.4	52.8
29	35	-	15.3	28.7	42.6	55.2
30	35.5	-	15.1	29.4	41.7	46.8
31	35.3	-	18.0	30.1	34.8	41.8

4. Discussion

The rigorous sanitation program implemented by washing most of the structure with pressurized water presented satisfactory results in terms of suppressing insect populations and a significant reduction on the level of infestation of the rice going to be processed.

The removal of crusted dust, broken kernels, whole kernels, sprouted seeds inside the metallic U shaped structure, washing the silos walls and the application of DE + deltamethrin were important to reduce migrant insect pressure from outside of the silos.

The treatment of the rice with powder DE+ deltamethrin as the silo was being filled, leveling the upper surface of the rice mass, and the use of artificial chilling combined with aeration with natural cold air showed to be a very efficient integration of technologies. After 8 months of storage no insects were found in the rice samples and screenings taken before processing.

The system to maintain rice quality during storage using natural cold air from the cold fronts especially during the winter months can bring great benefits such as, reduce insect infestation and consumers complains, and keep storage costs low. The temperature inside the rice grain mass was kept low; however, at the top layer of the rice mass it warmed quite quickly. The challenge in this situation is to maintain the temperature near the grain surface at a range that will make it possible to suppress insect development and prevent oviposition. Other measures will be taken, such as painting the roofs of the silos white, adding insulation, mechanical removal of the hot air, in order to find a way to control the temperature on the top layer of the grain mass.

New formulations and combinations of DE with other natural insecticides, such as Beauveria, spinosad, Metarhizium, growth regulators, and vegetable essences, should be tested as insect control measures.

The program to maintain rice quality at very large scale should be kept simple, and include efficient, safe and cost effective strategies, focusing on all the steps, from receiving to packing of the rice. For the 2009/10 crop season, heat treatment will be used by insufflating the heat through the aeration system of the silos to suppress any pest infestation before filling with new rice. Some roofs will be painted white to reduce the head space temperature, and a motorized fan with a thermostat will be placed on the very top of the roof of some silos to remove the moist hot air to avoid condensation. Before packing, white rice will be treated with infrared radiation, produced by a flameless catalytic heater to kill eggs, larvae, and pupae inside the polished rice kernels.

The technological package we are establishing is demonstrating very promising results towards the certification of insecticide-free rice for 2011 in this rice plant.

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Contact and fumigant activity of 1,8-cineole, eugenol and camphor against *Tribolium castaneum* (Herbst)

Liska, A.*#, Rozman, V., Kalinovic, I., Ivezic, M., Balicevic, R.
Faculty of Agriculture in Osijek, Department for Plant Protection, Josip Juraj Strossmayer University of Osijek, Trg Sv. Trojstva 3, 31000 Osijek, Croatia. Email: aliska@pfos.hr

* Corresponding author
# Presenting author

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Abstract

The red flour beetle, *Tribolium castaneum* (Herbst), holds a significant place in Croatia by causing considerably damages on stored products. This study was initiated in order to test contact and fumigant activity of three essential oil compounds (1,8-cineole, camphor and eugenol) for the control of adults of this stored-pest species. Contact toxicity of compounds was tested at four doses (0.2, 1.0, 5.0 and 10.0 µL/adult) and mortality was recorded every 2, 4 and 24 h after the application. The most effective compound was 1,8-cineole by its fastest action (2 h after application), with maximum mortality at the lowest dose (0.2 µL/adult). With its prolonged effect of 4 h, eugenol resulted in mortality of 87.5% at the dose of 0.2 µL/adult, while camphor obtained the highest mortality (78.5%) just after 24 h, and at the highest tested dose (10.0 µL/adult). Fumigant toxicity of compounds was tested at three doses (30, 60 and 120 µL/350 mL vol.) and after 48-h of exposure time; mortality was recorded every 24 h until the "end point mortality" (when no time-dependent changes in mortality occurred). The highest mortality (98.5%) had 1,8-cineole at the lowest dose (30 µL/350 mL vol.), followed by camphor (93.5%) at the highest dose (120 µL/350 mL vol.), while eugenol had no statistical significance in the control of *T. castaneum* adult by application of this fumigation method. Such investigations make positive contribution to new possible alternatives to conventional insecticides and fumigants used in protection of stored cereals.

Keywords: *Tribolium castaneum,* 1,8-cineole, Camphor, Eugenol, Contact and fumigant activity

1. Introduction

Synthetic insecticides and fumigants are being commonly used in stored products protection today. However, by reason of numerous side-effects such as toxic residue on cereals, environment pollution, pests' resistance, etc., many active compounds that have been used as fumigants are being withdrawn from the insecticide market. Since 2005, methyl bromide, a fumigant with broad range of activity, has merely been licensed for control of the pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle and the Asian long-horned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambyeidae) in wooden packaging for export purposes (Korunić, 2009); until 2015 its application will be permanently suspended (Montreal Protocol on Substances that Deplete the Ozone layer) (UNEP, 1994). Phosphine gas is another fumigant that is applied worldwide, mainly in fumigation process of cereals, dried fruit, nutmeg, cacao, rice and coffee, and presently being reassessed according to the regulations of EU and USA (Bell, 2000).

All these necessitate further findings on pest control methods that are effective and harmless for environment. Among them, botanical insecticides take an important role. Moreover, plant extracts contain substances that have varied influence on insects: ovicidal, repellent, toxic; they can act as sterilants or exhibit antifeedant effect (Nawrot and Harmatha, 1994). Many authors have reported their research studies into the insecticidal efficacy of the number of essential oils and plant extracts in the control of the major stored pests (Regnault-Roger et al., 1993; Pascual-Villalobos and Robledo, 1999., Huang et al., 2000; Papachristos and Stamopoulos, 2002).

One constituent of essential oil obtained from leaves of eucalyptus plant which can vary in quantity is 1,8 cineole (Gibson et al., 1991). As a natural plant extract it is of low toxicity for mammals and regularly used in many assessments of toxicity to stored pests. Prates et al. (1998) have proved that monoterpenes, 1,8 cineole (one of compounds of eucalyptus essential oil) and limonene (one of compounds of lemon essential oil) have significant insecticidal effect against *Rhyzopertha dominica* F. (Coleptera: Bostrichidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) by exhibiting contact and fumigant activity, and an antifeedant effect.

Owing to the low toxicity to warm-blooded mammals and high volatility, essential oils represent one of the alternatives to the fumigants that are presently used in stored cereal protection (Shaaya et al., 1991; Shaaya et al., 1997; Li and Zou, 2001). The aim of present study was to test contact and fumigant effects of the three essential oil compounds, 1,8-cineole, camphor and eugenol on the adults of red flour beetle, *T. castaneum.*

2. Materials and methods

2.1. Test insects

In this study we tested 2- to 4-wk-old *T. castaneum* adults of mixed sex. Test insects were reared on the medium of wheat rough flour and dried yeast at the ratio of 10:1, in a growth chamber at 30±1 ºC; 70-80% r.h., in darkness, following the method of Liu et al. (1999).

2.2. Contact toxicity

Determination of contact toxicity of essential oil compounds was done by the method of Huang et al. (2000). Compounds (1,8-cineole, eugenol and camphor) were purchased from "Sigma-Aldrich" (Export Division Grünwalder Weg 30 D-82041 Deisenhofen, Germany) and "Fluka" (Industriestrasse 25, CH-9471 Buchs, SG Switzerland); and tested at four doses (0.2; 1.0; 5.0 and 10.0 µL/adult). The compounds were applied with "Kartell" micropipette to the thorax of the adults. The insects were placed into Petri dishes and transferred in clime chamber at 30±1 ºC, and 70-80% r.h.; mortality was recorded every 2, 4 and 24 h after the application. Each treatment with 100 adults was replicated 4 times. Suspension of camphor was prepared by mixing camphor in crystal form with 96 % alcohol (ethanol) at the ratio of 1:1 and applied at 4 previously mentioned doses. Control samples relevant for the four suspension doses were 0.2; 0.5; 2.5 and 5.0 µL/adult, as follows. Control relevant for 1,8-cineole and eugenol was a sample without treatment.

2.3. Fumigant toxicity

Determination of fumigant activity of 1,8-cineole, camphor and eugenol was done by somewhat modified method described by Huang et al. (1997). Fifty *T. castaneum* adults per sample, 2 to 4 wks old, and of mixed sex, were placed into silk mesh cages containing mixture of some flour and yeast, and put into glass jars of 350-mL capacity. Each isolate was applied at three doses (30, 60 and 120 µL/350 mL vol.) with "Kartell" micropipette on to filter paper attached to the lids of the glass containers; and applied in 4 replications. After application tightly sealed containers were kept 48 h under controlled conditions at 30±1 ºC and 70-80% r.h., in darkness. Single adults of *T. castaneum* were placed in jars on new medium for 7 d under the same conditions and recorded for total mortality.

2.4. Statistical analysis

Statistical data analysis was done by analysis of variance (ANOVA) following the GLM model for test-insect mortality per compounds tested, and to test doses. Significant differences were identified by LSD tests at 5% level in SPSS 11.0 programme for Windows.

3. Results

3.1. Contact toxicity

Two hours after application at the lowest dose of 0.2 µL/adult, 1,8-cineole exhibited 100% mortality of *T. castaneum* adults, which proved the fastest activity at the lowest dose. Second by efficacy was eugenol (74.7%) with significantly higher differences in comparison to the control (0%) and camphor (5.0%). By dose increase of eugenol from 0.2 to 1.0 µL/adult 100% mortality was obtained, while camphor exhibited significantly lower mortality (7.7%) in comparison to the first two compounds, and significantly higher mortality in comparison to the control (0%). By dose increase of camphor to 5.0 and 10.0 µL/adult, after

2-h application no significant changes in mortality (7.2% and 6.2% respectively) were recorded in comparison to the 1,8-cineole and eugenol (Table 1).

Table 1 Mortality (%) of *T. castaneum* adults 2 h after contact toxicity test at four doses of 1,8-cineole, eugenol, and camphor.

Treatments	Average mortality* (%) ± SE			
	0.2 µL /adult	0.2 µL /adult	0.2 µL /adult	0.2 µL /adult
1,8-cineole	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
Eugenol	74.7 ± 5.1b	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
Camphor	5.0 ± 1.2c	7.7 ± 0.8b	7.2 ± 0.4b	6.2 ± 1.3b
Control 0	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c
Control 1**	0.0 ± 0.0d	0.5 ± 0.2c	0.0 ± 0.0c	0.5 ± 0.5c

*Means in the same column followed by the same letters are not significantly different (P>0.05) as determined by LSD test. **Control relevant for suspension of camphor at the following ethanol doses: 0.2, 0.5; 2.5 and 5.0 µL/adult

Four hours after application camphor exhibited significantly higher mortality at all four doses (13.2%, 20.2%, 19.0 and 18.0%, respectively) in comparison to the control, but was insufficiently effective to control adults of the test-species (Table 2).

Table 2 Mortality (%) of *T. castaneum* adults 4 h after contact toxicity test at four doses of 1,8-cineole, eugenol, and camphor.

Treatments	Average mortality* (%) ± SE			
	0.2 µL /adult	0.2 µL /adult	0.2 µL /adult	0.2 µL /adult
1,8-cineole	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
Eugenol	87.5 ± 5.6^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
Camphor	13.2 ± 3.4^b	20.2 ± 1.1^b	19.0 ± 0.7^b	18.0 ± 0.0^b
Control 0	0.0 ± 0.0^c	0.0 ± 0.0^c	0.0 ± 0.0^c	0.0 ± 0.0^c
Control 1**	0.2 ± 0.2^c	1.2 ± 0.6^c	0.0 ± 0.0^c	0.7 ± 0.7^c

*Means in the same column followed by the same letters are not significantly different (P>0.05) as determined by LSD test. **Control relevant for suspension of camphor at the following ethanol doses: 0.2, 0.5; 2.5 and 5.0 µL/adult

After 24 h contact activity of camphor the mortality values were obtained by 68.0, 74.7, 74.2 and 78.5% at the dose of 0.2, 1.0, 5.0 and 10.0 µL/adult, respectively (Table 3). The mortalities for camphor were significantly lower at all applied doses than those for 1,8-cineole and eugenol with 100% mortality. High contact activity against *T. castaneum* adults for camphor would be possible only with prolonged exposure time (after 24 h), and at the dose of 10.0 µL/adult or higher.

Table 3 Mortality (%) of *T. castaneum* adults 24 h after contact toxicity test at four doses of 1,8-cineole, eugenol, and camphor.

Treatments	Average mortality* (%) ± SE			
	0.2 µL/adult	0.2 µL/adult	0.2 µL/adult	0.2 µL/adult
Cineole	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
Eugenol	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a
Camphor	68.0 ± 2.1^b	74.7 ± 6.3^b	74.2 ± 3.3^b	78.5 ± 6.8^b
Control 0	0.0 ± 0.0^d	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^d
Control 1**	2.7 ± 1.1^c	4.75 ± 1.1^c	2.0 ± 1.0^c	3.0 ± 0.5^c

*Means in the same column followed by the same letters are not significantly different (P>0.05) as determined by LSD test. **Control relevant for suspension of camphor at the following ethanol doses: 0.2, 0.5; 2.5 and 5.0 µL/adult

3.2. Fumigant toxicity

Results of fumigant activity of three tested compounds were shown as total mortality of *T. castaneum* adults after 7-d observation. At the lowest dose (30 µL/350 mL vol.) the highest mortality was recorded with 1,8 cineole (98.5%) with significant differences in comparison to the control after 48 h (Table 4),

while the treatments with eugenol and camphor at the same dose showed no significant differences in comparison to the control. This proved application of 1,8-cineole at the dose of 30 µL/350 mL vol. valid for use by this method of fumigation.

Table 4 Total mortality (%) of *T. castaneum* adults resulting from 48-h laboratory fumigation at three doses of 1,8-cineole, eugenol, and camphor.

Treatments	**Total average mortality* (%) ± SE**		
	30 µL/350mL vol.	**30 µL/350mL vol.**	**30 µL/350mL vol.**
1,8-cineole	98.5 ± 1.5[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Eugenol	0.5 ± 0.5[b]	0.0 ± 0.0[c]	0.5 ± 0.5[b]
Camphor	3.5 ± 2.2[b]	7.0 ± 0.5[b]	93.5 ± 3.2[a]
Control 0	0.5 ± 0.5[b]	0.5 ± 0.5[c]	0.5 ± 0.5[b]
Control 1**	0.0 ± 0.0[b]	0.0 ± 0.0[c]	2.0 ± 0.8[b]

*Means in the same column followed by the same letters are not significantly different (P>0.05) as determined by LSD test. **Control relevant for suspension of camphor at the following ethanol doses: 0.2, 0.5; 2.5 and 5.0 µL/adult

By increasing dose of camphor from 30 to 60 µL/350 mL vol. mortality of 7.0% was recorded with significant differences in comparison to eugenol and the control, but still not effective enough in comparison to 1,8-cineole (100%). High fumigant activity of camphor on *T. castaneum* adults (93.5%) was obtained at dose of 120 µL/350 mL vol. while it was not significantly different from that for 1,8-cineole. At all the three doses tested mortality for eugenol was not significantly different from that for the control. This result indicated that eugenol had a weak fumigant activity on *T. castaneum* adults.

4. Discussion

Essential oils can consist of hundreds of different compounds, but only a few can be found in greater quantity in single oil, or in different oils. Certain compounds can exhibit significantly higher activity than the essential oil in its entirety (Tunç et al., 2000). Thus Rozman et al. (2006), on the basis of the results obtained by gas chromatography, came to the conclusion that 1,8-cineole was the principal active compound in four oils tested (oils of lavender, rosemary, thyme and laurel). By studying fumigant toxicity of plant essential oils from Myrtaceae family, Lee et al. (2004) also reported that the majority of the oils showing potential fumigant toxicity were rich in 1,8-cineole.

By examining fumigant toxicity of essential oil compounds from Lamiaceae and Lauraceae families Rozman et al. (2007) have proved *T. castaneum* adults as highly tolerant of the tested compounds. Not sooner than 7 d of exposure only 1,8-cineole at the highest dose (100 µL/720 mL) obtained acceptable efficacy (92.5%), against *T. castaneum* species, followed by camphor (77.5%) and linalool (70.0%).

In our studies by fumigating adults with 1,8-cineole, we obtained mortality of 98.5% even with the lower dose (30 µL/350 mL vol.).

Quintai and Yongcheng (1998) proved contact efficacy of camphor in the control of R. *dominica, Sitophilus zeamais* Motschulsky and *T. castaneum*, reporting that the isolate responded only as a repellent with no insecticidal effect against *T. castaneum*. Our investigations proved that camphor by its contact activity could exhibit insecticidal effect against the adults of the species, inducing 78.5% mortality, but only at the highest dose (10.0 µL) and prolonged exposure time to 24 h.

In general, based on above obtained results of contact and fumigant activity of the three compounds tested against *T. castaneum* adults the most effective one was 1,8-cineole. The other compounds were effective only in one method: eugenol in contact, and camphor in fumigant application.

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Penetration ability of *Holepyris sylvanidis* into the feeding substrate of its host *Tribolium confusum*

Lorenz, S.*, Adler, C.#, Reichmuth, C.
Julius Kühn-Institut (JKI) - Federal Research Centre for Cultivated Plants, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Königin-Luise-Str. 19, 14195 Berlin, Germany.
Email: sandra_lorenz03@web.de

* Corresponding author
# Presenting author

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Abstract

The bethylid wasp *Holepyris sylvanidis* (Brèthes, 1913) is an antagonist of the confused flour beetle *Tribolium confusum* Jacquelin du Val 1868, a severe pest in the food processing industry and in grain products, primarily in flour mills and bakeries. Females of the larval ectoparasitoid *H. sylvanidis* have to detect hosts that feed in different depths inside stored products like flour or grist. This study addresses the questions (1) whether successful host finding by *H. sylvanidis* is dependent on the location of *Tribolium* larvae in the substrate and (2) whether the type of substrate affects host finding. In laboratory experiments, *T. confusum* larvae in a Petri dish accessible to the wasps were placed 1, 2, 4 or 8 cm deep in either fine or coarse ground wholemeal grist of wheat (main particle size: <0.2 mm or 1.4 – 3 mm) in fifteen replicates per substrate and depth. Parasitoids were released onto the surface of the substrate. *Tribolium confusum* larvae were not able to leave the Petri dish, however they could be pulled outside into the grist by *H. sylvanidis*. Within the behavioural sequence of parasitisation, pulling away of host larvae is the typical behaviour preceding oviposition. In order to determine host finding success by the parasitoid, the number of missing host larvae was assessed 2 wks after release of the wasps. In fine grist larvae were attacked down to 4 cm depth; however, larvae placed deeper (8 cm) were not found anymore. In contrast, host larvae in coarse grist were still detected at 8 cm depth. The results suggest that host finding by *H. sylvanidis* is hindered by decrease in particle size of the substrate. Nevertheless, *H. sylvanidis* may be considered a promising candidate for biological control of *T. confusum* larvae feeding in coarse grist and in thin layers of fine grist.

Keywords: *Holepyris sylvanidis, Tribolium confusum*, Biological control, Penetration ability, Host finding

1. Introduction

The acceptance of synthetic, not naturally occurring chemical materials used for pest control decreases. The interest in organic food increases. Therefore, biological pest control becomes more important. *Holepyris sylvanidis* (Brèthes, 1913) is a larval ectoparasitoid of *Tribolium confusum* Jacquelin du Val 1868, a severe pest in the food processing industry and in grain products. *H. sylvanidis* was described by different authors and can be reared in the laboratory for some time (Abdella et al., 1985; Ahmed and Islam, 1988; Ahmed et al., 1997). In regard to the application of the parasitoid against the confused flour beetle parasitizing of *T. confusum* larvae in deeper layers of the substrate would be desirable. This investigation was carried out to elucidate whether successful host finding by *H. sylvanidis* is dependent on the location of *Tribolium* larvae in the substrate and whether the type of substrate affects host finding.

2. Materials and methods

2.1. Insects

The insects used for the tests originated from the laboratory insect cultures of the institute, where *T. confusum* is cultivated on fine ground wholemeal grist of wheat at 25 ± 1°C, 65 ± 5% r.h., *H. sylvanidis* is reared on larvae of *T. confusum* in hollow wheat kernels (hollowed out by *Sitophilus* spp.) at 25 ± 1°C, 57 ± 5% r.h. The fourth instar larvae of *T. confusum* were used for the tests since *H. sylvanidis* prefers this larval stage for parasitisation (Ahmed et al., 1997). At the beginning of the experiments the parasitoids had a maximum age of ten days and were mated at least once before.

2.2. Experimental setup

The tests were carried out with both fine ground wholemeal grist of wheat (milling level 1) and coarse ground wholemeal grist of wheat (milling level 7) (Table 1). The grist contained all compounds of the grain and was freshly prepared prior to each experiment (grist mill Billy 200, Hawo's Kornmühlen GmbH, Bad Homburg, Germany).

Table 1 Milling levels and appropriate particle compositions of the two types of wholemeal grist of wheat (fine and coarse) used in the experiments

Milling level [1)]	Particle sizes (µm)	Portion of the total quantity (%)	Maximum particle size (µm)
1	≤ 200	64.0	1400
	201 - 710	31.7	
	711 - 1400	4.3	
	> 1400	0	
7	≤ 200	9.4	2800 - 3000
	201 - 710	9.5	
	711 - 1400	17.1	
	> 1400	64.0	

[1)] Milling levels are given by the grist mill Billy 200 (Hawo's Kornmühlen GmbH, Bad Homburg, Germany)

Before performing the tests the grain had been stored at -18°C for at least 10 d in order to kill potential arthropod pests. After thawing the moisture content of the wheat grains was adjusted to 14 ± 1%.

Experiments with both types of grist were carried out to reveal if *H. sylvanidis* is able to penetrate 1, 2, 4 and 8 cm deep into the substrate. Each depth (or layer thickness of the grist) was investigated in a separate 2-L glass jar. Each jar contained a Petri dish in the middle of the bottom with ten larvae of *T. confusum* and 1 g wholemeal grist of wheat for feeding purpose. The lower part of the Petri dish (Ø 3.5 cm) was covered with a slightly larger lid (Ø 5.5 cm). A V-shaped wooden stick (Ø 3 mm) placed in the lid of the Petri dish was used as spacer to preserve a gap between the two parts of the Petri dish. Through this gap the wasps had access to the host larvae. The host larvae themselves were unable to leave the Petri dish. Subsequently, the Petri dish in the glass jar was covered with a 1, 2, 4 or 8 cm thick layer of grist. Jars were shaken carefully to obtain a uniform density of the grist. Ten female *H. sylvanidis* and two males were released onto the surface of the grist. A drop of honey on the wall of the glass jar above the grist was added as food for the wasps. Each jar was closed with a piece of cotton cloth and rubber bands and kept in continuous darkness for 2 wks at 25 ± 1°C, 57 ± 5% r.h. in a climatic chamber. Each set of experiments (each type of grist combined with each depth) comprised fifteen replicates.

2.3. Collected data

After 2 wks the grist and the Petri dish were removed. The number of the remaining larvae of *T. confusum* in the Petri dish was recorded. According to Abdella et al. (1985), Ahmed and Islam (1988) and Ahmed et al. (1997) *H. sylvanidis* paralyses the host larva and always transports it away to a potential hiding place prior to depositing an egg on the larva. Therefore, if the number of host larvae in the Petri dish was less than ten this test was judged as a successful host finding. In so far, there was no distinction between one or more removed larvae leading to a positive judgement. To establish the judgement the neighbouring grist around the Petri dish was investigated for removed and parasitized host larvae.

2.4. Evaluation of the test

For each set of experiments the frequency of host finding by females of *H. sylvanidis* was determined. The frequency indicates in how many of the 15 replicates the parasitoids achieved to find host larvae. Fisher's exact test served for statistical evaluation ($P < 0.05$, SigmaStat 3.11.0) of the frequency of host finding in relation to depths and types of grist.

3. Results

Experiments with grist of both types revealed the tendency that the frequency of host finding decreased with increasing depth: the deeper the host larvae were hidden in the grist, the more seldom they were removed from the Petri dish by *H. sylvanidis* (Fig. 1). Significant differences in frequency were detected in fine grist between the tested depths 1 cm vs. 4 cm and in coarse grist between 1 cm vs. 8 cm as well as 2 cm vs. 8 cm. Comparing the two types of grist for all tested depths, 1, 2, 4 and 8 cm, the frequency of host finding was always higher in coarse grist. At a depth of 8 cm the frequency of host finding in fine grist was zero - no wasp achieved to remove one of the ten offered larvae, while in coarse grist the frequency of host finding was still six.

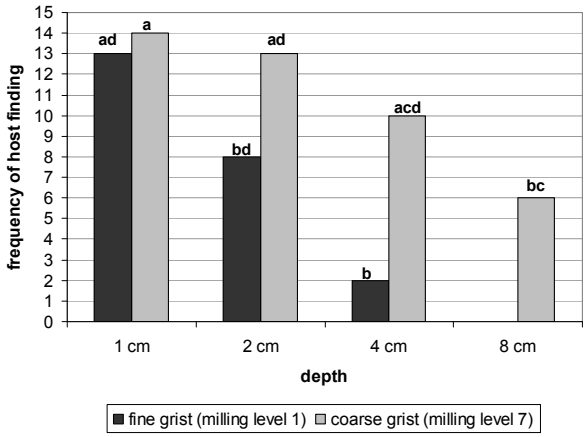

Figure 1 Frequency of host finding by *Holepyris sylvanidis* in all tested depths and types of wholemeal grist of wheat; tested locations of *Tribolium confusum* larvae: 1, 2, 4 and 8 cm deep in grist; tested types of substrate : fine grist (milling level 1) and coarse grist (milling level 7); number of replicates per depth and substrate: n = 15; bars (frequency of host finding) with different letters are statistically significant at $P < 0.05$ (Fisher's Exact Test); duration of the tests: 2 weeks; test conditions: 25 ± 1°C, 57 ± 5% r.h., continuous darkness.

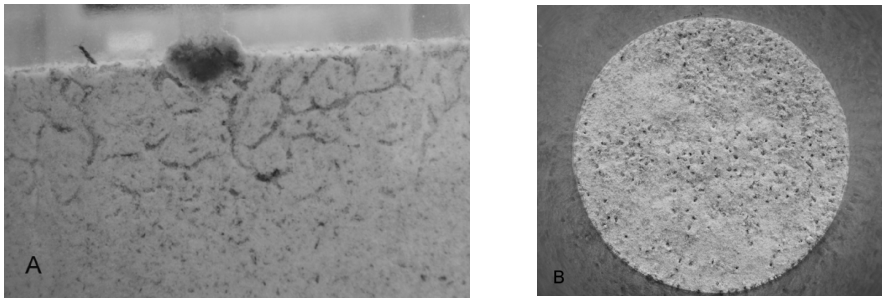

Figure 2 Small tunnels dug in the fine ground wholemeal grist of wheat (milling level 1) by *Holepyris sylvanidis*, (A) side view, (B) few from above.

Repeated observations of the single tests showed that females of *H. sylvanidis* were able to dig tunnels into the substrates (Fig. 2, A/B) and use them more than once. This was particularly obvious in fine grist.

4. Discussion

An important aspect for the optimisation of the application of biological control measures against *T. confusum* is the capability of a suitable antagonist to penetrate into the feeding substrate of its host. The experimental results showed that females of *H. sylvanidis* are able to penetrate differently thick layers of fine and coarse grist to find and parasitize hidden larvae of *T. confusum*. Host finding success

depended on the thickness of the grist layer and the type of the grist. The wasps penetrated easily through 1 and 2 cm fine grist, at 4 cm they were less successful and they were not able to penetrate 8 cm. In coarse grist the wasps seemed easily to be able to pass through 4 cm, whereas host finding clearly decreased at 8 cm thickness.

A possible explanation for the influence of the particle size of the grist on the penetration ability might be the magnitude of the hollow space between the particles. Larger particles offer larger hollow spaces and the material can not be so densely packed as fine grist. Therefore, the effort for the wasps to dig tunnels into coarse grist is much smaller than that into fine grist. Presumably more than one wasp uses a tunnel and one wasp uses a tunnel more than one time, respectively. This may explain why the maximum depth of penetration and the magnitude of the frequency are more pronounced in coarse grist.

The mostly prevailing particle size of the coarse grist was 1.4 mm – 3.0 mm (Table 1). The females of *H. sylvanidis* had a body length of 2.96 ± 0.27 mm (Frielitz, Berlin, personal communication). Due to their small body size the wasps seem to be able to use small crevices and hollow spaces to move through the grist. Presumably the wasps can penetrate deeper than 8 cm into coarse grist. Further experiments would be necessary to determine the maximum depth of penetration.

It can be assumed that the deeper the host larvae were hidden in the grist the more difficult it was for the parasitoids to locate them. Obviously the wasps use volatile substances emitted by the excrements of larvae of *T. confusum* (unpublished data). These substances diffuse through hollow spaces in the grist and can be detected by the parasitoids on the surface or in the upper layers of the substrate. Possibly, the orientation towards chemical cues is decreased by sorption due to the larger internal surface of fine grist.

Also *Trichogramma embryophagum* (Hartig) was able to parasitize eggs of a host, *Ephestia* spp., down to 5 cm of wheat (Schöller et al., 1996), *T. evanescens* Westwood even down to 55 cm (Schöller et al., 1994). Schöller (2000) determined a maximum penetration depth of 30 cm in rye for *Habrobracon hebetor* (Say). Al-Kirshi et al. (1997) found larvae of *Trogoderma granarium* Everts parasitized by *Laelius pedatus* (Say) in 90 cm depth in wheat. Steidle and Schöller (2000) showed the capability of *Lariophagus distinguendus* (Förster) to parasitize *Sitophilus granarius* larvae hidden in wheat kernels in 4 m depth.

In this study *H. sylvanidis* penetrated thinner layers of substrate because the grist consisted of much smaller particles and was, therefore, more difficult to be penetrated than whole kernels of grain.

On the other hand the capability to penetrate into a certain depth of fine grist is crucial for female *H. sylvanidis* since its host, the confused flour beetle, often lives hidden under more or less thick layers of milled grain in mills or food factories (Sokoloff, 1974). Often the insect hides in small cracks and crevices, in aeration ducts, in or under machinery, in areas that are difficult to clean.

In this study, it was clearly shown that the larval parasitoid *H. sylvanidis* is able to penetrate various kinds of grist to find and parasitize its host, the confused flour beetle *T. confusum*. On this ground *H. sylvanidis* is a promising candidate for biological control of this pest insect especially in cases where the larvae are hidden under thin layers of the substrate. As demonstrated in this study the survival strategy of these wasps comprises its capability of actively host searching and parasitizing even in deeper layers of flour and grist.

Acknowledgments

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Liotryphon punctulatus (Ratzeburg, 1848) (Hymenoptera: Ichneumonidae) – a parasitoid of *Ephestia kuehniella* larvae

Lukas, J.*#, Stejskal, V.
Crop Research Institute, Drnovska 507, 161 06 Praha 6 – Ruzyne. Email: lukas@vurv.cz, stejskal@vurv.cz

* Corresponding author
# Presenting author

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Abstract

Until now, there has been no record of *Liotrophon punctulatus* (Hymenoptera: Ichneumonidae) presence in the anthropogenic environment of mills, bakeries or pasta factories. This is the first report of the species parasitizing *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae. The host/parasitoid interaction was validated under laboratory conditions where fourth or fifth instar larvae of *Ephestia kuehniella* were provided ad libitum to *L. punctulatus* females. After two filial generations emergence, the validation process was considered to confirm the interaction.

Keywords: *Liotrophon punctulatus*, Parasitoid, *Ephestia kuehniella*

1. Introduction

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) is a cosmopolitan pest of cereal products and other stored foods. Larvae spin a web in flour, grain, or seeds, causing problems in milling or sorting. Until now, three species of parasitoids were known to attack *E. kuehniella* larvae in anthropogenic environments: *Bracon brevicornis* Wesmael, 1838, *Habrobracon hebetor* Say, 1836 (Hymenoptera: Braconidae) and *Venturia canescens* **(**Gravenhorst, 1829) (Hymenoptera: Ichneumonidae) (Schöller and Flinn, 2000). Of these, *H. hebetor* is used in some European countries for biological control against *E. kuehniella* (Schöller, 2001).

2. Materials and methods

Larvae of the Mediterranean flour moth search actively for crevices where remains of flour accumulate. Sentinel traps baited with third to fifth instar larvae of *E. kuehniella* were exposed to parasitatization inside of mills, bakeries and pasta factories in the Czech Republic (locality of Prague, Kladno, České Budějovice, Plzeň, Prostějov) from the spring of 2002 until the autumn of 2007. Based on previous experiences, the traps were placed near windows, as parasitoids are attracted there by light. The traps were replaced every 20 d. After their removal, traps were placed into plastic boxes to control and record parasitoid emergence.

3. Results and discussion

Unknown specimens was found among individuals of *H. hebetor* and *V. canescens* repeatedly during the late spring months in every year of the study. Both males and females emerged from the sentinel traps. The unknown species was identified by Josef Šedivý as *Liotryphon punctulatus* (Ratezburg) (Hymenoptera: Ichneumonidae) (Fig. 1) and placed in the insect collection of the Research Institute of Crop Production, Prague.

The host/parasitoid interaction was validated under laboratory conditions where fourth or fifth instar larvae of *E. kuehniella* were provided *ad libitum* to *L. punctulatus* females. After two filial generation emergences, the validation process confirmed the interaction.

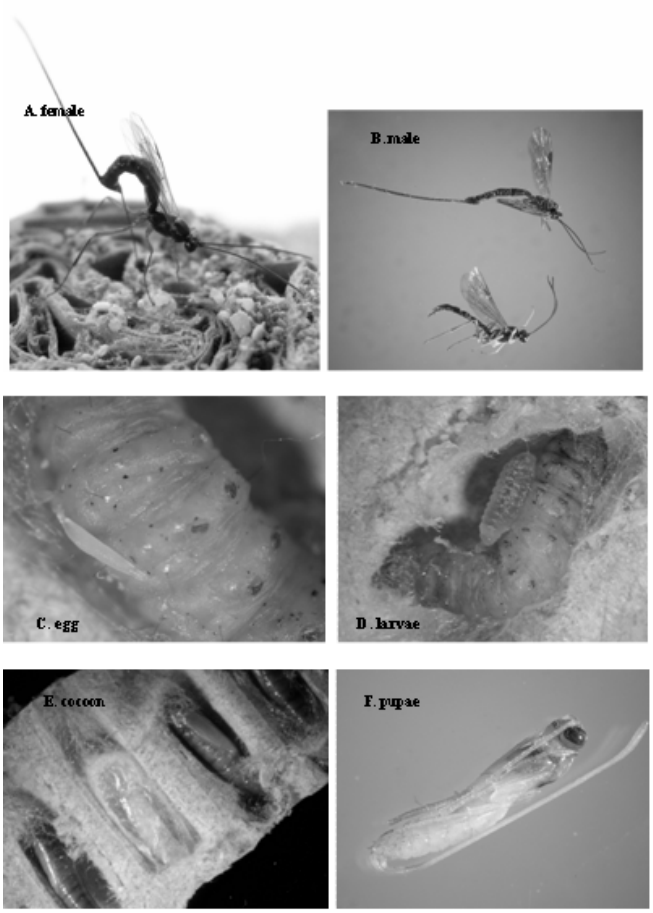

Figure 1 *Liotrophon punctulatus* life cycle (**A.** female and male, **B.** egg laying, **C.** egg, **D.** larvae, **E.** cocoon, **F.**pupae)

The biology of *L. punctulatus* is rather fragmentary (Lyngnes, 1960; Kühlhorn, 1964; Kazakova, 1971). *Liotryphon punctulatus* is an external solitary idiobiont parasitoid of cocooned larvae that actively search in cryptic habitats. It measures in size to 1.5 cm, the ovipositor somewhat longer. The period elapsing between emergence and first oviposition is 10-19 d at 25°C and 20-30 d at outdoor temperatures during the early part of the year (Rosenberg, 1934). Eggs that are deposited during the latter portion of the oviposition period of the female were consistently different from those first laid, being markedly wider in relation to the length. A portion of the eggs of this species are devoid of contents when laid, and the number of these is greater after a period of rapid oviposition and during the latter portion of the oviposition period. Many adult female ichneumonids feed on the body fluids of the host stages that they parasitize; this is either incidental to oviposition or entirely independent of it. The feeding may have no relation to oviposition, and the punctures are often enlarged by use of the mandibles. Not only the fluids but the entire body contents may be consumed; and the feeding habit, instead of being incidental to, and associated with oviposition, has developed into a distinctly predaceous habit, independent of the reproductive activities, though very probably essential to oögenesis (Clausen, 1962). The adult parasitoid paralyzes and oviposits on its host, upon which the parasitoid larva feeds. After feeding externally on the host larva and killing it, the fully developed parasite larva spins a cocoon inside the host cocoon and emerges afterwards (or overwinters as a diapausing larva). Medvedev (1981) reports *L. punctulatus* parasitizing the seciids *Pennisetia hylaeiformis* (Laspeyres, 1801), *Synanthedon culiciformis* (L., 1758), *Synanthedon myopaeformis* (Borkhausen, 1789), *Synanthedon spheciformis* (Denis and Schiffermüller,

1775), *Synanthedon tipuliformis* (Clerck, 1759), and the tortricids *Archips oporana* (L., 1758), *Cydia pactolana* (Zeller, 1840), *Cydia pomonella* (L., 1758) and *Retinia resinella* (L., 1758)**.**

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Using aeration and insulation to reduce grain temperature in China grain warehouses

Lu, J.*#[1], Zhu, Q.[2], Jia, S.[3], An, X.[4], Wang, F.[3]
[1] Grain trading company Ltd. Tianjin Tanggu District, China, P.O.: 300450, Grain Trading Company of Tianjin Tanggu District, China. Email: tjtglsgx@163.com
[2] Tianjin Grain Bureau of China, Tianjin Hedong District, 8 Wei Street 207, 300171.
[3] National Grain Reserve of Tianjin Tanggu, Tianjin Tanggu District, Xing Bei Gong Street 7275-344, 300453.
[4] State Central Grain Reserve, Ji County Storage, Tianjin Ji County YingLiu Township, 301914.

* Corresponding author
# Presenting author

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Abstract

Reducing grain temperature is a safe, effective and economic way to minimize damage to grain. It suppresses the activity of all life forms in a grain storage ecosystem. As a result, it stabilizes the grain during storage, reduces loss and keeps freshness. We conducted trials on grain storage facilities (2800-8000 t) in Tianjin, China. The technologies are based on the following factors: the characteristics of local climate (cold dry winters and hot humid summers), large scale storage structure and grain special requirements. Based on these factors, the researchers devised comprehensive and targeted solutions. For example, the insulation of existing large flat top storage was increased, by using various insulation materials in exterior roofing, interior ceiling, walls, vents, windows and doors, using efficient yet economic insulation material in new storage construction. In winter, we used ventilation by natural and forced aeration to maintain low temperatures. In summer, we used insulation and cooling to achieve temperature control. The technology can effectively reduce grain losses and maintain quality by reducing grain respiration, insect and microbial activities. At the same time, it reduces and avoids chemical pollution, protects the grain and the environment from pollution. It achieves low grain loss, low environmental impact, lower cost; high grain quality, high grain nutrient and high efficiency. It is becoming a new direction in scientific grain storage.

Keywords: Temperature controlled grain storage, Tianjin, China

1. Introduction

Temperature is a key factor in preserving grain. The current study of temperature controlled grain storage technology was based on the following factors: the climate of Tianjin, improvement of storage structure insulation, aeration cooling in winter, temperature control in the summer. It is designed to maintain low temperature throughout the storage period, to reduce losses due to grain respiration, moulds and insects. Temperature controlled grain storage technology is safe, economic, environmentally-friendly and effective (Fields, 1992; Mason and Strait, 1998; Burks et al., 2000). It has been a trend in grain storage technology.

We were interested in looking at low temperature grain storage technology for the following reasons. Pests include insects, microorganisms can greatly damage the grain. Pests are active within certain temperature range. Controlling storage temperature can effectively suppress pest growth and reproduction, and as a result, it maintains grain quality during storage. Temperature greatly influences the grain ecosystem. High temperature will accelerate grain respiration, end-use quality degradation and dry weight loss. Controlled temperature grain storage technology can reduce grain respiration and other activity. As a result, it maintains grain quality during storage.

Storage facilities should have good thermal insulation and temperature control, which protects the grain from external heat. Various insulation materials were retrofitted to the existing facilities to control temperature. The climate: Tianjin (39° 8' 32" N / 117° 10' 36" E) has long, cold, dry winter. It lasts approximately 160 days. Air temperature is around -8 to 6°C. There are around 100 days below 0ºC. In summer, it is hot and humid. Air temperature is around 20 to 34ºC. There are around 50 days above 30ºC. There are approximately 80 days above 80% r.h. Based on this climate, the facilities use aeration cooling in winter and air conditioning in summer to control temperature.

2. Case studies

Four studies were conducted; 1: aeration cooling; 2: large sandwich panel insulation; 3: magnesium board insulation; 4: self cooling. All studies were performed in close to Tianjin, China.

2.1. Case study 1: Aeration

Two methods or aeration cooling were used: natural aeration and forced aeration with fans. Natural aeration cooling took advantage of the cold climate in winters, maintained low temperature without using additional powered ventilation system. Specifically, it controlled temperature by opening, closing the windows and vents using timers. Ventilation was operated between 13 October 2006 and 29 January 2007 (Table 1).

Table 1 The effect of aeration, natural and forced aeration (centrifugal fan) on large flat wheat storages in China, Study 1. Warehouses had walls made of brick and concrete (500 mm thick), and concrete roofs.

Method	Location Storage Facility Storage number	Building length, width (m)	Flow rate (m^3/h)	Pressure (pa)	Power (kW)	Duration of aeration (days, total hours)	Wheat Grade	Size of grain bulk (t)	Average moisture content (%)	Average Temperature before aeration		Average Temperature After Aeration	
										Level	(°C)	Level	(°C)
Natural aeration cooling	Hangu district DongFeng #81	30, 21	NA	NA	NA	100, 1000	3	3069	12.5	Upper	21.7	Upper	5.3
										Middle	17.1	Middle	9.9
										Mid 2nd	14.0	Mid 2nd	8.8
										Lower	14.4	Lower	8.0
Forced aeration cooling	Tanggu National Grain Reserve #17	54, 24	3420-4020	1340-2040	5.5	8, 168	3	5015	12.5	Upper	5.9	Upper	5.8
										Middle	20.0	Middle	5.7
										Mid 2nd	21.0	Mid 2nd	5.2
										Lower	16.0	Lower	5.8

The forced aeration cooling used mobile centrifugal fans mounted to external lower vents of the warehouse. The compressed air traveled from centrifugal fans through lower vent, ducts, to grain, then exited through upper vents. The study was conducted from mid to late December 2005, approximately 20 h/d, 168 h total. Four fans were used. The unit ventilation rate is calculated as m^3h/t of grain. The result showed significant improvement in both natural and force aeration.

2.2. Case study 2: Sandwich panel insulation

Large insulated sandwich panels were used to insulate the storage from warm outside summer temperatures to maintain low grain temperature. The insulated site was National Grain Reserve, Tianjin Tanggu district, #2 storage. The storage is 54 m long, 24 m wide. Load bearing structures are steel beams, columns and trusses. The roof and walls are double layered composite sandwich panels, with heat insulating fiber glass wool core. The roof panel: 0.8 mm thick external skin, 0.6 mm internal skin, 150 mm fiber glass fiber wool core. External-internal wall panel: 0.5 mm skin, 75 mm thick fiber glass wool core. The uninsulated facility was National Grain Reserve, Tianjin Tanggu district, Yujiabao #8 storage. It is 50 m long, 20 m wide. The walls were constructed of 370 mm thick prefabricated concrete frames and bricks. The roof was concrete construction.

Grain information for case studies are shown in Table 2. Temperatures were taken periodically during the study. In summer, the warehouse insulated with large sandwich panel construction storage had room air temperatures from 3º to 6 ºC lower (Fig. 1) and grain temperatures from 2ºC to 5ºC lower (Fig. 2) than uninsulated conventional storage. The insulation had significant benefits.

Table 2 Conditions of storage of storage facilities, temperatures given in figures.

Study	Storage facility number	Aeration	Insulation added	Wheat grade	Size of grain bulk (t)	Grain moisture content (%)
2	2	No	Yes	3	3800	12.2
2	8	No	No	3	2800	12.0
3	16	No	Yes	-	8299	12.5
3	7	No	No	-	7202	11.0
4	64	Yes	Yes	2	6140	12.4
4	38	No	No	2	3017	11.4

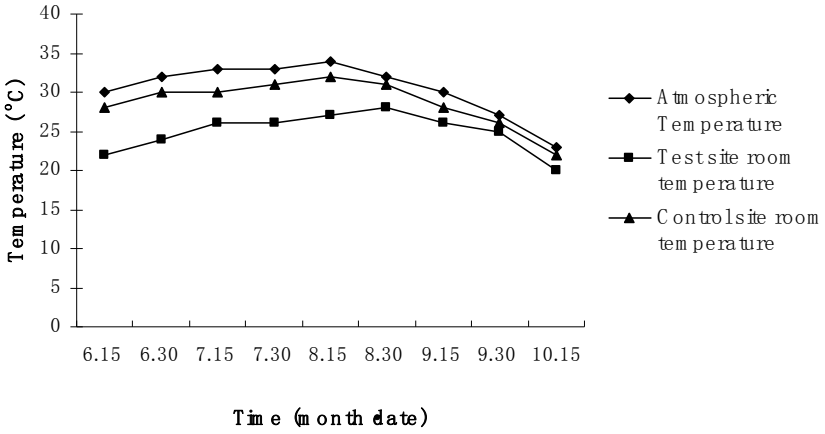

Figure 1 Air temperature compared to test (insulated) and control (uninsulated) grain, Study 2.

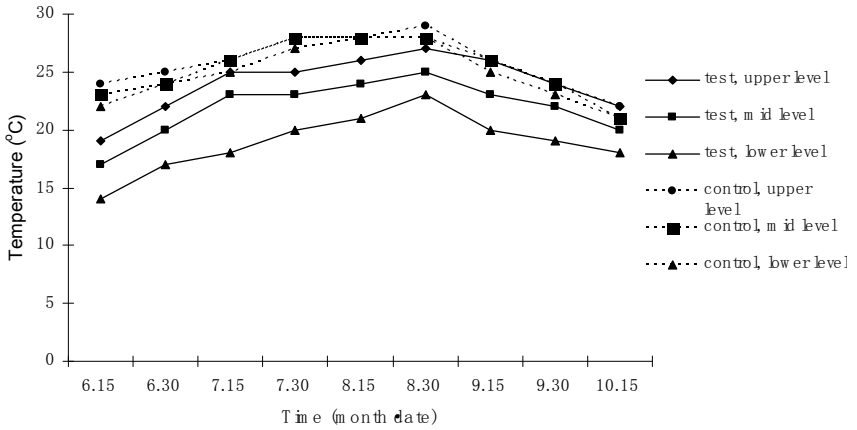

Figure 2 Grain temperatures by level for test (insulated) and control (uninsulated) grain, Study 2.

2.3. Case study 3: Magnesium board insulation

Magnesium board insulation was used to lower the temperature in large flat top storage. The insulated site was Central Grain Reserve, Tianjin Ji County storage. It was 54 m long, 30 m wide. The warehouse was constructed of brick and concrete. The wall was 500 mm thick. In summer, due to the lack of insulation from the concrete roof, the upper level room temperature can reach as high as 31ºC. The upper level grain temperature can reach between 28º and 31ºC. This had an adverse effect on the grain. Insulation improvements were made to #16 storage by installing magnesium composite insulation boards. First, the exterior of the storage was water sealed with 4 mm SBS layer. Scaffold was built on the exterior of the storage using waterproof magnesium trusses (60 mm X 80 mm X 2400 mm). The magnesium insulation boards (30 mm X 900 mm X 1800 mm) were glued to the scaffold. The scaffolding between the roof and the insulation board forms a gap, promoting ventilation cooling by natural aeration. The insulation boards were painted with white protective paint. It reflects light and repels water. The uninsulated site, #7 storage, was 48 m long, 30 m wide.

Compared to the uninsulated, the insulated storage, room temperature was 5º to 6.4ºC lower (Fig. 3), upper level grain temperature was 4.2º to 6.7ºC lower; mid and lower level grain temperature was 2.2 to 3.1ºC lower (Fig. 4). The insulation improvement had a significant effect on the temperature.

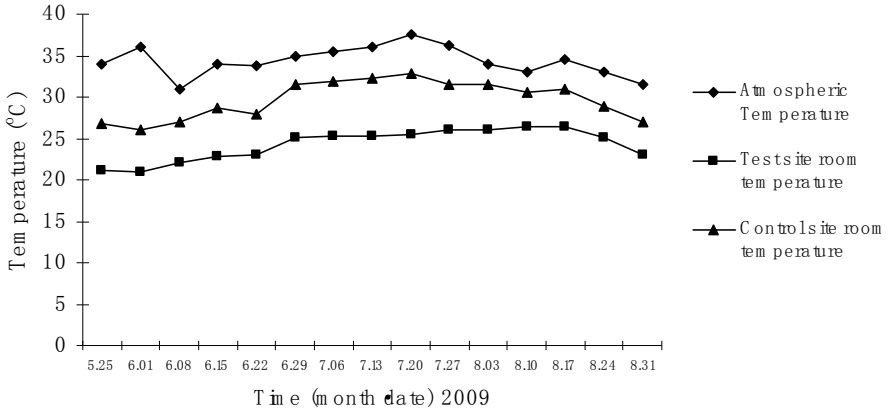

Figure 3 Air temperature compared to test (insulated) and control (uninsulated) grain, Study 3.

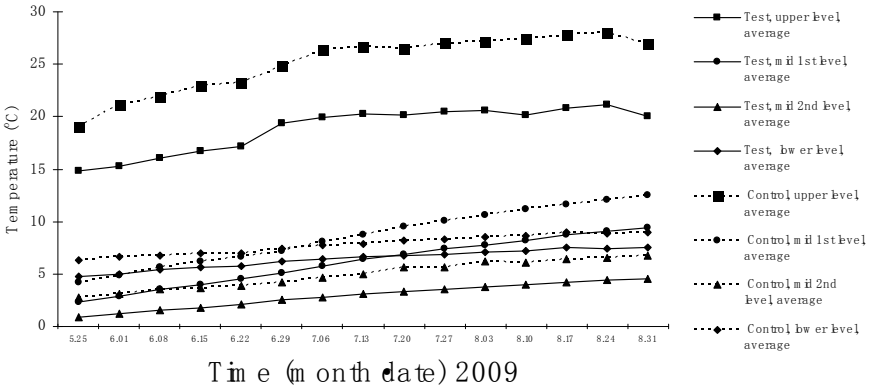

Figure 4 Temperatures by level for test (insulated) and control (uninsulated) grain, Study 3.

2.4. Case study 4: Self cooling

The self cooling study, the lower level cooler grain in the large flat top storage was used as a cold source to lower the temperature of the upper level grain. The aerated and insulated site was Central Grain Reserve at Tianjin Ninghe County. It was brick and concrete construction with 500 mm thick wall. The aerated site, #64 storage was 54 m long, 23 m wide. The following insulation improvements were made. Insulation sandwich panels were suspended from the interior ceiling. The panels were 75 mm thick with steel skins and polystyrene thermal plastic foam core. The external of the storage was covered 46mm thick polystyrene thermal plastic foam panels. Storage doors, windows and vents were sealed with the same material.

In large flat top storage, open air circulation in winter can reduce the grain temperature to between 0 and 5ºC during winter. During summer, the upper level grain temperature can reach between 28º and 30ºC. However, the mid/lower level grain temperature was between 2º and 15ºC. This technique utilized the cooler grain at the mid/lower level to lower the upper level temperature. Methods: From mid June to mid September, two to four air blowers were setup at the upper level. The air intake was 4 to 5 m below the grain surface. It was used to circulate the mid/lower level cool air to the top. Depending on the temperature condition, 2 to 4 blowers were operated 8 to10 h/d, a total of 264 blower/operations, equivalent of 2090 operating hours. The fan was a JW8X1 model, with a flow rate of 630 m^3/h, a pressure of 1400 Pa and used 0.55 kW. The unaerated and uninsulated site was #38 storage. It was 23 m long, 27 m wide.

The result of the study 4 showed that the maximum room temperature in summer was below 23ºC. The maximum grain temperature was below 22ºC. It was between 4º to 7ºC lower than the unaerated and uninsulated warehouse. The maximum grain temperature was 7°C lower in the upper level, 9ºC lower in the mid/lower level. There was a significant reduction in temperature.

The following techniques were also experimented and achieved good results: Pressure seal the storage, water spray cooling on the roof, external wall, windows and door shading.

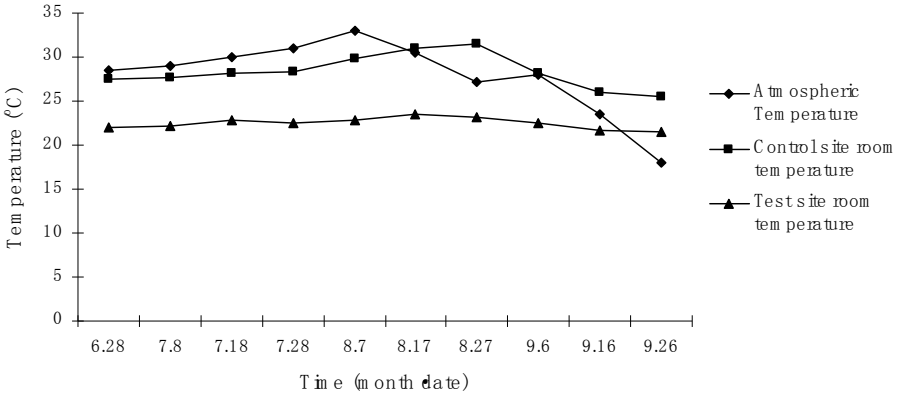

Figure 5 Air temperature compared to test (aerated and insulated) and control (unaerated and uninsulated) grain, Study 4.

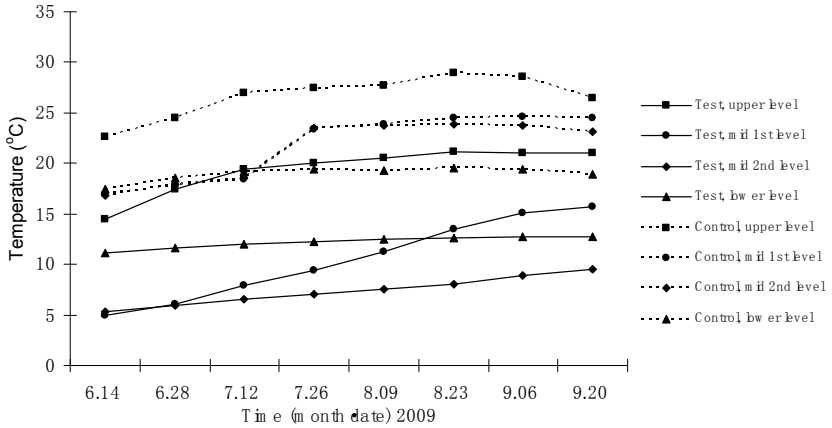

Figure 6 Temperatures by level for test (aerated and insulated) and control (unaerated and uninsulated) grain, Study 4

3. Conclusions

Using aeration and insulation to reduce grain temperatures can effectively suppress pest activity, such as the reproductive activity of insects and microorganisms. It can control grain respiration, reduce solid matter loss. Based on the climate, grain condition, ventilation cooling by natural and forced aeration can be used. The grain storages should be built or remodeled with insulation materials that are strong, durable, economic, environmental friendly, to achieve the cooling effect. An optimum solution should be designed with the consideration of climate, storage condition/specification and grain condition. Temperature controlled grain storage technology has proven to be safe, economic, environmentally-friendly and effective. It should be widely applied to storages with similar conditions.

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Insecticidal effect of anisaldehyde against *Acanthoscelides obtectus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae)

Ndomo, A.F.[1,2,3]#, Tapondjou, L.A.*[1], Tchouanguep, F.M.[2]
[1] University of Dschang, Department of Chemistry, Faculty of Science, Laboratory of Applied and Environmental Chemistry, P.O. Box 183, Dschang, Cameroon. Email tapondjou2001@yahoo.fr
[2] University of Dschang, Faculty of Science, Department of Biochemistry, Laboratory of Medicinal plants, Science and Nutrition, P.O. Box 67, Dschang, Cameroon
[3] Julius Kuehn-Institut, Federal Research Centre for Cultivated Plants/ Institute for Ecological Chemistry, Plant Analytics and Stored ProductProtection, Königin Luise Straße 19, 14195 Berlin, Germany

* Corresponding author
# Presenting author

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Abstract

In the present study, anisaldehyde, a compound found in the essential oil of *Clausena anisum-olens*, was tested for its insecticidal activities against *Acanthoscelides obtectus* and *Callosobruchus maculatus*. The amounts of anisaldehyde applied were 0, 0.5, 1, 2 and 4 µL diluted in 1mL of acetone and applied to 40 g of either beans or cowpeas corresponding to the doses of 0, 0.008, 0.016, 0.033 and 0.066 µL/g of seed. Additionally, adsorbent clay was used as a carrier of this product in order to increase the persistence of its insecticidal activity over time. This clay was mixed with the aforementioned volumes of anisaldehyde to form a powder formulation. Furthermore, to assess the insecticidal effect over time, the F_1 progeny production was also evaluated. These two products caused significant mortality in the two tested insects. Nevertheless, *C. maculatus* was more susceptible than *A. obtectus* at tested doses. The progeny production decreased with the increasing doses of anisaldehyde and ACP with 0 % at the highest dose (0.066 µL/g). According to the LD_{50}, LD_{95} and their confidence intervals, the toxicity of ACP was significantly different (P<0.05) to anisaldehyde at the tested doses towards *A. obtectus* adults. However, there was no significant difference observed between the effects of these two products towards *C. maculatus*. These preliminary results suggest that anisaldehyde and ACP could be used in stored-product protection, but this needs further research. Research is also needed to determine its toxicity on rats in order to assess its potential hazards for workers and consumers.

Keywords: Anisaldehyde, Clay, Contact toxicity, Bruchids

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walpers) and beans (*Phaseolus vulagris* (L.) (Fabacaea) are important crops for many subsistence farmers in the tropics, especially in Africa, because they contain a high level of protein (20 to 25% and 23 to 30% respectively), and are used as human food (Broughton et al., 2003). In tropical and subtropical countries, dry and ripe seeds of these legumes are currently destroyed by *Callosobruchus maculatus* (F.) and *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), respectively (Delobel and Tran, 1993). Physical, biological and chemical methods have been developed in order to control stored-product insects. In addition, plants extracts (essential and vegetable oils, organic and aqueous extracts) have insecticidal, fungicidal and bactericidal properties (Boeke et al., 2001, Kuiate, 1993). As an insecticide, plant extracts can acts as a contact insecticide, fumigant, and antifeedant or as a repellent (Boeke et al., 2001).

Some constituents of essential oils are insecticidal (Boeke et al., 2001; Hinman, 1954; Burditt et al., 1963; Hammond et al., 2000) and may inhibit growth of insects (Huang et al., 2002). On the other hand, some clays are recognised by some traditional societies for their ability to control insects (Ramaswamy et al., 1995). The use of such powders, aromatised with essential oils or insecticidal pure compounds could have a combined effect of mechanism action and insecticidal action (Ramaswamy et al., 1995; Ndomo et al., 2008).

The family of Rutaceae contains plants with very strong aroma, and their essential oils contain constituents with which the insecticidal activity has already been studied. The study of the essential oil of *Clausena anisum-olens* (Blanco) Merrill (Rutaceae) shows that it contains anethole and methyl chavicol as major components and anisaldehyde as a minor component, (Molino, 2000). Anethole shows insecticidal properties against *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) by inhibiting its reproductive activity (Bazzoni *et al*., 1997) and against *C. maculatus* (Tapondjou et al., 2002). Generally, the biological activity of essential oils is due to the synergy of its major and minor components (Kuiate, 1993). However, constituents of many extracts are now tested individually in order to find out the active component in an extract (Prates et al., 1998, Huang et al., 2002; Tapondjou et al., 2002). In view of the toxicity of many pure compounds, the present study investigates the insecticidal effect of anisaldehyde, a pure compound found in essential oil of *C. anisum-olens* on *A. obtectus* and *C. maculatus*; also to use a clay as a support of this chemical in order to increase the persistence of its insecticidal activity over time.

2. Materials and methods

2.1. Insects

The legume pests *A. obtectus* and *C. maculatus* were obtained from our stock cultures maintained in 5-L glass jars held in a controlled temperature chamber at 27 ± 2°C, 75 ± 5% r.h. and photoperiod of LD 12:12 (hours light:dark) on beans and cowpea seeds as culture medium, respectively.

2.2. Chemicals

Anisaldehyde (anisic aldehyde, 4-methoxybenzaldehyde, $C_8H_8O_2$, MW=136.15; 99.5% purity, Sigma-Aldrich Chemicals GmbH Company, Taufkirchen, Germany) discovered in essential oil of *C. anisum-olens* (Mollino, 2000) was diluted with acetone to prepare a series of concentrations. Quantities of 0, 0.5, 1, 2 and 4 µL of anisaldehyde were diluted in 1 mL of acetone and applied to 40 g of either beans or cowpea corresponding to doses of 0, 0.008, 0.016, 0.033 and 0.066 µL/ g of grain.

2.3. Preparation of aromatized clay powder (ACP)

The mineral material used was fine white clay of smectitic nature and montmorillionitte type already investigated by Tonle (2004) and present in our laboratory in the form of powder with particles less than 106 µm diameter. Preliminary tests were carried out in order to choose the non toxic quantity of clay to insects, and which must be able to remain as a powder not a paste after admixture with tested volumes of anisaldehyde. Four different samples of ACP were prepared by mixing separately 0.5, 1, 2 and 4 µL of anisaldehyde with 0.05 g of clay. The mixtures were manually stirred for 5 min to obtain a homogenous mixture called aromatised clay powder (ACP). The control consisted only of 0.05 g of clay powder without anisaldehyde.

2.4. Biological tests

2.4.1. Contact toxicity of anisaldehyde

In order to determine the contact toxicity of anisadehyde towards insects, the method used by Tapondjou et al. (2003) was followed. *Acanthoscelides obtectus* and *C. maculatus* assays were conducted on beans and in cowpea seed, respectively. Forty gram samples of grain contained in 270 cm^3 glass jars were mixed with each of the previous test solutions by tumbling for 5 min to ensure even spread of the material over the surface of the grain. In the control jars, grain was treated only with acetone (1 mL) and all jars were manually stirred for 5 min and kept open for 15 min to allow complete evaporation of solvent. The grain was then infested with 1-day-old unsexed adult insects (25 per jar) and each jar was covered with fine porous cloth held with rubber bands; jars were placed in a chamber conditioned at 27 ± 2°C and 75 ± 5% r.h. and a photoperiod of LD 12:12 (hours light:dark). Each treatment was replicated three times. Mortality counts were made daily up for four days.

2.4.2. Contact toxicity of ACP

The contact toxicity of ACP was carried out as previously described with anisaldehyde and in the same experimental condition, however, instead of anisaldehyde, every jar containing 40 g of grain was treated with one sample of ACP previously prepared and manually stirred so that all grain were uniformly coated. Thus lead to the following doses: 0.008, 0.016, 0.033 and 0.066 µL/g of grain (volume of

anisaldehyde per quantity of grains). However, in control jars, the grain was treated with non ACP (0.05 g of clay powder without anisaldehyde).

2.4.3. Effect of anisaldehyde and ACP on F_1 progeny production

After counting mortalities in the above contact toxicity tests on the fourth day, the remaining living adult insects were removed and the glass jars kept under the same experimental conditions until the emergency of F_1 progeny adults. Based on the life cycle of untreated insects (Delobel and Tran 1993), the counting period of F_1 progeny (38 d after treatment) was established so as to avoid an overlap of generations. Percentage of reduction in adult emergence or inhibition rate (% IR) was calculated with the formula used by Tapondjou et al. (2003) as:

$$\%IR = \frac{Cn - Tn}{Cn} \times 100$$

Where *Cn* is the number of newly emerged insects in the untreated (control) jar and *Tn* the number of insects in the treated jar.

2.5. Data analysis

Data obtained from each dose-response bioassay were subjected to probit analysis in which probit-transformed mortality was regressed against log-transformed dose (Finney, 1971); LD_{50} and LD_{95} values were calculated after each day of exposure using the software Poloplus version 2.0. One-way analysis of variance was performed to compare the effect of dose tested for each exposure period. Means were separated using a subsequent Waller-Duncan at 5% significance level with the Software SPSS, 2000 (Steel and Torrie, 1980).

3. Results

3.1. Adults of A. obtectus

The mortality of adult *A. obtectus* increased with increased doses and time (Table 1). There was a significant difference (P<0.05) between the mortalities induced by the highest doses (0.033 and 0.066 µL/g of beans) and the lowest (0.008 and 0.016 µL/g of beans) after 4 d exposure. For anisaldehyde, the LD_{50} and LD_{95} were 0.052 (0.041-0.075) µL/g and 0.196 (0.118-0.563) µL/g of beans respectively, after 2 d exposure.

Table 1 Effect of different doses of anisaldehyde and ACP (aromatized clay powder) on mortality of *Acanthoscelides obtectus* adults

pExposure time (day)		Mortality ± SD (%) Dose (µL/g of grains)					LD_{50} (µL/g of grains)	LD_{95} (µL/g of grains)
	Product	**0.000**	**0.008**	**0.016**	**0.033**	**0.066**		
1	Anisaldehyde	0.0±0.0a	2.7±2.3ab	2.6±2.3ab	5.3±4.6b	32.0±4.0c	0.125 (0.083-0.319)	0.750 (0.302-6.854)
	ACP	0.0±0.0a	5.3±2.3a	5.3±2.3a	37.3±4.6b	72.0±2.3c	0.043 (0.034-0.061)	0.151 (0.094-0.417)
2	Anisaldehyde	0.0±0.0 a	2.7±2.3 a	8.0±4.0ab	25.3±4.6 b	64.0±7.3c	0.052 (0.041-0.075)	0.196 (0.118-0.563)
	ACP	0.0±0.0a	8.0±4.0a	10.7±2.3a	46.7±4.6b	82.7±4.6c	0.034 (0.028-0.045)	0.117 (0.078-0.257)
3	Anisaldehyde	0.0±0.0a	6.7±2.3a	9.3±2.3a	30.7±10.0b	86.7±6.1c	0.038 (0.031-0.047)	0.117 (0.082-0.218)
	ACP	0.0±0.0a	13.3±2.3b	17.3±6.1b	70.7±2.3c	92.0±4.6d	0.025 (0.021-0.030)	0.076 (0.057-0.122)
4	Anisaldehyde	0.0±0.0a	10.7±4.6b	10.7±2.3b	33.3±4.6c	94.7±4.6d	0.033 (0.026-0.044)	0.101 (0.067-0.231)
	ACP	0.0±0.0a	14.7±2.3b	20.0±4.0c	82.7±2.3d	97.3±2.3e	0.021 (0.019-0.024)	0.054 (0.046-0.069)

For a given row means followed by the same letter are not significantly different (p>0.05) at Waller-Duncan test.

The effect of the ACP based on the mixture of clay with different doses of anisaldehyde on beans was dose dependent (Table 1). The highest dose (0.066 µL/g of beans) induced 72.0% mortality after 1 d exposure. This mortality increased to 97% after 4 days whereas the lowest dose (0.008 µL/g of beans)

induced 15% of mortality. No mortality was recorded in the control jars after 4 d. There were significant differences (P<0.05) between mortalities induced by the doses of 0.034 (0.028-0.045) and 0.117 (0.078-0.257) µL/g of beans during the 4 d of assay. The LD_{50} and LD_{95} values of ACP were 0.034 µL/g and 0.057 µL/g of beans, respectively, after 2 d. Additionally, anisaldehyde and ACP were significantly different (P<0.05).

3.2. Adults of C. maculatus

As with *A. obtectus*, there was a dose-dependent evolution in mortality of adults of *C. maculatus* in cowpeas treated with anisaldehyde (Table 2). The mortalities induced by the highest doses 0.033 and 0.066 µL/g were significantly different (P<0.05) during the 4 d of exposure. The LD_{50} and LD_{95} were 0.031 (0.026-0.039) µL/g and 0.132 (0.088-0.263) µL/g of cowpeas after 2 d.

Table 2 Effect of different doses of anisaldehyde and ACP (aromatized clay powder) on mortality of *Callosobruchus maculatus* adults

Exposure time (day)		Mortality ± SD (%) Dose (µL/g of grains)					LD_{50} (µL/g of grains)	LD_{95} (µL/g of grains)
	Product	0.000	0.008	0.016	0.033	0.066		
1	Anisaldehyde	0.0±0.0a	0.0±0.0a	5.3±4.6a	45.3±12.8b	72.0±10.6c	0.041(0.035-0.049)	0.117 (0.088-0.188)
	ACP	0.0±0.0a	4.0±0.0ab	10.0±2.0b	42.7±8.3c	76.0±4.0d	0.039 (0.034-0.045)	0.132 (0.101-0.197)
2	Anisaldehyde	0.0±0.0a	15,3±5.0ab	20.0±6.9b	49.3±16.2c	82.7±2.31d	0.031 (0.026-0.039)	0.132 (0.088-0.263)
	ACP	0.0±0.0a	14.0±3.4b	21.3±2.0b	50.±5.2c	86.7±4.6d	0.030 (0.026-0.034)	0.119 (0.089-0.183)
3	Anisaldehyde	0.0±0.0a	16.0±4.0b	24±13.8b	61.3±15.1c	94.7±2.31d	0.026 (0.021-0.033)	0.095 (0.065-0.188)
	ACP	0.0±0.0a	21.3±2.3b	26.7±3.4b	64.7±6.4c	98.7±2.3d	0.022 (0.018-0.026)	0.072 (0.053-0.120)
4	Anisaldehyde	0.0±0.0a	37.3±2.3b	41.3±15.1b	78.7±6.1c	98.7±2.3d	0.016 (0.013-0.019)	0.062 (0.045-0.108)
	ACP	0.0±0.0a	34.7±2.3b	42.0±3.4b	80.0±8.0c	100.0±0.0d	0.016 (0.013-0.018)	0.055 (0.042-0.086)

For a given row means followed by the same letter are not significantly different (p>0.05) at Waller-Duncan test.

The mortality of *C. maculatus* adults increased with the dose of ACP applied (Table 2), and is more pronounced than with anisaldehyde applied alone. Also, there were significant differences (P<0.05) between the lowest doses (0.008 and 0.016 µL/g) and the highest (0.033 and 0.066 µL/g). The LD_{50} and LD_{95} were 0.030 (0.026-0.034) µL/g and 0.119 (0.089-0.183) µL/g of cowpea, respectively, after 2d. However, according to the LD_{50}, LD_{95} and their confidence intervals during the 4 d exposure, there was no significant difference between the effect of anisaldehyde and ACP at tested doses.

3.3. F_1 progeny production

Anisaldehyde and its ACP reduced the production of progeny of *A. obtectus* and *C. maculatus* (Table 3). The percentage of inhibition of these adults insects at F_1 increased with the dose of anisaldehyde or ACP applied. However, at lowest doses the inhibition of F_1 progeny production of these beetles induced by ACP is more pronounced than that caused by anisaldehyde at the same doses.

Table 3 Inhibition rate of anisaldehyde and ACP (aromatized clay powder) on F_1 progeny production of the two bruchids

Dose (µL/g of seeds)	Reduction in F1 progeny production (% of untreated)			
	A. obtectus		*C. maculatus*	
	Anisaldehyde	ACP	Anisaldehyde	ACP
0	0	0	0	0
0.008	59.6	88.3	38.1	45.4
0.016	76.9	90.9	71.4	90.9
0.033	90.4	100.0	90.5	100.0
0.066	100.0	100.0	100.0	100.0

4. Discussion

Anisaldehyde as well as its ACP were toxic against adults of *A. obtectus* and *C. maculatus*. The current results are in agreement with the report of Kuiate (1993) who mentioned that even minor components, alone or in association with other components, could have biological activity. Many pure compounds, especially terpene have been evaluated for their insecticidal activities against stored-product insects (Prates et al., 1998; Huang et al., 2002). The study of some aldehydes has also attracted the attention of some researchers in their use as insecticides (Ferguson and Pirie, 1948; Hinman, 1954; Burditt et al., 1963; Hammond et al., 2000). Our results corroborate with those of Hammond et al., (2000), who found that propanal, (*E*)-2-pentenal, and 2-methyl-(*E*)-2-butenal have excellent potential as post-harvest insect control agents. These chemicals killed 100% of aphids with little or no detectable harm to a majority of the commodities tested (naked and wrapped iceberg lettuce, green and red table grapes, lemon, grapefruit, orange, broccoli, avocado, cabbage, pinto bean and rice). This could explain the toxicity of anisaldehyde tested in this study.

Based on the LD_{50} and LD_{95} level during the 4-d exposure, ACP was more toxic than anisaldehyde towards *A. obtectus* adults. In fact, the adsorbent properties of this particular clay powder have been tested by Ndomo et al. (2008) who have used this clay to maintain the persistence of insecticidal essential oil of *C. anisata* against *A. obtectus* over the course of time; However, *C. maculatus* was more susceptible than *A. obtectus* at tested doses. The differences in response by different insect species could be attributed to the morphological and behavioural differences between the species (Delobel and Tran, 1993)

The use of such clay powders aromatized with essential oils or chemicals has a two-fold advantage due to the combined effects of the mechanism of action by the powder, which blocks the insect movement, filling intergranular spaces at high doses, and insecticidal action itself due the chemical (Ramaswamy et al., 1995). Additionally, Tonle (2004) mentioned that the adsorbent character of a clay powder is inversely proportional to the diameter of the particles. Consequently, the use of clay particles powder of very small diameter could increase its capacity of fixing anisaldehyde and ,thus, increase persistence of the insecticidal activity of this product over time. This could provide a solution for Ferguson and Pirie (1948), Hinman (1954) and Burditt et al. (1963) who concluded in their studies that the low to moderate toxicity of aldehydes with three or more carbons made them too weak for commercial insecticide applications.

In spite of the efficiency of anisaldehyde as insecticide against *A. obtectus* and *C. maculatus* adults in this study, further research is needed using other pest insects, in order to broaden its spectrum of action. Research must also determine the toxicity of both anisaldehyde and ACP on rats in order to assess its potential hazards for workers and consumers.

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Distribution of insect pests and their natural enemies in a barley pile

Belda, C.*#, Riudavets, J.*
IRTA (Institut de Recerca i Tecnologia Agroalimetaris), Entomology, Ctra Cabrils km 2, E-08348 Cabrils (Barcelona), Spain. Email: consuelo.belda@irta.es

* Corresponding author
# Presenting author

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Abstract

The distribution and abundance of stored product pests and their natural enemies infesting the upper layer of a barley pile was assessed in this study. Sampling was carried out on a monthly basis from February to December 2009. Insects were sampled with a grain trier and pitfall traps. Yellow sticky traps were also used to capture flying insects. Most abundantly captured species were, in order of abundance: *Rhyzopertha dominica*, *Sitophilus granarius*, and *Latheticus oryzae*. Fewer individuals of other species were occasionally captured, such as *Cryptolestes ferrugineus*, *Oryzaephilus surinamensis*, *Lasioderma serricorne* and *Stegobium paniceum*. Concerning Hymenoptera, the species *Anisopteromalus calandrae* and *Cephalonomia waterstoni* were abundantly captured with yellow and pitfall traps, while lower numbers were captured with the grain trier trap. Comparing pitfall and grain trier captures, in the former one, natural enemies were abundantly captured, whilst captures with the grain trier were punctual. The predator *Withius piger* (Pseudoscorpionida: Withiidae) was captured both with grain trier and pitfall traps. Its captures peaked in August-September. The abundance of coleopteran pests varied among the different depths sampled and the time of the year. The highest captures of pests occurred in March and May; the natural enemy *A. calandrae* peaked in April, and *C. waterstoni* was scarcely captured. The three species of natural enemies were occasionally found in the deepest samples, thus, they were able to penetrate around 80-cm deep in the grain searching for their hosts.

Keywords: Grain, Sampling, Biological control, Host finding.

1. Introduction

Pest control of stored products is based on the application of chemical products, fumigants and residual insecticides. However, the maximum residue limits of pesticides allowed in the final food products is becoming more and more restrictive. Therefore, alternatives are needed, and biological control is one possible alternative (Schöller et al., 2006). In previous studies, natural enemies have been found in several stored-product companies manufacturing different food products and in warehouses (Riudavets et al., 2002). Moreover, other studies have focused on how coleopteran pests and natural enemies distribute vertically through a grain bulk (Buchelos and Athanassiou, 1999; Steidle and Schöller, 2002; Steidle et al., 2002). In this study we present the results of sampling a barley pile that had been placed next to a heavily infested 1-year-old barley pile. The main objectives of our study was to search for natural occurring parasitoids, to assess how insects (both pests and natural enemies) colonize a new pile from the very first week, to evaluate which are the first species to arrive and how do they invade and distribute through the pile, i.e. their vertical movement.

2. Material and methods

Trials were carried out in a warehouse of the IRTA (Institut de Recerca i Tecnologia Agroalimetaris) Research Centre, located in Caldes de Montbui, Barcelona, Spain. This warehouse is normally used to store cattle food for winter and sowing machinery. There was an old barley pile in the warehouse that had been stored for one year, since harvesting in summer 2007. The second pile of barley was harvested the summer 2008, and it was placed adjunct to the old barley pile on February 2009. The new pile contained 2 t of barley that had not been treated, as it came from organic crops. It was 180-cm high, 3-m wide and 3-m long. Sampling was conducted on a monthly basis from February 2009 till December 2009. To sample the grain pile for coleopteran pests (Consultores cerealistas S.A., Castelldefels, Barcelona, Spain), a 1.50-m long aluminum grain trier was used (Fig. 1). There are eight elongated holes (each 10-cm long) on the grain trier, with a separation of 2.5 cm between holes. The trier was introduced in the barley pile three times per sampling date, one time on each side of the pile and one in the front

side. So, three replicates per month were carried out. Barley was sampled at about 80 cm from the top surface, corresponding with five levels of the grain trier (0, 20, 40, 60 and 80 cm), each with a capacity of 70 g for a total of 350 g of grain. All adults were sieved out, identified and counted. We focused on the grain trier because we wanted to study the vertical distribution of the species present. Other traps were used for sampling the barley pile, this being six pitfall traps (Killgerm S.A., Viladecans, Barcelona, Spain) distributed at two heights (on the surface and 15-cm deep), and three yellow traps (20 x 20 cm, Sanidad Agrícola Econex S.L., Santomera, Murcia, Spain), two located around the pile never higher than 1.5 m, and one hanging 20 cm over the pile. All traps were checked and changed monthly.

Figure 1 Devices used for sampling: grain trier, pitfall trap and yellow sticky trap.

3. Results and discussion

There was a rapid colonization from the older pile to the new pile. In the first month of sampling all species had reached and had distributed among the different depths of the pile. The most abundant coleopteran.species through all the sampling period captured with the grain trier and pitfall traps were *Rhyzopertha dominica* (F.) (Bostrichidae), *Sitophilus granarius* (L.) (Curculionidae), and *Latheticus oryzae* (Waterhouse) (Tenebrionidae) in this order. They were captured with the grain trier in the deepest levels already in the first month of sampling. Fewer individuals of other species were occasionally captured both with grain trier and pitfall traps, such as *Sitophilus oryzae* (L.), *Tribolium confusum* Jacquelin du Val (Tenebrionidae), *Tribolium castaneum* (Herbst), *Cryptolestes ferrugineus* (Stephens) (Laemophloeidae) and *Oryzaephilus surinamensis* (L.) (Silvanidae) (Table 1). Yellow sticky traps also had high captures of coleopteran species.

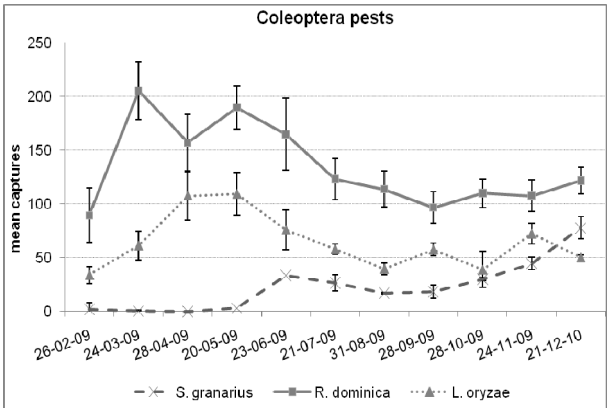

Figure 2 Temporal distribution of Coleoptera pests in one year of sampling with grain trier.

During the sampling period, from February to December 2009, the main coleopteran species captured were relatively abundant (Fig. 2). *Rhyzopertha dominica* had two small peaks in March and May, with its population decreased after that and maintained a relatively constant level. For *L. oryzae*, there was an increase from February to April-May, after that the population decreased until August and stayed fairly constant. *Sitophilus granarius* was, in comparison to the other two species, the last one to increased its population in the new pile. Its population did not increase until June, from where it maintained the levels of captures and slowly increased up to December.

Table 1 Pests and parasitoids captured, abundance levels: 1: abundant, 2: present, 3: occasional.

			Abundance /Trap		
Order	**Family**	**Species**	**Grain trier**	**Pitfall trap**	**Yellow trap**
Coleoptera	Curculionidae	*Sitophilus granarius*	1	1	2
	Bostrichidae	*Rhyzopertha dominica*	1	1	1
	Tenebrionidae	*Latheticus oryzae*	1	1	1
	Laemophloeidae	*Cryptolestes ferrugineus*	3	1	3
	Silvanidae	*Oryzaephilus surinamensis*	3	3	-
	Anobiidae	*Lasioderma serricorne*	-	-	3
Hymenoptera	Pteromalidae	*Anisopteromalus calandrae*	2	1	1
	Bethylidae	*Cephalonomia waterstoni*	3	2	1
Pseudoscorpionida	Withiidae	*Withius piger*	1	1	3

Regarding the natural enemies, the main parasitoids found to infest the barley pile were *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae), *Cephalonomia waterstoni* Gahan (Hymenoptera: Bethylidae) and the predator *Withius piger* (Simon) (Pseudoscorpionida: Withiidae) (Table 1). *Anisopteromalus calandrae* is a cosmopolitan parasitoid of coleoptera species that infest stored products (Schöller et al., 2006). Captures of this pteromalid were sporadic; its population had a very small increase in April (Fig. 3). *Cephalonomia waterstoni* is a bethylid parasitoid, very host-specific (Finlayson, 1950a, b), found to parasitize late-instar larvae of *Cryptolestes* species (Rilett, 1949). In our study, captures of this parasitoid happened just in October and November (Fig. 3). *Withius piger* is a natural predator found in rice in Spain (Pascual-Villalobos et al., 2005), but it is the first time to be found on barley in Spain. It was relatively abundant from July to September (Fig. 3). Among all the levels sampled a total of 38 *A. calandrae* were collected, of which three individuals were collected from the deepest sample. Six *C. waterstoni* were captured, only one reached 80-cm deep. Regarding *W. piger,* a total of 131 individuals was collected, and 10 were found in the deepest sample. Hence, there are natural occurring parasitoids present, and all the species sampled are able to penetrate deep in the pile searching for their hosts. Focusing on parasitoids getting captured on yellow traps, *A. calandrae* was abundant from April to August, while *C. waterstoni* was abundant from August to November. Populations seemed to complement each other, as when temperatures got colder *C. waterstoni* replaced *A. calandrae*.

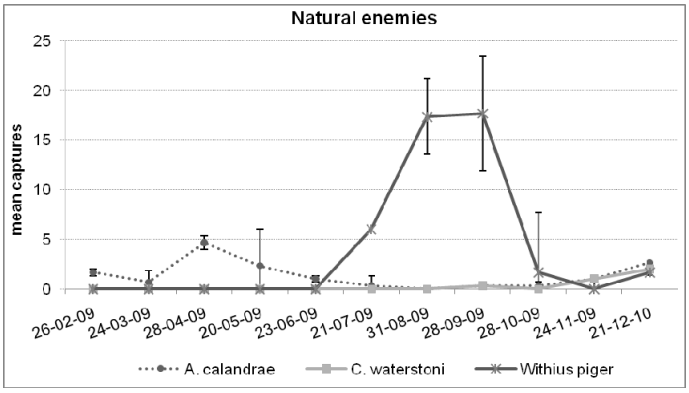

Figure 3 Temporal distribution of natural enemies in one year of sampling with grain trier.

For illustrating the vertical distribution of the main coleopteran pests captured, we present one month representing each season (Fig. 4). For the first month sampled (February 2009) the distribution of *R. dominica* remained in the lower levels of the grain pile, where it's warmer than in the surface and temperature variations are less extreme. *Latheticus oryzae* was more abundantly distributed along the upper levels, though it is a species usually present at high temperatures, as well as *R. dominica*. This might happen because the grain pile has just been placed in the warehouse, so the grain was probably too cool. Also the primary species might have not destroyed the grain at deeper levels enough to allow the entrance of a secondary pest. Other factors affecting the species' distribution was probably their

phototactism, mobility or population densities (Hafeez and Chapman, 1966; Buchelos and Athanassiou, 1999). Colonization of *S. granarius* in this first month was low (Fig. 4a).

In April, there was a change in the distribution of insects. The abundance of *R. dominica* increased, and it was also more abundantly distributed in the upper levels. In spring, air temperatures became warmer, and the grain was warming up as well. That may be why insects are found at top grain levels. For *L. oryzae,* captures were probably increasing due to the degradation of grain that *R. dominica* had done at every level and the mild temperatures (Fig. 4b). In July, pests were found at all depths, although there were fewer at the surface, possibly due to the high temperatures. Populations of *R. dominica* were lower, perhaps due to competition with an increasing population of *S. granarius* (Fig. 4c).

In October, more insects were found at the deeper levels, as air temperatures were getting colder. The grain temperature at the surface would be cooler, with the core of the pile being warmer and the insects would probably be attracted to the warmer grain (Flinn and Hagstrum, 1998). With lower temperatures, *S. granarius* got more abundantly distributed, reaching superficial levels, as it is more resistant to colder conditions (Fig. 4d).

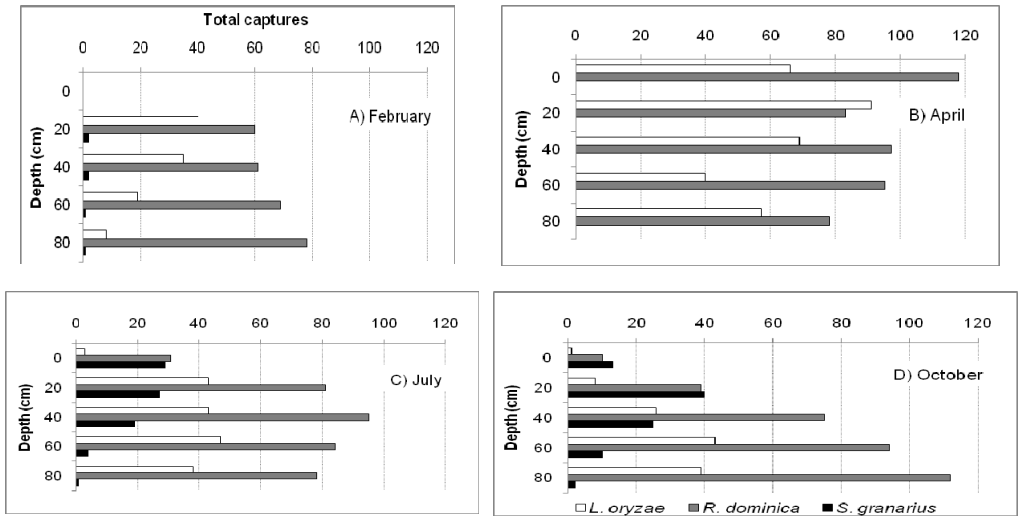

Figure 4 Vertical distribution of the total coleopteran pests captured in the barley pile, sampled with grain trier.

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Biological activity of essential oils of *Alpinia conchigera* rhizome against *Sitophilus zeamais* and *Tribolium castaneum*

Suthisut, D. *[2], Fields, P.G. #[1], Chandrapatya, A.[2]
[1] Cereal Research Centre, Agriculture & Agri-Food Canada, 195 Dafoe Road, Winnipeg, Manitoba, R3T 2M9, Canada, paul.fields@agr.gc.ca
[2] Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, 10900, dsuthisut@yahoo.com, chandrapatya@yahoo.com

* Corresponding author
# Presenting author

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Abstract

Research dealing with plant products is a new direction as an alternative to conventional insecticides for stored-product insect control (Shaaya et al., 1991, 1997). *Alpinia conchigera* Griffin (Zingiberaceae) is a native plant in southern Thailand, and it has a wide variety of the essential oils (Ibrahim et al., 2009). The toxicity and repellency of the water distilled essential oils from rhizome of *A. conchigera* was evaluated against the major stored-product insect pests, maize weevil, *Sitophilus zeamais* Motschulsky and red flour beetle, *Tribolium castaneum* (Herbst) 1-14 day-old adults at 29±2 °C and 65±5% r.h. In fumigation trials (Liu and Ho, 1999), the mortality was assessed at concentrations ranging from 74 to 667 µL/L in air with exposure times ranging from 3 to 24 h. There was complete mortality of *S. zeamais* at 222 µL/L after 24 h, whereas 593 µL/L for 24 h was required for complete mortality of *T. castaneum*. *Sitophilus zeamais* adults ($LC_{50,}$ fiducial limits: 121, 114-129 µL/L) were more susceptible to essential oils *of A. conchigera* than *T. castaneum* (295, 203-369 µL/L) (Table 1). Contact toxicity was assayed by topical application to insect thoraxes (Liu and Ho, 1999) at different concentrations (10 to 40%). *Sitophilus zeamais* adults ($LC_{50,}$ 27, 18-40 µg/mg) had the same mortality as *T. castaneum* ($LC_{50,}$ 34, 28-47 µg/mg) (Table 2). A filter paper choice bioassay (Ko et al., 2009) of essential oils of *A. conchigera* in 100% ethanol showed that *T. castaneum* has repelled more than *S. zeamais* (Table 3).

Keywords: Alpinia conchigera, Sitophilus zeamais, Tribolium castaneum, Essential oils, Toxicity

Table 1 Fumigation toxicity of essential oils from *Alpinia conchigera* rhizome against *Sitophilus zeamais* and *Tribolium castaneum* at 29 °C after 24 h.

Insect	LC_{50} (µL/L)	95% confidence Intervals (µL/L)	LC_{95} (µL/L)	95% confidence Intervals (µL/L)	Degrees of freedom	Chi-square
S. zeamais	121	113-128	180	168-196	8	0.395
T. castaneum	294	203-368	417	350-658	8	170.09

Table 2 Contact toxicity of *Alpinia conchigera* rhizome essential oils against *Sitophilus zeamais* and *Tribolium castaneum* at 29 °C after 24 h.

Insect	LC_{50} (µg/mg)	95% confidence Intervals (µg/mg)	LC_{95} (µg/mg)	95% confidence Intervals (µg/mg)	Degrees of freedom	Chi-square
S. zeamais	26	18-39	51	38-103	3	18.04
T. castaneum	34	28-46	60	47-101	3	9.33

Table 3 Percent repellency (PR) of *Alpinia conchigera* rhizome essential oils against *Sitophilus zeamais* and *Tribolium castaneum* using treated filter paper test*

Insect	Oil (µg/cm²)	PR (Mean% ± SD) Time after insect release (h)					
		1	2	3	4	5	PR (Mean%)
S. zeamais	0.16	32±59 **b**	36±59 **b**	56±38 **b**	68±41 **a**	60±47 **a**	50
	0.31	88±11 **a**	68±61 **ab**	76±26 **ab**	80±14 **a**	60±20 **a**	74
	0.47	96±9 **a**	96±9 **a**	88±18 **a**	72±30 **a**	72±33 **a**	85
	0.63	100±0 **a**	100±0 **a**	96±9 **a**	72±30 **a**	48±39 **a**	83
	0.79	100±0 **a**	96±9 **a**	96±9 **a**	80±45 **a**	60±14 **a**	86
T. castaneum	0.16	80±20 **a**	100±0 **a**	92±11 **a**	76±26 **b**	52±30 **b**	80
	0.31	72±18 **a**	80±25 **b**	92±11 **a**	80±14 **ab**	84±22 **a**	82
	0.47	84±17 **a**	96±9 **ab**	92±11 **a**	92±11 **ab**	100±0 **a**	93
	0.63	92±18 **a**	100±0 **a**	96±9 **a**	96±9 **ab**	80±28 **ab**	93
	0.79	96±9 **a**	96±9 **ab**	96±9 **a**	100±0 **a**	88±18 **a**	95

*Five replicates of 10 insects in each replication, for each insect, means in same column followed by the different letters are significantly (P>0.05) Duncan's multiple range test (DMRT).

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Mass trapping of *Ephestia kuehniella* Zeller in a traditional flour mill

Trematerra, P.*# , Gentile, P.
Department of Animal, Plant and Environmental Science, University of Molise, Via De Sanctis, 86100 Campobasso, Italy. E-mail: trema@unimol.it

* Corresponding author
# Presenting author

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Abstract

Results obtained by mass trapping method, using the synthetic pheromone (*Z*,*E*)-9,12-tetradecadienyl acetate (TDA), to control the population of *Ephestia kuehniella* Zeller in a large traditional flour mill are reported. The surveys were carried out over a period of five years. Forty-two funnel traps, each baited with 2 mg of TDA, were placed in the mill on March 2004 and kept until November 2008. Eight additional traps were located around the exterior of the facility, especially in the wheat silo area and near loading equipment. In almost five years, the pheromone traps attracted a total of 54,170 male *E. kuehniella*. Considering only the catch data obtained from the traps located in the internal departments of the mill, 28,360 specimens were captured. Outside the plant, 1,975 males were trapped. From the trap counts obtained it was possible to identify the locations of the main foci of infestation. With regard to the pest control attained by mass trapping techniques, trap catches of *E. kuehniella* inside the mill revealed a conspicuous decrease in the population density (of about 92.2%) comparing the data obtained in 2008 with that from 2004. The population density of the pest outside the mill also decreased from the first until the last year of the surveys. The infestation was maintained at a low level, especially during the last two years of the study, when the Integrated Pest Management program applied in the plant did not include general fumigations but only localized insecticide treatments and careful cleaning of the various departments (wheat storage bins, processing and packaging areas, milling products warehouses and the loading zone) and the interior of all equipment.

Keywords: Mediterranean flour moth, Mass trapping, IPM, Flour mill, Italy.

1. Introduction

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) is one of the major pests in European cereal warehouses and food processing industries. If not controlled in the flour mills its infestations, it can be so abundant as to clog the flow of products in equipment. Furthermore, the presence of larvae and webbing in the end product is unacceptable to consumers.

In Italy, before the Montreal Protocol that limited methyl bromide use, control of insect pests inside mills was typically realized by one or two general fumigations per year and several contact insecticide treatments, especially during the summer period (Trematerra and Gentile, 2006). Methyl bromide has usually been replaced by sulfuryl fluoride or, in only a small number of industrial facilities, by non-chemical alternatives, generally in combination with spot insecticide treatments. In this context, for the majority of flour mills the number of chemical treatments using contact insecticides (natural pyrethrins and synthetic pyrethroids but also organophosphates) has been increased, whereas preventive measures, such as good hygiene procedures, even if widely accepted as important in pest control, are often not adopted by mill managers.

Several studies have been carried out in the last two decades to find effective alternatives to methyl bromide and conventional chemical treatments or, in any case, of limiting their use. Among them have been investigation on the potential of pheromone-based methods (mass trapping, attract and kill or mating disruption techniques) to control indoor populations of *E. kuehniella* (Trematerra and Battaini, 1987; Trematerra, 1988, 1990, 1994a and 1994b; Süss et al., 1996 and 1999; Anderbrant et al., 2007; Ryne et al., 2007; Trematerra and Gentile, 2010). Considering the mass trapping method, Trematerra & Battaini (1987) demonstrated that integrated control of *E. kuehniella* can be achieved by this technique in limited environments. Furthermore, Trematerra (1988; 1990) reported results obtained in an entire large flour mill: the practical application of mass trapping to control the infestation of *E. kuehniella* led to a

reduction in chemical treatments, and as a consequence the mill obtained economic and qualitative advantages by protecting milling products from pesticide residues and improving the image of the firm.

In the present paper, the results of applying the mass trapping method to contain the Mediterranean flour moth infestation in a traditional flour mill are reported. Our researches focused on the effectiveness of mass trapping, combined with other pest control techniques, at improving the procedures applied to combat infestation by *E. kuehniella* in an Integrated Pest Management (IPM) approach.

2. Materials and methods

The surveys were carried out in a flour mill situated in Central Italy, over a period of almost 5 years, from March 2004 until November 2008. The plant is a building of 11,500 m3 with four floors, and it produces about 70 tons of flour per day from processing spring wheat or hard wheat.

Funnel traps (Mastrap type) with rubber dispensers baited with 2 mg of (Z, E)-9, 12-tetradecadienyl acetate (or TDA) (daily release of 13 µg) were used. The dispensers remained effective for about 2 months at which point they were replaced (traps and dispensers were supplied by Novapher, Italy). According with Trematerra and Battaini (1987), 42 traps were positioned in the mill, about one every 270 m3, placed 2 to 2.5 m above the floor and 3 to 3.5 m from the walls. Eight traps were located at the exterior of the mill, especially in the wheat silo area and near loading equipment, sectors that are frequently covered with grain, debris or dust. Trap captures were recorded weekly. Pipe joints were left open whenever processing was temporarily halted, i.e., during the holidays, so that the pheromone could act on moths inside machinery. On the occasion of structural fumigations and chemical treatments with contact insecticides, traps were removed and then reinstalled after 1 wk.

For every sampling date, visual inspections were carried out to observe the presence of E. kuehniella free adults, larvae, pupae or their traces, such as the larval silken webbing. These evidences, recorded as qualitative data, were reported every week to management personnel of the flour mill together with the number of the trapped moths. This was to assist them decisions making regarding measures to perform against any critical situation found. Moreover, these observations, in addition to the trap catch data, were also used in our study to evaluate the effect of mass trapping techniques and other IPM procedures applied in the mill.

3. Results and discussion

The environmental conditions found inside the flour mill (monthly mean temperatures between 15°C and 31°C from April to October, and between 8°C and 21°C from November to March) allowed the continuous development of the Mediterranean flour moth during eight to nine months every year, with approximately one generation every two months (Bell, 1975).

The captures obtained by the funnel traps positioned inside and outside the mill for every month throughout the five years of the trial are represented in Figure 1. In the entire survey period, pheromone traps attracted a total of 54,170 male Mediterranean flour moths. The insect pest was present in the mill on almost all sampling dates. The traps located in the internal departments of the mill trapped 28,360 specimens during 2004, 5,856 in 2005, 8,992 in 2006, 2,235 in 2007 and 2,218 in 2008. Outside the facility, 1,975 males were captured in 2004, 1,405 in 2005, 1,005 in 2006, 1,010 in 2007, and 1,114 in 2008.

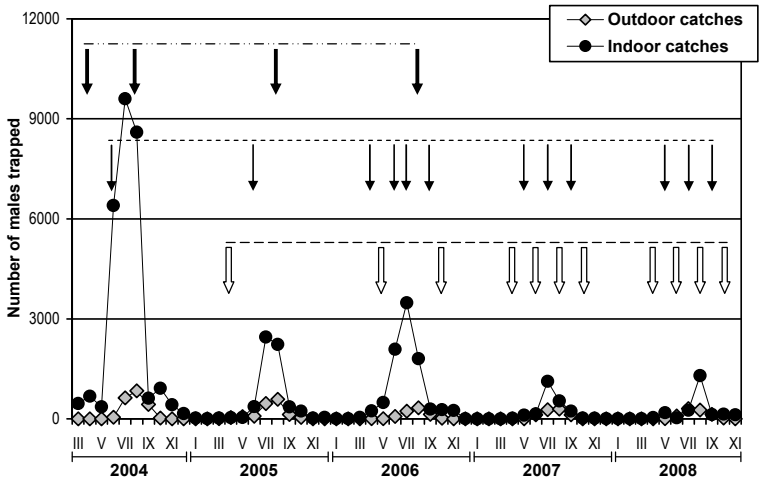

Figure 1 Cumulative monthly trap catches of *Ephestia kuehniella* males inside and outside the flour mill.

The main peaks occurred during the summer months, July or August, whereas the population remained at low levels from November until March, with few trap catches found. The increases recorded in the spring period, especially in May and June, are considered a consequence of emergence from the cocoon of the portion of the pest population that overwinters as mature larvae or pupae.

After structural fumigations with both methyl bromide (carried out twice in 2004: in April and in August, and once in August 2005) and sulfuryl fluoride (carried out in August 2006), trap catches were almost totally annulled for 2-3 weeks, however a rapid recolonization of the mill occurred, especially after of the spring fumigation of 2004.

The highest numbers of males were recorded in traps positioned near machinery with 'critical points', where fairly constant large amounts of food resources or favorable environmental conditions are to be found. High levels of infestation also occurred in rooms where silos of milling products (flour, semolina or bran) were located. Visual inspections confirmed this particular distribution of the pest. The position of the main infestation foci of *E. kuehniella* in critical areas could be due to various causes: microclimatic suitability, interaction with biotic factors, processing practices, presence of doors and windows and other physical attributes of a facility. The presence of the pest is a more frequent problem in all the departments where the possibility of finding flour, semolina, damaged grain, wheat debris or dust is regularly greater than that in other areas. Critical points such as the roll stands, the screen conveyors, the spouts, the plansichters, and the dust collectors, must be regularly and accurately inspected and cleaned. Other studies carried out in similar contexts indicate that there is a significant correlation between some of these factors and the spatial distribution of several insects in food processing facilities and in flour mills (Trematerra & Sciarretta, 2004; Trematerra and Gentile, 2006).

During the investigation, a higher presence of Mediterranean flour moth was observed on the ground floor compared to the other sectors of mill, especially in sampling dates following fumigations or contact insecticide chemical treatments. The assumption that the outdoor population might reinfest the flour mill seems to be well founded because in this sector of the plant, during the summer period, it is easier for insects to enter from outdoors and colonize the indoor departments. Investigations into the incidence of stored-product moths of the genera *Ephestia* and *Plodia* outside warehouses and food-processing factories were carried out at various locations (Wohlgemuth et al., 1987; Trematerra, 1988 and 1990; Süss et al., 1996; Doud and Phillips, 2000; Campbell and Mullen, 2004; Campbell, 2007). The results show that during summer adults fly in the outdoors near these types of structures. Several surveys suggest that a population of *E. kuehniella* outside storage facilities can potentially migrate inside.

Although *E. kuehniella* is primarily associated with stored foods and is not considered to be a pest in crop fields, the immediate areas outside warehouses or food processing factories can represent important

sources of infestation. Trematerra (1990) captured a great number of *E. kuehniella* males outside a flour mill in pheromone-baited traps. Trematerra (1990) and Süss et al. (1996) recorded rapid reinfestations by *E. kuehniella* in flour mills after fumigation with methyl bromide, and attributed these increases to immigration by the outdoor population. Campbell and Arbogast (2004) found similar results when assessing seasonal trends in *E. kuehniella* trap captures in a flour mill, the relationships between catch data inside and outside the plant, between the number of trapped moths and product infestation, and the impact of fumigation on the pest population.

In our case, immigration by outdoor adult specimens has to be a limited phenomenon, since infestation remained at low levels without further important increases. On the other hand, the exiguous number of *E. kuehniella* adults observed by means of visual inspections in the inner departments of the plant, and consequently, the low level of presence of free females, was in concordance with other mills controlled using a mass trapping method (Trematerra and Battaini, 1987; Trematerra, 1990). Indeed, the pheromonal substance present inside the structure could induce the Mediterranean flour moth females to leave the internal areas in favour of the outdoor zones, and the absence of males could also stimulate dispersal.

With regard to the pest control attained by mass trapping techniques, the trap catches of *E. kuehniella* inside the mill revealed a conspicuous decrease in the population density (of about 92.2%) comparing data obtained in 2008 with that from 2004. The population density of the pest outside the mill also decreased from the first until the last year of the surveys, even though this reduction was smaller being about 44.6%.

Further IPM strategies were employed in the flour mill during the hot seasons of the last three survey years. Mass trapping was accompanied by careful cleaning of the various departments (especially wheat storage bins, processing and packaging areas, milling product warehouses and loading zone) and of the equipment interiors (in May and October 2006; April, June, August and October 2007; April, June, August and October 2008). This was done in tandem with localized chemical treatments with contact insecticides of the critical sectors of the facility (in April, June, July and September 2006; May, July and September 2007; May, July and September 2008) (Figure 1). These chemical treatments consisted of spot surface spraying or space fogging of single infested rooms by means of synergized pyrethrum or, in areas with unusual problems, pyrethroids such as deltamethrin, permethrin or bioallethrin.

The mass-trapping method accompanied with other pest control procedures was able to remove so many *E. kuehniella* specimens as to ensure a low infestation level from the first year of the survey on. This prevented an increase in the residual population. It follows that the prolonged presence of funnel traps led to a drastic reduction of insect presence in the entire facility. As reported by Knipling and McGuire (1966), we can likewise assume that the effectiveness of mass trapping was such that about 85-90% of males were captured. The effectiveness of the IPM program carried out in the mill during our research rendered unnecessary the second general fumigation in 2005 and 2006. Afterwards, in 2007 and 2008, when the IPM program applied in the plant included regular cleaning procedures and localized insecticide treatments, no fumigation treatment was carried out; there was no increase in pest problems. Conversely, the fumigation treatments did not appear to impact trap captures of *E. kuehniella* for a long time, probably because of high rates of immigration from the exterior.

Considering the mass trapping method alone, its effectiveness is above all conditioned by the density of the population present in a structure. In our case assuming that a highly efficient trapping system has been designed and an adequate trapping regime established, the problem of accurately assessing the effects of the mass trapping treatment as a component of pest control still remains. IPM, in the strictest sense, includes the establishment of thresholds, at least at the level of economic injury. This is difficult to determine in any environment where it is neither possible to measure the exact size of the pest population nor to quantify the economic damage caused by a specific actual population size. In particular, for the Mediterranean flour moth, an independent measurement of population density is still lacking (Ryne et al., 2006). Furthermore the risks of attack by this pest are often underestimated (Süss et al., 1996; Campbell et al., 2002). This means that IPM strategies in flour mills, to a much higher degree than in other processing food plants or stored product areas, may be dependent on a strategy which includes a number of preventive elements.

The impressive reduction in the population density of *E. kuehniella* obtained in our surveys raises the question of whether "insectistasis" (Levinson and Levinson, 1985) can be obtained in a flour mill by mass trapping alone. Extrapolation of data recorded suggests that use of pheromone traps in a traditional mill for a longer time should dilute the population density of the Mediterranean flour moth even further. However, it was not possible to eliminate infestation, or even reduce the level of "insectistasis", if trapping was not accompanied by insecticide treatment and general cleaning of the mill, particularly in the corners and inside the machinery where the insects can hide and reproduce undisturbed. If such measures are not observed, the mass trapping will, at best, only reduce the number of insecticidal treatments.

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Comparing flower nectar and artificial diet on the longevity and progeny production of *Trichogramma turkestanica*

Tuncbilek, A.S*#, Cinar, N., Canpolat, U.
Erciyes University, Faculty of Arts & Sciences, Dept. of Biology, 38039 Kayseri, Turkey.
Email: tunca@erciye.edu.tr

* Corresponding author
# Presenting author

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Abstract

The efficacy of natural enemies as biological control agents is sometimes limited by phenological asynchrony with their host or prey populations or by climatic intolerance during portions of the season. In some circumstances, such limitations can be overcome by augmentative releases of insectary-reared natural enemies. Nectar and honeydew usually function as fuel for adult metabolism or as complementary food rather than as complete diets.

To test the capacity of *T. turkestanica* individuals to parasitize when reared on different food sources, a recently emerged (0-24h) *T. turkestanica* females introduced and held for 24 h. In order to test palatability of nectar from flowers, we offered different floral nectars (dead nettle, willow, dog-fennel, plum, dandelion,) in glass tubes. Control tubes contained water only and this experiment was repeated on artificial diets (honey, grape molasses, raisins which wetted with water before offering to parasitoid, beet molasses, glucose%10 and sucrose%10 syrups, egg yolk + honey (1:1, w/w) and egg yolk+honey+water (1:2:1, w/w). Flowers were collected daily and spread on white paper to check insects under a lamp and then offered to the parasitoid. These flowers were offered simultaneously to a single female of *T. turkestanica* for 24 h in glass vials (together with an egg card).

The mean fecundity or parasitism (offspring of both sexes), directly observed, was between 15,5 and 30,9 per female; the values diet 12 (Sucrose) was significantly different from diet 5, 9, and 15. All of the diets had nutritional qualities that allowed complete development of the parasitoid, indicating that there is a potential for rearing this insect on artificial and floral nectars. Adult emergence was greater on the diet 12 (99,5%), it was significantly different from diet 9 and 15.

Female emergence differed significantly among diets, especially diet 12 which significantly differ from diet 5 and 15. The greatest female emergence was found on diet 12 and 14 with 87.8 and 86.8% female emergence. Male emergence did not differ significantly among diets. It was greater on diet 5 (without food) (46.6%). The number and sex ratio of progeny emerging from hosts parasitized by either fed or unfed females did differ significantly.

The longevity of males and females of *T. turkestanica* was influenced by the diet used. Females lived longest when provided honey, live adults were evident for 14 days, but, shortest when provided only water; all adults of *T. turkestanica* eclosed from host eggs died within 4 d. Therefore, we conclude that all diets are the most adequate for rearing *T. turkestanica*, except diet 5, 9 and 15, based on the biological parameters of parasitization, adult emergence, and female longevity.

Keywords: *Trichogramma turkestanica*, Flower nectar, Artificial diet, Parasitization, Insect nutrition longevity, Fecundity.

Suppression of *Sitophilus zeamais* Motschulsky by the ectoparasitoid, *Anisopteromalus calandrae* (Howard)

Visarathanonth, P., Kengkanpanich, R., Uraichuen, J.*#, Thongpan, J.
Postharvest and Product Processing Research and Development Office, Department of Agriculture, Bangkok 10900, Thailand. E-mail: waew29@hotmail.com

* Corresponding author
# Presenting author

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Abstract

The use of natural enemies to control insect pests has been developed to reduce using insecticides for safety of human, animals and the environment. Most of the biological control practices are aimed for control of insect pests in the field and there is very little work done for post-harvest sector. *Anisopteromalus calandrae* (Howard) is an ectoparasitic wasp that is found throughout Thailand. The goal of this study is to describe the biology of *Anisopteromalus calandrae* and determine if it can control stored rice insect pests. An experiment on biology of this parasitoid wasp was tested for the wasp progenies in the *Sitophilus zeamais* when fed with milled rice at 32.5°C and 70% r.h. The egg laying stage observed is 1 d, 4.1±0.7 d for larvae, 6.3±0.9 d for pupae and 9.6±1.0 d. for adults. The life span from eggs to adults was 11.4 d. Female parasitoid wasps laid on average 37±14 females and 42±14 males for a total of 79±13 insects. The duration for egg laying is 11 d with the peak 12±5 insects on the 5th day. The parasitoid wasp sex ratio for female to male was 0.88: 1. Maize weevil larva at 21 d old reared on brown rice at 21d gave the highest yield of wasps (65±17). The number of the wasps produced was reduced to 57±17, 56±21 and 40±22 when raised with the weevil larva ages, 19, 23, and 25 d, respectively. The weevil larva age at 21 days was the most economic stage for the parasitoid wasp mass rearing. To obtain 1,124±236 wasps will use 220 g brown rice after 33 d rearing. The efficacies of the reared parasitoid wasps on controlling the *S. zeamais* was conducted by releasing 1,000 and 800 wasps into 25 kg rice at Rachaburi Rice Experiment Station yielded good control of the *S. zeamais* and with good rice quality. The rice without the parasitoid was heavily damaged and it had poor quality.

Keywords: *Sitophilus zeamais*, Ectoparasitoid, *Anisopteromalus calandrae*, Life cycle, Control efficacy

1. Introduction

Rice is an economic important cash crop for Thailand, both for exporting and for local consumption. Milled rice in storage is often faced with stored product insect infestation. The maize weevil (*Sitophilus zeamais* Motschulsky) is a common insect that infests rice in Thailand (Sukprakarn, 1985; Visarathanonth and Sukprakarn, 1988). Natural enemies have been extensively studied to control the stored product insect pests. The parasitoid wasp (*Anisopteromalus calandrae*) is a natural enemy of many stored product insects and it has been reared commercially in many countries (Perez-Mendoza et al., 1999; Haines, 1991; Hayashi et al., 2004; Subramanyam and Hagstrum, 1995). Amed (1996) reported the life cycle of this parasitoid wasp on lesser grain borer (*Rhyzopertha dominica* (F.)) fed with wheat kernels. This parasitoid wasp is widely distributed in stored products in Thailand (Konishi et al., 2004), so there is potential for its populations to be artificially reared and released to parasitize and control the stored rice insect pests in warehouses.

The goal of this study was to determine if the parasitoid wasp (*A. calandrae*) can be used to control *S. zeamais*, a major stored pest of the rice grains in Thailand.

2. Materials and methods

2.1. Biology of parasitoid wasps

A pair of parasitoid wasps, *Anisopteromalus calandrae* (male and female) was reared with 21-d-old larvae of *S. zeamais* previously fed with 50 grains of milled rice in each plastic box at 32.5°C and 70% r.h. The wasps were released into new boxes of *S. zeamais* larvae after every other day until the wasps died. The new generations or progenies of the parasitoid wasps from each plastic box were checked for total ratios of the resulting male and female insects.

2.2. Effect host age on wasp production

One hundred *S. zeamais* were reared with 50 g of milled rice in each plastic box at 32°C and 70% r.h. for 1 d before removal all of the weevils. The infested rice in all plastic boxes was separately kept for 19, 21, 23, and 25 days before five pairs of wasps were released in each treatment. The wasp progeny were checked for male and female insect ratios.

2.3. Mass rearing of wasps

Three hundred *S. zeamais* were reared with 200 g of brown rice for 7 d, then all adults were removed and the rice stored for another 21 d at 31 - 35°C. Fifty pairs of the wasps were then released into the *S. zeamais* infested rice. After 14 -15 d, the number of emerged wasps was counted.

2.4. Large scale trial

Five hundred 2-3 wk old *S. zeamais* adults were released into 25 kg of rice in jute bags placed in plastic boxes with lids. The *S. zeamais* were left to lay eggs for 7 d, and then all adults were removed. Rice was left for another 21 d, allowing the *S. zeamais* to grow to the ideal larval stage for wasp reproduction. Five hundred *A. calandrae* were released into the *S. zeamais* infested rice and left for 14 days before removal all of the wasps. Three doses of the wasps were released; 1,000, 800, and 0 wasps/box. Each treatment had 4 replications. The wasps, at the various levels, were released into the rice every month for one year. At the same time at each month wasp released, a 250 g sample was taken from the 25-kg rice bulk and the number of *S. zeamais* adults counted.

Another set of experiments was conducted at the same time and inside the 25-kg rice bulks. Each month, twelve bottles with 200 g rice infested *S. zeamais* infested were prepared. One bottle was placed in each of the boxes within the 25 kg of rice. No additional parasites were added to these bottles. Bottles had lids that allowed the parasites to enter and leave at will but prevented the *S. zeamais* adults from entering or leaving the bottles. The bottles were replaced each month at the same time of 250-g rice samples were taken from the 25-kg rice bulk. The rice was shaken and the number of adult *S. zeamais* was counted.

The experiments were conducted at the Stored Product Insect Research Lab, Postharvest and Product Processing Research and Development Office, Department of Agriculture, Bangkok and the Rachaburi Rice Experiment Station in Rachaburi Province, Thailand, during October 2003 – September 2005.

3. Results and discussion

3.1. Biology of parasitoid wasps

The life cycle study of parasitoid wasp (*Anisopteromalus calandrae*) was studied by rearing this insect with 21-d-old- *S. zeamais* larvae previously fed with milled rice at 32.5°C and 70% r.h. The result indicated that *A. calandrae* egg, larva, pupa, and adult periods are 1, 4.1, 6.3, and 9.6 d, respectively (Table 1) for a total time from egg to adult of 11 d.

Table 1 The duration of eggs, larvae, pupae, and adults of parasitoid wasps (*Anisopteromalus calandrae*) reared with 21-d-old *Sitophilus zeamais* fed with milled rice at 32.5°C and 70% r.h., n=20.

Insect stage	Duration Mean ± SD (d)	Range (d)
Egg	1.0 ± 0	1-1
Larva	4.1 ± 0.7	3-5
Pupa	6.3 ± 0.9	5-8
Adult	9.6 ± 1.0	7-11

The *A. calandrae* egg incubation periods on fully grown larvae of *R. dominica* are 36 h at 26°C and 27 h at 30°C (Amed, 1996).The larval stage, pre-pupal, and pupa lasted 6.9 and 5.4, 23.6 and 17.8, 5.4 and 4.6 d at 26 and 30°C (Amed,1996), respectively. The total time from egg to adult lasted 18.9 days at 26°C and 14.6 days at 30°C. However, the life cycle from the eggs to adults of this parasitoid wasp on the *S. zeamais* in this study was approximately 11 d, this shorter time is likely due to the slightly higher temperature, 32.5°C, used in our experiments.

The duration for female parasitoid wasps (*A. calandrae*) to lay eggs was 11 d with 7.7 eggs at the first day and peaked at 12.4 eggs on the fifth day. The wasp progenies were then reduced afterward to the11th

day on the last day of insect died. One female *A. calandrae* could lay 90.4 insects, 43.9 females and 46.5 males (Table 2). The sex ratio of female to male is 0.88:1. Amed (1996) has conducted a similar study to this study with *R. dominica* and could obtain 150 wasps at 26°C and 133 at 30°C with the insect ratio 2.1 and 2.3 at 26°C and 30°C, respectively. The wheat- *R. dominica* combination produced more wasps than our system using rice and *S. zeamais*. However, wheat costs more than rice in Thailand, therefore more work is needed to find the most cost effective production that still produces high numbers and high quality wasps.

Table 2 Numbers of parasitoid wasp (*Anisopteromalus. calandrae*) progeny per female, n=20.

Age (d)	Number of female progeny (Mean ± SD)	Number of male progeny (Mean ± SD)	Total wasps (Mean ± SD)
1	2.1 ± 2.2	5.6 ± 2.2	7.7 ± 3.1
2	2.4 ± 1.5	6.9 ± 2.2	9.2 ± 2.1
3	3.8 ± 3.3	7.1 ± 3.7	10.9 ± 4.4
4	5.1± 3.2	5.8 ± 3.5	10.8 ± 3.7
5	6.5 ± 4.3	5.9 ± 3.7	12.4 ± 5.0
6	5.6 ± 4.6	4.2 ± 4.6	9.8 ± 4.5
7	4.5 ± 2.6	2.5 ± 2.5	7.0 ± 2.4
8	3.4 ± 3.0	2.2 ± 2.6	5.5 ± 3.0
9	3.0 ± 2.2	1.6 ± 1.8	4.5 ± 2.3
10	2.8 ± 2.4	2.8 ± 2.6	5.5 ± 4.2
11	4.7 ± 3.3	1.9 ± 2.7	5.6 ± 2.5
Total	43.9 ± 32.6	46.5 ± 32.1	90.4 ± 64.7

3.2. Effect host age on wasp production

The progeny of wasps when raised on the *S. zeamais* previously fed with brown rice from all of the treatments were shown to have higher female ratios than male. This could benefit on using the female wasps for increasing new insect progenies. The 21-d-old larvae *S. zeamais* yielded 65.4 wasps, the highest number of wasp progenies for all ages of the host (Table 3).

Table 3 Effects of *Sitophilus zeamais* age on the progeny production of the parasitoid wasps (*Anisopteromalus calandrae*), n=20.

S. zeamais **age (day)**	**Number of parasitoid wasp progeny**			
	Female (mean ±SD)	**Male (mean ±SD)**	**Total**	**Sex ratio (Female: Male)**
19	32.1 ± 12.4	24.7 ± 8.9	56.8 ± 21.3	1.3
21	43.1 ± 14.3	22.3 ± 7.4	65.4 ± 21.7	1.9
23	31.3 ± 13.3	24.3 ± 11.0	55.6 ± 24.3	1.3
25	26.2 ± 17.2	13.9 ± 6.8	40.1 ± 24.0	1.9

3.3. Mass rearing of wasps

The 21-d-old *S. zeamais* larvae reared on brown rice yielded on average 1,124 wasps with a minimum of 708 wasps and a maximum of 1543 wasps per 200 g brown rice in the plastic boxes.

3.4. Large scale trial

After 6 mo to one year 1000 wasps/25 kg rice reduced *S. zeamais* adults in large rice bulks more than releasing 800 wasps/25 kg, while the treatment without any wasps had the highest infestation of the *S. zeamais*. At the end of one year, the number of *S. zeamais* adults was 3, 6 and 111 weevils/250 g when treated with 1000, 800, and 0 parasitoid wasps, respectively (Fig.1). The weevil infested rice in the bottles at the same period of storage time yielded 24, 26, and 1465 insects when treated with 1000, 800, and 0 parasitoid wasps, respectively (Fig. 2).

Our results show that the parasitoid wasp, *A. calandrae* gave good control of *S. zeamais* in both large and small sizes of the rice samples in this study. The quality of the parasitoid wasps released rice remained

good with minor weevil infestations in a few replications. The results indicate that it may be possible to replace chemical insecticides for control of *S. zeamais* and to apply in the integrated control programs in the near future. The use of this biological control agent could reduce insecticide residues on rice, require fewer fumigants of rice in warehouses thereby improving worker safety. It could also reduce the development of insecticide resistance developing in insect populations and protect the other beneficial insects found in grain storages for both better stored product quality and quantity.

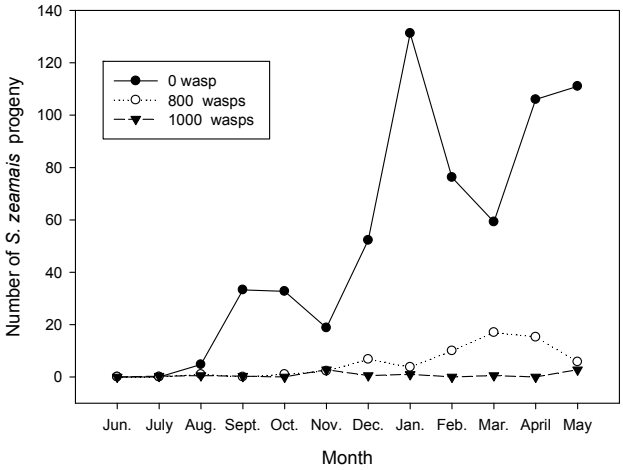

Figure 1 Monthy numbers of *Sitophilus zeamais* adults in 250 g rice samples taken from 25-kg rice sacs at three densities of the parasitoid, *Anisopteromalus calandrae.*

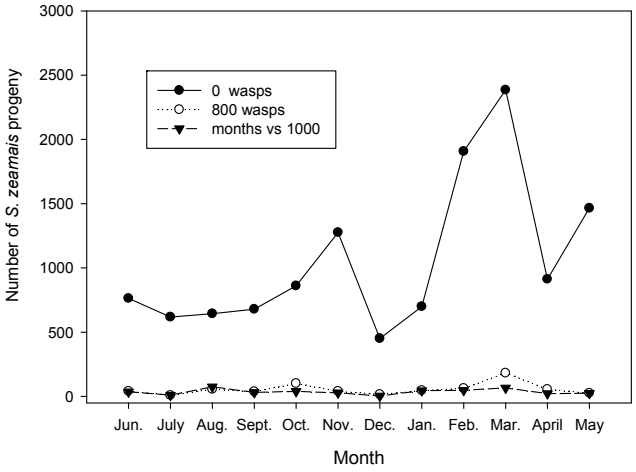

Figure 2 Monthy numbers of *Sitophilus zeamais* adults in 200 g rice samples taken bottles placed in a 25-kg rice sacs with three densities of the parasitoid, *Anisopteromalus calandrae.*

Acknowledgements

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Progress in the development of a biopesticide for the structural treatment of grain stores

Wakefield, M.E.[1]*#, Moore, D.[2], Luke, B.[2], Taylor, B.[2], Storm, C.G.[3], Collins, D.A.[1], Grammare, P.[4], Potin, O.[4]
[1] The Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ.
Email: maureen.wakefield@fera.gsi.gov.uk
[2] CABI Europe-UK, Bakeham Lane, Egham, Surrey, TW20 9TY
3 Exosect Ltd, Leylands Business Park, Colden Common, Winchester, SO21 1TH
4 Somycel-SylvanBio, Route de Mauvières, Zi Tivoli, 37600 Loches, France

* Corresponding author
# Presenting author

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Abstract

Chemical insecticides are used to protect stored grain from infestation by stored product insects and mites. In the UK only a limited number of products are available for application and there are concerns about safety, pest resistance and environmental impact of these conventional pesticides. Biological control offers an alternative to the use of chemical insecticides. The potential for biological control of storage pests in the UK using an insect-specific fungus, *Beauveria bassiana*, to treat the structure of the stores, has previously been established. However, this study also highlighted areas where improvements were needed; specifically to improve the uptake of the fungal conidia by the pests and to improve their germination and penetration into the pests. In addition it was necessary to ensure that potential formulations had a good shelf-life and to develop a mass production method to consistently produce high quality fungal conidia. A four year project has recently been completed examining these areas in detail. The work has concentrated on two different fungal isolates of *B. bassiana*, both of which were found from insects in UK grain stores. Optimisation of production methods, formulation and delivery systems has resulted in prototype formulations that exhibit good viability over periods up to one year and that have good efficacy against a range of storage insect pests under conditions that are likely to be found in UK grain stores. Pilot scale trials using three species of stored product beetle have shown that significant levels of control can be achieved. An overview of the key findings is presented. The study has made a significant contribution to the development of a biopesticide as a structural treatment for grain storage areas in the UK.

Keywords: Biological control; *Beauveria bassiana*; *Oryzaephilus surinamensis*; Structural treatment; Biopesticide

1. Introduction

Cereals are an important component in the human diet and are similarly important in livestock feedstuffs. Cereals, whilst in storage, are at risk of infestation by insects, resulting in quality deterioration and losses. Pesticides are commonly used for control of stored product insects; in 2002 over 9 tonnes of active ingredients were used as fabric treatments to protect the harvest in Great Britain (Dawson et al., 2004a, b). Concerns about the safety of some insecticides, in particular organophosphates, have resulted in proposed changes to EU legislation, which would result in removal of some currently approved pesticide products (Pesticides Safety Directorate, 2008). Concerns have also arisen with regard to insect resistance to commonly used products. Resistance has been reported for all insecticide classes for one of more key pest species (Whalon et al., 2008), including stored product insects. With an increasing emphasis on food security, alternative approaches for control of stored product insects are needed.

Biological control of storage pests is one such novel approach. While bacteria, fungi, protozoa and viruses all have potential as natural microbial control agents (biopesticides), it is the insect-specific (entomopathogenic) fungi such as *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin for which most is currently known and that are the best candidates. Research to investigate the potential of entomopathogenic fungi for control of stored product insects has increased over the past decade, with studies examining the potential of both *M. anisopliae and B. bassiana* on a range of species. The majority of these studies have examined the efficacy when the fungus is applied to grain (Lord, 2001; 2005; 2007a, b; Akbar et al., 2004; Vassilakos et al., 2006; Michalaki et al., 2006; Kavallieratos et al., 2006; Cherry et al., 2007; Hansen and Steenberg, 2007; Athanassiou et al., 2008).

The potential of *B. bassiana* as a structural treatment for UK stores was investigated by Cox et al. (2004) and, although 100% mortality of one or more species of stored product insect and mites was achieved in laboratory studies, it was concluded that it would be essential to improve the germination of the conidia on the insect pest and the uptake of the fungal conidia by the pests. In particular it had been found that high levels of insect mortality could only be achieved under conditions of high humidity and when insects were treated directly with the conidia (Cox et al., 2004). The current project therefore focussed on improving efficacy by overcoming these conditions. The studies were undertaken to achieve three objectives (1) to improve the efficacy of the entomopathogenic fungus when in contact with insects (2) to improve the delivery of the entomopathogenic fungus to the insects and (3) to demonstrate that prototype biopesticides formulations are effective under practical conditions. Initially several different isolates of *B. bassiana* that had been isolated from insects found in UK grain stores (Cox et al., 2004) were used. From these, two isolates, IMI 386243 and IMI 389521, were chosen for further studies, and a single isolate was used to demonstrate the potential of prototype formulations under practical UK conditions.

2. Materials and methods

2.1. Optimization of the conidia

The first part of this study aimed to improve the pathogenicity and viability of *B. bassiana* isolates by manipulating the conditions under which the conidia were mass produced. The effect of four different rice treatments on the production level of conidia was assessed with eight different fungal isolates, which had previously been shown to be pathogenic to the saw-toothed grain beetle, *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) (Cox et al., 2004). Conidia were produced using a two stage method, whereby inoculum were grown in a liquid broth initially and then inoculated onto sterile rice (Jenkins et al., 1998). Levels of water added to the substrate were varied, as it was hypothesised that conidia grown in conditions of water stress would be more viable at lower humidities. Isolates were tested for their ability to germinate at lower water availability levels on polyethylene glycol (PEG) adjusted agar. The method was a simulation to test the viability of conidia at lower levels of humidity. The effect of the different production methods on the pathogenicity of the conidia was assessed in bioassays using *O. surinamensis*.

2.2. Formulation studies

Formulation can play a key role in the efficacy of a fungal biopesticide as it may enhance the infectivity of the fungal conidia and allow a product to be stored over a prolonged period of time. The viability of conidia of isolates IMI 389521 and IMI 386243 in various formulating agents was examined. A range of formulations were considered including both liquid and dust formulations, various bulking agents and liquid emulsions. In addition the effect of the formulations on insect mortality was also assessed against *O. surinamensis* in laboratory bioassays.

2.3. Improving contact between insects and conidia

Optimising the efficacy of the conidia when in contact with insects is an important factor, but, to achieve adequate control, it is also essential that a sufficient number of conidia are brought quickly enough into contact with the target insects, which may be hiding in cracks and crevices. Improving the delivery of the conidia can be achieved in two ways 1) by moving the insects to the conidia and 2) by moving the conidia to the insects. Moving the insects to the conidia can be achieved in two ways; either a repellent could be used to treat cracks and crevices to remove insects from these locations in order to make contact with treated surfaces, or the insects can be attracted to areas where the conidia are present. The ability of a diatomaceous earth (DE) and pyrethrins to act as repellents and remove insects from refuges was examined by creating artificial crevices containing the test compound. To determine whether insects could be attracted to areas treated with the conidia, 'bait stations' either with or without dry conidia powder and the presence or absence of a lure, developed to attract several species of stored product beetle, were used.

The ability to improve contact by moving the conidia to the insects was also investigated by examining the uptake and behavioural responses of *O. surinamensis* to an electrostatically chargeable powder, Entostat™. This is a processed plant wax and has been identified as a potential carrier for active ingredients to be delivered to cracks and crevices in food facilities.

2.4. Pilot scale trial

In order to establish the effectiveness of the biopesticide under practical conditions a larger scale experiment was undertaken to ensure that efficacy was maintained for a reasonable period of time under the fluctuating environmental conditions that would typically be encountered in a UK grain store. The pilot scale trial examined the effect of two prototype formulations at two target concentrations on the mortality of three species of stored product beetle when applied to plywood arenas housed within a grain store. A comparison with a currently registered chemical pesticide, pirimiphos methyl, was also made.

3. Results

3.1. Optimization of the conidia

Generally fewer conidia were produced per gram of conidiated rice when the rice had received no prior treatment and therefore had the lowest moisture content. The other three treatments resulted in similar levels of conidia produced for each isolate. The viability of the conidia produced for each of the four treatment methods and the ability of the conidia to germinate at lower water activities (corresponding to lower relative humidities) was assessed for two of the isolates. Isolate IMI 389521 proved to have better viability than isolate IMI 386243, showing no significant decrease in germination over time. The production method did not affect the pathogenicity of the conidia at a low humidity; treatment with conidia produced by the four methods resulted in similar levels of insect mortality, which was very low. To ensure that the production methods had not diminished the ability of the isolates to cause insect mortality, conidia of isolate IMI 386243 produced by the four production methods were tested under conditions of high humidity for the first 24 hours. Good levels of mortality were achieved under these conditions; conidia produced under conditions with the greatest water stress had the higher level of pathogenicity (Table 1). This study demonstrated that the production method can have an affect on the level of mortality caused by the conidia.

Table 1 Mean % mortality (± S.E.) of *Oryzaephilus surinamenisis* 14 d after treatment with a solution containing $1x10^7$ conidia/mL^{-1} *Beauveria bassiana*. Conidia were produced using a method in which different quantities of water were added. The amount of water added is indicated by the figures 1-4, with 1 being the lowest and 4 the highest.

Water added	1	2	3	4
% mortality	74 ± 5	73 ± 4	44 ± 6	50 ± 3

3.2. Formulation studies

The formulation studies demonstrated that in an appropriate formulation, at 5°C, the isolates retained excellent germination over a period of 365 d. In general IMI 389521 performed better than IMI 386243 with higher initial germination and more reproducible results in experiments. At 25°C the experiments showed that, in general, good germination was retained after 301 d of storage in an appropriate formulation, with viability remaining above 70%. Water based formulations were not suitable for either isolate as viability was lost very rapidly at 25°C and less rapidly at 5°C.

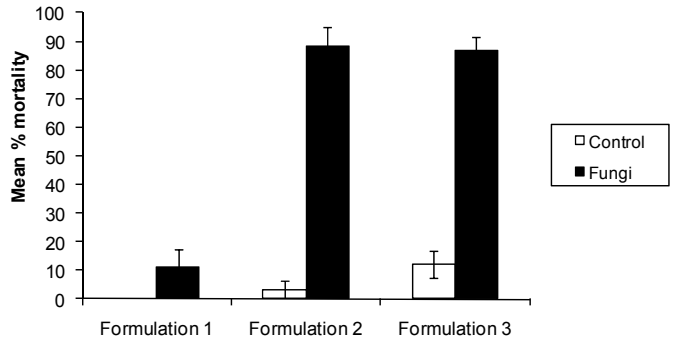

Figure 1 Mean (± S.E.) % mortality of *Oryzaephilus surinamensis* 14 d after treatment with *Beauveria bassiana* formulated in different carriers at a concentration of $1x10^9$ conidia/mL^{-1}.

The oil formulations resulted in greater mortality of *O. surinamensis* in comparison with the water-based formulation (Figure 1). The powder based formulations also showed potential. In conclusion, based on the viability and efficacy results, oil and powder based formulations look to be good candidates for future use as commercial mycoinsecticide formulations.

3.3. Improving contact between insects and conidia

The ability of DE and pyrethrins to remove insects from refuges was examined by creating artificial crevices containing the test compound. At the concentration tested the diatomaceous earth, Silico-sec, reduced the number of insects that were present in the refuge. This was noticeable 1 h after the insects were introduced to the arena (Table 2). Pybuthrin also reduced the number of insects present in the refuge, but not to the same extent as the diatomaceous earth (Table 2). The work has shown that, on a small scale it is possible to reduce the number of insects present in a refuge at a given time. However, the ability to achieve this at a larger scale remains to be determined and practical issues with regard to treatment of all potential refuges may preclude this as a practical measure to improve uptake by the insects.

Table 2 The mean percentage of *Oryzaephilus surinamensis* observed in arenas containing refuges with different treatments at various time points after introduction (n = 5).

Elapsed	Control			Silico-sec			Pybuthrin		
Time (h:m)	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.
00:15	89.4	98.0	76.0	79.0	92.0	72.0	87.4	94.0	84.0
00:30	85.6	96.0	80.0	86.4	90.0	82.0	84.6	90.0	76.0
00:45	82.2	94.0	72.0	84.0	92.0	78.0	84.2	88.0	78.0
01:00	80.2	94.0	74.0	81.0	88.0	74.0	81.0	86.0	70.0
01:30	59.8	80.0	28.0	74.4	82.0	60.0	59.8	68.0	38.0
02:00	42.4	56.0	16.0	61.2	78.0	44.0	49.4	68.0	34.0
02:30	39.8	56.0	18.0	61.6	70.0	56.0	46.2	62.0	32.0
03:00	36.2	50.0	14.0	60.6	66.0	52.0	49.0	66.0	34.0
04:00	33.6	46.0	18.0	53.8	68.0	40.0	45.2	58.0	34.0
05:00	35.2	54.0	20.0	54.0	64.0	46.0	43.8	62.0	28.0
24:00	21.2	32.0	14.0	54.4	64.0	36.0	36.6	52.0	20.0

There was a significant difference in the mortality of *O. surinamensis* between treatments with and without the conidia in the bait station; mortality was significantly higher in treatments with the conidia (Figure 2). Mortality in the treatment with the lure in the bait station with the conidia was significantly greater than for the conidia without the lure. This study has shown that insects will enter an area where the conidia are present and will pick up a lethal dose of the dry conidia powder. Bait stations containing an appropriate formulation of the fungal isolate therefore offer potential of targeted delivery of the conidia to the insects.

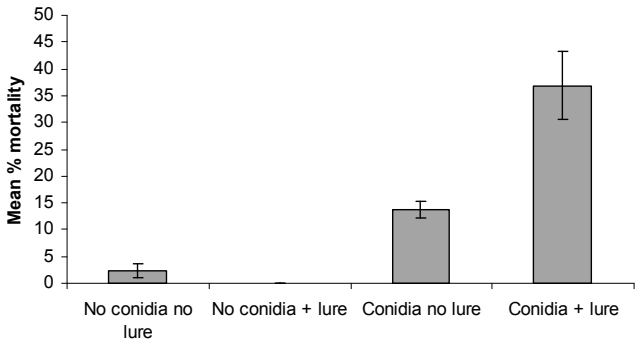

Figure 2 Mean (± S.E.) % mortality of *Oryzaephilus surinamensis* 14 d after exposure to different treatments in 'bait stations' (n = 10).

Entostat uptake and retention by *O. surinamensis* 24-72 h after exposure to rolled oats mixed with Entostat was quantified. SEM images showed that Entostat adhered to all body parts, including joints, between body segments, and at insertions of body hairs (Nansen et al., 2007). Choice experiments were used to determine whether *O. surinamensis* individuals were repelled by Entostat. The results suggest that considerable amounts of Entostat were taken up even when beetles were offered a choice between treated and untreated cracks (Nansen et al., 2007). The addition of Entostat therefore provided a means by which contact between the insect and the conidia could be improved.

3.4. Pilot scale trial

The chemical pesticide, Actellic (pirimiphos-methyl) when applied at the recommended concentration caused rapid death of all three species of insect. Large numbers of knock down or dead insects were observed within 2 h of the introduction of the insects to the treated surface and 100% mortality was recorded for insects recovered from the rings after 14 d. The biopesticide formulations also caused a significantly greater level of mortality (45-95% dependant on species, concentration and formulation) than was observed for the control treatments. The viability of the conidia on realistic surfaces, as determined by the % germination, remained high throughout the trial indicating that under the test conditions isolate IMI 389521 retained the potential to infect insects and may therefore have residual activity.

4. Discussion

During the course of the project significant progress was made; of particular note is that enhancement of the production and formulation of the conidia has negated a need for a period where the humidity needs to be close to 100% and that the conidia do not have to be directly applied to the insects to achieve good efficacy. In addition mass production methods resulting in consistent, high quality production of conidia with excellent viability and virulence have been determined and significant control of insect populations under practical conditions has been demonstrated.

This research has made a significant step towards the development of a biopesticide, based on *B. bassiana*, as a structural treatment in UK grain stores. Candidate formulations have been identified but further work will be needed to fully establish the most appropriate formulation. The mass production process has been optimised, but until the most appropriate formulation and dose rate have been established it will remain to be seen whether cost effective production can be realised. The project has made significant progress in the development of a novel structural treatment that would be a benefit to farmers and storekeepers.

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Study on the insecticidal activity compounds of the essential oil from *Syzygium aromaticum* against stored grain insect pests

Zeng, L.*#, Lao, C.Z., Cen, Y.J., Zeng, L., Liang, G.W.
[1] Guangdong Institute for Cereal Science Research, No.222 North Yuexiu Road, Guangzhou, Guangdong, 510050, P. R. China. Email: zengl@tom.com
[2] Laboratory of Insect Ecology, South China Agricultural University, Wusan Road, Guangzhou, Guangdong, 510640, P. R. China

* Corresponding author
# Presenting author

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Abstract

Insect pests are a major cause of damage in stored grain around the world. To control the stored grain insects, synthetic insecticides have been used extensively for many years, resulting in insect populations that are resistant to insecticides. Consequently there is an interest to find alternatives to chemical pesticides. The essential oil from *Syzygium aromaticum* (clove oil) has a number of bioactive compounds. The chemical constituents of the clove oil were analyzed by GC-MS, and 9 of 18 compounds were identified. The main compound (83%) was 2-methoxy-4-(2-propenyl)-phenol the second most common compound (12%) was trans-caryophyllene. These two pure compounds and clove oil were tested for toxicity and repellency against *Rhyzopertha dominica, Sitophilus oryzae and Tribolium castaneum*. The pure compounds were tested at the dosages found in clove oil. The mortality from 2-methoxy-4-(2-propenyl)-phenol was not significantly different from clove oil, suggesting that the activity of clove oil was solely due to this major compound. The repellency results were more complex. 2-methoxy-4-(2-propenyl)-phenol was more repellant than clove oil. Trans-caryophyllene was less toxic and less repellant than both clove oil and 2-methoxy-4-(2-propenyl)-phenol. The potential for these compounds to be used to control stored product insects is discussed.

Keywords: Essential oils, *Syzygium aromaticum*, Clove oil, Insecticidal activity compounds, Stored grain insects

1. Introduction

Rhyzopertha dominica (F.) (lesser grain borer), Sitophilus oryzae (L.) (rice weevil), *Tribolium castaneum* (Herbst) (red flour beetle) are cosmopolitan pests of grain. In China, these insects damage paddy, wheat, maize, potato and their processed products (Li, 2004). These pests not only cause damage in the warehouse, but also in rice processing factories. In southern China, they are active throughout the year.

At present, the major method to reduce damage caused by stored grain pests is chemical control (Liang, 1994; Yang, 2004; Collins, 2006). Chemical control has several advantages; fast acting, the low cost, controls most insect pests, but the "3R" (Resistance, Resurgence and Residue) problems have become more and more serious (Li, 1994; Hu, 2001).

Based on the current problems cause by the chemical pesticides, the environmentally friendly pesticides against stored products pests are being developed to replace chemical pesticides. Developing plant resources and extracting essential oils from plants as grain protectants is an area of considerable recent research (Cao, 2002; Hu, 2001; Hou,2001; Yan, 2007). Plant essential oils have several advantages; low mammalian toxicity, low resides on grain, and novel chemical structures. Given that essential oils are very different in chemical structure than the currently used stored-product insecticides, we do not expect the insects that have resistance to the commonly used insecticides to also be resistant to the essential oils. Therefore, essential oils fulfill the requirements of pesticides in the 21st century and they may be widely used to stored product pest control (Zhang, 2004).

The essential oil from *Syzygium aromaticum* (clove oil) possesses many compounds with biological activity, and it is used to control insects, fungus, mildews in stored grains (Kong, 2004; Han, 2006; Lou, 2006; Shang, 2007). There are several compounds in clove oil, but the specific activities of these various compounds against stored-product pests has never been examined. The objective of this study was to examine the insecticidal activity of compounds from the clove oil against the major stored grain insect pests.

2. Materials and methods

2.1. Culturing insects

Insects were cultured at 25° ± 1°C and r.h. 70%-80%. *Rhyzopertha dominica* and *S. oryzae* were cultured on wheat, and *T. castaneum* was reared on a mixture of crushed wheat, oatmeal, yeast in the ratio of 3:3:1. After 7 d, the adults were removed. All of the experimental insects were the newly emerged adults 1 to 14 d old. The wheat grain was from China Grain Reserves Corporation, oatmeal and yeast were purchased from the market. In order to kill all insects, the wheat was heated to 60°C for 2-3 h before use and moisturized after heating until the content of water was about 14%. The paddy used for experiments was the Five-star Mew Rice with water content of about 13%. It was produced in Xinhui city and Lianshan County, Guangdong Province.

2.2. Essential oils

The clove oil was provided by Guangzhou Gaoshangmei Fine Chemical Co., Ltd. The compounds from clove essential oil used for tests were shown in Table 1.

Table1 The compounds from essential oils used for test.

No.	Compounds	Purity (%)	Formula	CAS number	Relative content in essential oil (%)
D5	2-methoxy-4-(2-propenyl)- phenol	99.0	C10H12O2	97-53-0	83.13
D6	trans-caryophyllene	98.5	C15H24	87-44-5	12.42

* The compounds were provided by Happy & Excited Guangzhou Biotech Ltd. Co.

2.3. GC-MS analysis

The compounds from essential oils were analyzed by Finnigan TRACE Gas Chromatography - Mass Spectrometry. The oils sample was diluted 10 fold with ethanol, and then 0.3 μL of that was taken to inject in GC.

Conditions: DB-1 column: 30m × 0.25mm; Ionization mode EI: 70 eV; Mass range: 35-395 amu; Operating temperatures: maintain 60°C for 1 min, then heat to 90°C at 100C/min, to 150°C at 5°C/min, to 300°C at 10°C/min. and at the end, maintain 3000C for 5 min.

2.4. Repellent activity

The repellent activity was examined by insecticide-impregnated filter paper, following the methods described by Jilani (1990). A filter paper of 9 cm in diameter was cut in half. One half was dipped in the acetone diluted solution containing essential oils, the other half as a control was dipped in acetone solution. After the acetone evaporated form both two halves, they were fixed together again by a transparent plastic at the bottom. A stainless steel ring of 9 cm in diameter was placed on the filter paper. The wall of the ring was wiped by a layer of Teflon to prevent escape. Thirty adults of the pests were put into one ring. Five replications were used for each concentration. The distribution of insects on the two halves of the filter paper was examined at 12, 24, 36, 48, 60 and 72 h after treatment. Percentage repellency was calculated as follows.

$$PR(\%)=(Nc - Nt)/ Nc\times 100\% \qquad (1.1)$$

PR=Percentage repellency, %;

Nc=Average number of insects on the untreated area after the 6 exposure intervals;

Nt=Average number of insects on the treated area after the 6 exposure intervals;

2.5. Toxicity

The essential oil was dissolved with acetone in order to get different concentrations of solutions. Respectively, the solutions and a certain amount of paddy were well-mixed. After the volatilization of acetone, 50 g paddy and 30 adult insects were put into a jar of 250 mL. Each treatment had five replications and the control was treated with acetone. The mortality of insects was examined 3, 7, 10 and 14 d after treatment. The morality and corrected mortality was calculated as follows.

$$M\ (\%) = Nd / Nt \times 100\% \quad (1.2)$$

$$CM\ (\%) = ((Mt - Mc)/(1 - Mc)) \times 100\% \quad (1.3)$$

M=Mortality, %;
CM=Corrected mortality, %;
Nd=Number of dead pests;
Nt=Number of pests used for test;
Mt=Mortality of treatment, %;
Mc=Mortality of control, %.

3. Results

3.1. Component analysis of the essential oil from S. aromaticum

GC-MS technology was used to analyze the components of the essential oil from S. aromaticum (Fig. 1). Eighteen components were detected (Fig. 1, Table 2). Nine main components (SI> 800) were identified, of which the peak area was 98.68% of the total ion peak area. The most important component was 2-methoxy-4-(2-propenyl)-phenol (83.13%), followed by trans-caryophyllene (12.42%). The proportions of the other 7 components were all above 1%. The content of a-caryophyllene (SI> 800) was about 1.69%.

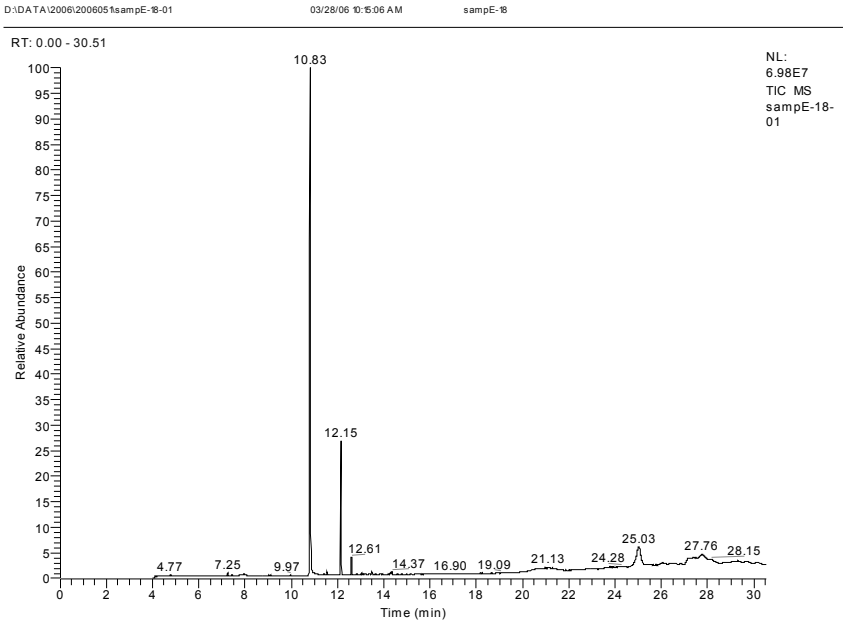

Figure 1 Total ion current chromatogram of the essential oil from *S. aromaticum*

Table 2 Components of the essential oil from *S. aromaticum* detected by GC-MS.

Peak no.	Retention time (min)	Compound	Formula	Similarity	Relative content in essential oil (%)
1	7.25	(2S-trans)-5-methyl-2-(1-methylethyl)-cyclohexanone	C10H18O	908	0.29
2	7.44	(2S-trans)-5-methyl-2-(1-methylethyl)-cyclohexanone	C10H18O	807	0.07
3	7.55	menthofuran	C10H14O	711	0.03
4	7.74	L-(-)-menthol	C10H20O	830	0.11
5	7.83	L-(-)-menthol	C10H20O	799	0.18
6	7.96	2-hydroxy-benzoic acid, methyl ester	C8H8O3	741	0.25
7	9.02	chavicol	C9H10O	793	0.07
8	9.11	2h-1-benzopyran	C9H8O	702	0.06
9	10.83	2-methoxy-4-(2-propenyl)- phenol	C10H12O2	920	83.13
10	11.13	2-methoxy-4-(2-propenyl)- phenol	C10H12O2	709	0.23
11	11.43	1,2-dimethoxy-4-(2-propenyl)- benzene	C11H14O2	699	0.18
12	11.54	cedrene	C15H24	814	0.34
13	12.15	trans-caryophyllene	C15H24	943	12.42
14	12.61	a-caryophyllene	C15H24	902	1.69
15	13.04	2-methoxy-4-(2-propenyl)-phenol	C10H12O2	702	0.16
16	13.48	tau-cadinol	C15H26O	803	0.31
17	14.27	25-desacetoxy-bccurbitacin b	C30H44O6	727	0.16
18	14.37	caryophyllene oxide	C15H24O	841	0.32

3.2. Repellency

We tested the repellency and toxicity of the essential oil of S. aromaticum and its two main chemical components: 2-methoxy-4-(2-propenyl)-phenol (Code: D5) and trans-caryophyllene (Code: D6). The essential oil, D5 and D6 were repellent *R. dominica*, *S. oryzae* and *T. castaneum* (Table 3). D5 had highest repellency grade on all 3 tested pest species. The mean repellency rates of D5 on R. dominica, S. oryzae and *T. castaneum* were 93, 71 and 97% respectively, which was significantly higher than that of D6 on all 3 pest species, and significantly higher than that of the clove oil on S. oryzae and *T. castaneum*. The percentage repellency of D6 on *R. dominica* was significantly lower than that of the clove oil, but significantly higher than the clove oil with *S. oryzae*. This suggests that D5 was the main ingredient with repellent activity.

Table 3 Repellency of the essential oil of *S. aromaticum* and its two main compounds against stored grain insect pests.

Insect	Essential oil and compound	Repellency + SE (%) Duration (h) 12	24	36	48	60	72	Mean repellency (%)
R. dominica	*S. aromaticum*	79.1 ± 0.5 a	66.0 ± 18.4 a	70.5 ± 11.6 a	60.9 ± 14.7 ab	68.5 ± 9.2 ab	64.5 ± 19.9 a	68.2 ± 11.6 a
	D5	96.5 ± 0.0 a	95.2 ± 3.3 a	91.5 ± 1.3 a	92.9 ± 0.0 a	91.3 ± 2.6 a	93.7 ± 4.7 a	93.5 ± 0.7 a
	D6	11.1 ± 11.1 b	0.0 ± 0.0 b	16.7 ± 16.7 b	19.1 ± 19.1 b	38.5 ± 16.7 b	11.1 ± 11.1 b	16.1 ± 9.6 b
S. oryzae	*S. aromaticum*	4.2 ± 4.2 c	0.0 ± 0.0 b	4.2 ± 4.2 b	21.5 ± 6.2 b	33.8 ± 19.7 a	42.9 ± 0.8 b	17.8 ± 2.2 c
	D5	94.0 ± 1.3 a	17.4 ± 1.2 ab	92.3 ± 4.6 a	81.2 ± 5.9 a	75.3 ± 2.7 a	68.8 ± 3.8a	71.5 ± 2.1 a
	D6	59.1 ± 12.6 b	35.9 ± 11.4 a	71.9 ± 12.4 a	63.9 ± 16.6 a	44.4 ± 11.2 a	42.5 ± 11.3 b	52.9 ± 3.4 b
T. castaneum	*S. aromaticum*	13.0 ± 13.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	2.2 ± 2.2 b
	D5	92.8 ± 2.2 a	100.0 ± 0.0 a	100.0 ± 0.0 a	98.9 ± 1.2 a	95.3 ± 1.2 a	97.7 ± 1.2 a	97.4 ± 0.5 a
	D6	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b

* Tested dosage: oil was 600 µg/cm2, D5 was 500 µg/cm2, D6 was 240 µg/cm2; Means followed with same letters in the same insect species within the same column are not significantly different at 0.05 level by Duncan's multiple range test.

3.3. Toxicity

All compounds were toxic to the three species tested (Table 4). The lowest mortality was with D5 and D6 against *T. castaneum* after 3 d. As expected, mortality increased with the time. There was no significant difference in mortality against all 3 pest species between D5 and the clove oil, except for D5 against *T. castaneum*. But the mortalities of D6 on all pests were very low, and were lower than the clove oil against *R. dominica* and *S. oryzae*. After 14 d, D5 and the clove oil had similar mortality, whereas mortalities of D6 were lower than that of D5 and the clove oil treatments. As with the repellency tests, D5 was the compound responsible for the mortality of the clove oil.

Table 4 Toxicity of the essential oil of *S. aromaticum* and its two main compounds against stored grain insect pests.

		Mortality + SE (%)			
		Duration (d)			
Insect	**Essential oil and compound**	**3**	**7**	**10**	**14**
R. dominica	*S. aromaticum*	33 ± 6 a	41 ± 7 a	60 ± 11 a	80 ± 6 a
	D5	27 ± 5 a	31 ± 6 ab	49 ± 10 a	67 ± 5 a
	D6	6 ± 2 b	13 ± 2 b	16 ± 1 b	19 ± 1 b
S. oryzae	*S. aromaticum*	1 ± 1 a	35 ± 2 a	44 ± 2 a	51 ± 2 a
	D5	4 ± 3 a	37 ± 8 a	43 ± 6 a	51 ± 5 a
	D6	1 ± 1 a	9 ± 5 b	14 ± 8 b	21 ± 7 b
T. castaneum	*S. aromaticum*	1 ± 1 a	3 ± 1 a	11 ± 2 a	22 ± 4 a
	D5	0 ± 0 a	1 ± 1 a	4 ± 1 b	21 ± 5 a
	D6	0 ± 0 a	1 ± 1 a	2 ± 1 b	10 ± 2 a

* Tested dosage: oil was 2000 mg/kg (0.2%W/W), D5 was 1660 mg/kg, D6 was 250 mg/kg; Means followed with same letters in the same insect species within the same column are not significantly different at 0.05 level by Duncan's multiple range test.

4. Conclusions

Two-methoxy-4-(2-propenyl)-phenol (D5) is the major compound of clove oil, with a proportion of 83%, followed by trans-caryophyllene (D6), which is 12%. The clove oil and both its two components had repellent and toxicity activity on the 3 important stored grain insect pest species, *R. dominica, S. oryzae* and *T. castaneum*. For mortality, there was no significant difference between clove oil and 2-methoxy-4-(2-propenyl)-phenol and very little mortality with trans-caryophyllene. Therefore it can be concluded that 2-methoxy-4-(2-propenyl)-phenol (D5) is responsible for the mortality of clove oil and the compounds did not act synergistically together. The repellency is more complicated to interpret, with different species reacting differently; D5 was more repellant than the clove oil for S. oryzae and *T. castaneum*, suggesting that some of the other compounds in clove oil may mask the repellency of D5. D6 also had some activity alone suggesting that not all the repellency in the clove oil was due to D5.

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Combination of *Bacillus thuringiensis* and *Habrobracon hebetor* for the biological control of *Plodia interpunctella*

Akinkurolere R.O., Zhang, H.*#,
[1] Institute of Urban Pests, and Hubei Key Laboratory of Insect Resource Application and Sustainable Pest Control, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, P. R. China. Email: hongyu.zhang@mail.hzau.edu.cn
[2] Laboratory of Food Storage Technology, Biology Department, Federal University of Technology, P. M. B. 704, Akure, Nigeria.

* Corresponding author
# Presenting author

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Abstract

In this paper, we review our work on biological control of *Plodia interpunctella* (Hubner) (Zhang et al., 1995) in our laboratory over the years. We isolated, screened and evaluated *Bacillus thuringiensis* strains collected from warehouses against *P. interpunctella* and determined the lethal concentration for 50% of the population (LC_{50}) of highly toxic isolates and compositions of crystal proteins and the genotypes of these isolates; evaluated the role of host-instar and refuge on the parasitization behavior of *Habrobracon hebetor* Say (Hymenoptera: Braconidae); investigated the effect of combining *B. thuringiensis* with *H. hebetor* for management of *P. interpunctella* infestation and assessed the influence of *B. thuringiensis* on *H. hebetor* during this combination treatment. The results showed that three strains of *B. thuringiensis* (IMM130, IMM368 and IMM408) were highly toxic to *P. interpunctella* among 122 *B. thuringiensis* isolates obtained from 413 field samples (Zhang et al., 2000a); Isolate IMM408 with LC_{50} 1.24 µg/g diet, was most potent (Akinkurolere et al., 2007). It belongs to H_7 serotype and contains ~135kDa crystal proteins and *cry1Ab9, cry1Ca1, cry1Da1* and *cry2* genotypes (Zhang et al., 2000b). It was observed that *H. hebetor* could parasitize all larval stages of *P. interpunctella,* but significantly fewer first and second instars were parasitized under choice and no-choice conditions (Akinkurolere et al., 2009a). Parasitized fourth instars were more profitable to *H. hebetor* irrespective of refuge or choice factors, as significantly more adult parasitoids emerged from host instars. Therefore, *H. hebetor* females consistently showed high preference for late instars of *P. interpunctella* when they were offered a choice between early and late host instars. Refuge significantly hindered *H. hebetor* from locating the early instars, but not the late instars (Akinkurolere et al., 2009a). *Bacillus thuringiensis*-parasitoid combination treatment significantly evoked more *P. interpunctella* mortality than either treatments (*B. thuringiensis* or parasitoid) when used singly (Akinkurolere et al., 2009b). *Bacillus thuringiensis* or *H. hebetor* alone caused 42% and 35% *P. interpunctella* larval mortality, respectively. The *B. thuringiensis*-parasitoid combination treatment significantly evoked more *P. interpunctella* mortality (86%) than other single treatments. Progeny development of parasitoid wasp was dependent upon its susceptibility to *B. thuringiensis* contaminated hosts. *H. hebetor* was able to successfully complete its development on the hosts although, fewer wasps emerged from *B. thuringiensis*-parasitoid combined treatment than in none *B. thuringiensis* treatments (Akinkurolere et al., 2009b). *H. hebetor* showed positive response to acetone and hexane extracts from frass and larvae of *P. interpunctella*, and the active compounds are mostly hydrocarbons (unpublished data).

Keywords: *Bacillus thuringiensis*, *Habrobracon hebetor, Plodia interpunctella*, Pest management, Parasitoid.

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Section: Residual Insecticides – Synthetic and Botanical

Residual insecticides, inert dusts and botanicals for the protection of durable stored products against pest infestation in developing countries

Obeng-Ofori, D.*#
College of Agriculture & Consumer Sciences, Department of Crop Science, University of Ghana, Legon, Accra, Ghana. Email: dobeng@ug.edu.gh

* Corresponding author
# Presenting author

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Abstract

Insect pests associated with durable grains and processed food cause considerable quantitative and qualitative losses throughout the world. Insect infestation can occur just prior to harvest, during storage in traditional storage structures, cribs, metal or concrete bins, and in warehouses, food handling facilities, retail grocery stores as well as in-transit. Many tools are available for managing insects associated with grains and processed food. Although pest management strategies are changing to meet consumer's demand for food free of insecticide residues, address concerns about safety of insecticides to humans, delay insecticide resistance development in insects and comply with stricter insecticide regulations, the use of synthetic residual insecticides will continue to be a major component of stored-product pest management programmes. Selective use of residual insecticides requires a through understanding and evaluation of risks, costs and benefits. The use of plant and inert materials may be a safe, cost-effective and environmentally friendly method of grain preservation against pest infestation among low-resource poor farmers who store small amounts of grains. There is a dearth of information on the use of plant materials by rural farmers in Africa for stored-product protection. The most promising candidate plant materials for future utilization as grain protectants are *Azadirachta*, *Acorus, Chenopodium, Eucalyptus, Mentha, Ocimum, Piper* and *Tetradenia* together with vegetable oils from various sources. Neem is the only plant from which several commercial products have been developed worldwide. However, unlike synthetic insecticides these alternatives often do not provide effective or rapid suppression of pest populations and may not be effective against all species of pests. These alternatives are also more expensive than synthetic insecticides, and have not been tested extensively under field conditions in the tropics. This paper focuses on the current state of the utilization of residual insecticides, inert dusts and botanicals by resource-poor farmers for protection of durable stored produce against pest infestation in Africa. A major research priority is a well designed on-farm trials to validate the efficacy of botanicals and inert dusts for stored-product protection using standard procedures and formulations that can be transferred to other communities.

Key words: Botanicals, Residual insecticides, Inert dusts, Grain storage, Storage pests, Stored products

1. Introduction

Presently the world's population stands at about 6.5 billion and it is projected to increase at 2.2% per year to around 11.5 billion by 2100, with 87% living in the developing countries of Africa, Asia and Latin America and only 13% in the developed countries of North America, Europe and Far East (Penning de Vries, 2001). The high population growth rate, particularly in the developing countries, and the changing diets will lead to a much higher food demand by 2020 (Penning de Vries, 2001). The attainment of food security in sub-Saharan Africa and Asia can only be realized from increase in productivity through the use of sustainable good agricultural practices (GLOBALGAP) and prevention of losses caused by pests in the field and along the value chain.

It is estimated that between 60-80% of all grain produced in the tropics is stored at the farm level (Golob et al., 1999). Grains (cereals, legumes, oilseeds) contribute the bulk of the world's calories and protein. The reduction of postharvest grain losses, especially those caused by insects, microorganisms, rodents, and birds, can increase available food supplies, particularly in less developed countries where the losses may be largest and the need is greatest. Postharvest losses are recognized as a major constraint in Africa

with reports of losses averaging 30% of durable stored grains (Golob and Webley, 1980). In the developing countries, the greatest losses during storage to cereals and grain legumes are caused by insect pests. Insect pest control in durable stored agricultural produce at farm level is increasingly relying on the use of synthetic insecticides by farmers who lack technical knowledge in the safe handling and use of such products. The misuse of synthetic pesticides has led to accidental poisoning, the development of insect resistance and other adverse environmental and health hazards. Furthermore, the development of synthetic insecticide-based techniques for grain protection in traditional stores in Asia and Africa has been partially caused by the high cost, unavailability or erratic supply of safe insecticides (Obeng-Ofori, 2007). In many developing countries availability of suitable and safe pesticides is poor, and often dangerous, highly toxic or persistent chemicals, such as fenthion, lindane and DDT, may be used to the detriment of the health of applicators and consumers and the environment as a whole (Golob et al., 1999).

However, the protection of the consumers of treated produce and education of the users of the chemicals is imperative. The phase-out of the fumigant methyl bromide has begun. In addition, many stored-product pests have developed resistance to the phosphine, due to its wide application for insect control in stored grains. It is most likely that residual insecticides will continue to constitute the dominant and valuable tool in stored-product pest management programs (White and Leesch, 1995).

Moreover, residual insecticides can be applied easily without specialized equipment, are compatible with international grain trade and global restrictions for zero insect tolerance, are generally less expensive than fumigants or biopesticides and are effective against a wide range of storage pests. Chemical pest control methods, if carried out intelligently and knowledgeably, can be both effective and safe. It is therefore important for users to have good knowledge of the classification, mode of action, properties, metabolism and residues of the pesticides, to enable them make proper appraisal of the benefits and potential hazards of the pesticides. Thus, they should be able to choose insecticides judiciously and formulate efficient control measures in any particular set of circumstances. There is also, the need for continuous education and training on selective and appropriate use of safe residual insecticides to ensure human safety and environmental protection.

In Africa, most of our agricultural produce is produced by poor resource farmers who cannot easily afford the cost of safer synthetic pesticides. It has therefore become necessary to search for other alternatives such as inert dusts and botanical insecticides, which are environmentally friendly and cost effective at the small-scale farmer level (Niber, 1994; Bekele et al., 1997). In many systems utilizing chemical pesticides, resistance is the rule rather than the exception; operator hazards are very real; environmental and consumer concerns cannot be ignored; and the proponents of IPM have to be taken seriously in order to develop sustainable systems for protecting stored products against pest infestation (Haines, 2000).

Inert dusts are non-toxic materials that can be mixed with the produce to control stored-product insect pests. Inert dusts can also be used to disinfect storage facilities before new produce is brought for storage. These dusts do not deteriorate or break-down and, therefore, provide long-term control of insect pests and are non-toxic, and therefore completely harmless to humans and mammals. In India during the 1960, about 70% of the grain was treated with activated Kaolin clay. Egypt also used rock phosphate as a grain protectant. Some local farmers in West Africa use ashes, lime and sand dust as grain to protect grains against pest infestation (Obeng-Ofori and Boating, 2007).

Botanicals are traditional and non-synthetic protectants derived from plants. Traditionally, many different types of plant parts are used for the protection of agricultural produce. These plants are available in many developing countries and contain several active ingredients and act in different ways under different circumstances (Schmutterer, 1990; Isman, 2006). Botanicals break down rapidly to harmless metabolites and appear less likely to build up genetic resistance in targeted species. They can also less harmful to mammals and other beneficial organisms. Botanicals can be used reliably and safely to treat cereals and grain legumes when stored in small quantities at the farm level.

The wide-scale commercial use of plant extracts as insecticides began in the 1850s with the introduction of nicotine from *Nicotiana tabacum,* rotenone from *Lonchocarpus* sp, derris dust from *Derris elliptica* and pyrethrum from the flower heads of *Chrysanthemum cinerariaefolium* (Golob et al., 1999). Several other traditionally used plant preparations and constituents of many aromatic plants used for flavouring

or medicinal purposes have been found to possess insecticidal properties (Tanzubil, 1986; Bell et al., 1990; Obeng-Ofori et al., 1997). The use of locally available plants avoids the need to establish complex mechanisms and structures for pesticide distribution and other related issues (Golob et al., 1999). The community can collect or grow the plants itself; the technique is therefore sustainable for rural farmers in Asia and sub-Saharan Africa.

2. Current status of the use of residual insecticides

The most important curative measure in stored-product pest control is the application of synthetic residual or contact insecticides. There are general principles that underlie selective use of residual insecticides to control storage pests. While there is great number of products against field pests there are only few products available which meet the special requirements of pest control during storage. None of the existing products, however, will entirely fulfill all of them. However, it is user's responsibility to select the correct insecticide that meet most of these specific requirements:

- Effective against most storage pests (broad-spectrum effect)
- Long persistence and stable under various climatic conditions
- Low toxicity to warm-blooded animals and low tendency to create insect resistance
- No harmful residue left in stored produce
- No influence on the smell or taste of the stored produce
- No chemical reaction with the ingredients of the stored produce (proteins, fats, etc.)
- Simple to use and low price

2.1. Groups of active ingredients in storage pest control

There are two main groups of active ingredients commonly used in stored-product protection. These are organophosphorous compounds and pyrethroids (Tables 1 & 2). Organophosphorous compounds are effective against most storage pests, although less against the Bostrichidae *(Rhyzopertha dominica* (F.), *Prostephanus truncatus* (Horn), *Dinoderus* spp.). Pyrethroids are very effective against Bostrichidae, though less against other species of beetles. Combined products, also known as "cocktails", containing an organophosphorous compound and a pyrethroid have been used as broad spectrum contact insecticides against mixed insect infestation (Table 3). The selection of insecticides for treatment of edible commodities is based mainly on the toxicological data (low mammalian toxicity), the effectiveness and persistence under certain storage conditions and absence of side effects such as discolouration, flavour alternation and odour. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) collect toxicological information and give advice on overall acceptable tolerance levels (Table 4).

2.2. Insecticide resistance and its management

The frequent and indiscriminate use of insecticides as a substitute rather than as a supplement to non-chemical management techniques has resulted in the failure of these chemicals to effectively control storage insect pests (Subramanyam and Hagstrum, 1996). Almost all the economically important stored-product insect pests throughout the world are resistant to most of the insecticides commonly used to protect commodities against inset infestation and damage (Subramanyam and Hagstrum, 1996). Resistance is the ability in individuals of a species to withstand doses of toxic substances that would be lethal to the majority of individuals in a normal population. This means that the target pests are no longer controlled by the originally recommended application rate of an insecticide. Resistance may be suspected under the following circumstances:

- If higher doses are required to achieve a constant mortality of insects
- If there is a significant decrease in insect susceptibility to a fixed amount of the insecticide
- If it takes longer to obtain a fixed mortality of insects
- If the mortality of field populations of a species frequently exposed to insecticides is significantly less than mortality of the same species that has little or no insecticide exposure

Table 1 Common organophosphorous contact insecticides used for stored product pest control.

Active Ingredient	Brand names
Chlorpyrifos-methyl	Reldan
Dichlorvos (DDVP)	Nuvan, Vapona
Fenitrothion	Folithion, Sumithion
Iodofenphos	Nuvanol
Malathion	Malathion, Malagrain etc
Methacrifos	Damfin
Phoxim	Baythion
Pirimiphos-methyl	Actellic
Tetrachlorvinphos	Gardona

Table 2 Common pyrethroid contact insecticides used for stored product pest control.

Active Ingredient	Brand names
Cyfluthrin	Baythroid
Deltamethrin	K-Othrin
Fenvalerate	Sumicidin
Permethrin	Permethrin

Table 3 Combined contact insecticides commonly used against mixed insect infestation in storage.

Active ingredients	Brand names
Fenitrothion + Cyfluthrin	Baythroid Combi
Fenitrothion + Fenvalerate	Sumicombi
Pirimiphos-methyl + Deltamethrin	K-Othrine Combi
Pirimiphos-methyl + Permethrin	Actellic Super

Table 4 Acute oral LD (mg/kg body wt. rate) of insecticides used for storage pest control.

Insecticide	Rat Oral LD50 (mg/kg)	Rat Dermal LD50 (mg/kg)
Malathion	1375-2800	4000-4800
Pirimiphos methyl	2050	2000
Chlorpyrifos methyl	1650-2100	3000
Tetrachlorvinphos	4000-5000	5000
Bromophos	4000-8000	2188
Dichlorvos	80	107
Fenitrothion	250-500	3000
Diazinon	300-850	2150
Iodofenphos	2100	-
Phoxim	1845	7100
Etrimfos	1800-2040	-
Methoxychlor	5000-7000	2820-6000
Pyrethrum	1500	1800
Bioresmethrin	9000	10.000
Deltamethrin	1290	2940
Fenvalerate	450	3700-5000
d-Phenothrin	5000	5000
Resmethrin	1500	3040
Permethrin	4000	4000
Methoprene	5000	Relatively nontoxic
Piperonyl butoxide	Relatively nontoxic	Relatively nontoxic

The rate of evolution of resistance depends on several factors. In general, the rate of selection for resistance increases with increase in the dose, coverage, frequency of application, and persistence of an insecticide. The most effective way to delay the development of resistance is to use integrated pest management (IPM) approach which emphasizes on the use of non-chemical methods and selective insecticide treatments. Resistance management strategy for stored-product insects should therefore rely heavily on non-chemical methods because of the limited number of safe insecticides available to practitioners. Monitoring of resistance is important for making resistance management decisions and diagnostic tests that distinguish between resistant and susceptible individuals must be used instead of dose-response tests. The following measures can prevent or delay the development of resistance:

- Change the active ingredient regularly (if possible once a year). The use of different insecticides to which the insects are not cross resistant can contribute substantially to slowing the development of resistance.
- Two insecticides can be used sequentially, as mixtures, in rotation or as mosaics (some areas treated with the first insecticide and other areas with the second insecticide. Applying insecticides in rotation is generally the preferred method because susceptible genotypes generally have a reproductive advantage over resistant genotypes in the absence of an insecticide. The frequency of susceptible genotypes may increase during the periods when an insecticide is not used.
- Use insecticides only under good hygiene conditions
- Ensure that dosage and application method are correct
- Do not use insecticides on calendar basis but only when it is necessary
- Apply insecticides efficiently using the correct application equipment to minimize wastage
- Increasing the amount of insecticide is no solution as it promotes further resistance. This approach is also uneconomical and not permitted because of lethal stipulations of maximum residue limits. It has been suggested that the risk of resistance be incorporated into pesticide registration requirements and that resistance management be used as justification for the registration of insecticide mixtures.

3. Current status of the use of inert dusts for stored-product pest control

The use of inert dusts is one of several innovative, reduced-risk or biorational and physical methods for stored-product insect pest management (Subramanyam and Roesli, 2000). Inert dusts are non-toxic dry powders of different origins that are chemically un-reactive in nature and, which can be mixed with the produce to control stored-product insect pests. Inert dusts can also be used to disinfect storage facilities before new produce is brought for storage. Inert dusts do not deteriorate or break-down and, therefore, provide long-term control of insect pests and are completely harmless to humans and mammals. Clays were used as grain protectant in North America and Africa over thousands of years ago (Ebeling, 1971; Golob and Webley, 1980). The research on inert dusts against storage pests started in the 1920s (Headlee, 1924) and there have been several reviews and research papers on the subject since then (Ebeling, 1971; Fields and Muir, 1995; Golob, 1997; Korunic, 1998; Subramanyam and Roesli, 2000). The main advantages of inert dusts are that they are non-toxic and provide continued protection of produce. They do not affect baking quality when applied to grains and are compatible with other control techniques such as heat treatment, fumigants and aeration (Bridgeman, 2000) and host-plant resistance (Chanbang et al*.,* 2008) (Table 5). Inert dusts are suitable for disinfesting empty storage facilities and for grain treatment.

3.1. Types of inert dusts

There are four main types of inert dusts available for use against stored products. These are (earth, diatomaceous earth, silica aerogels, and non silica dusts.

3.1.1. Earth

Earth includes clays, sand, paddy husk ash, wood ash and volcanic ash (Subramanyam and Roesli, 2000). These materials have been applied traditionally in some developing countries as stored-product protectants and are usually used as a layer on top of stored seeds (Golob and Webley, 1980). These materials are effective at high rates ($\geq$10 g per kilogram of gram (Subramanyam and Roesli, 2000). Local farmers in West Africa including Ghana, Benin, Senegal, Niger and Mauritania still use varying levels of fine sand and ashes from different plants to protect stored grain against insect pest infestation (Obeng-

Ofori, 2007). Research is being carried out in many parts of Africa to replace these traditional dusts with more effective synthetic silica dusts that work at lower rates (Golob, 1997; Obeng-Ofori, 2007).

Table 5 Percentage insect-damaged kernels caused by *Rhyzopertha dominica* exposed on the different rough-rice varieties treated with 0 to 1000 mg/kg diatomaceous earth (DE) and held at 32°C and 75% r.h. for 56 days.

Rice variety	**Insect-damaged kernels (%)**				
	DE (mg/kg)				
	0	**250**	**500**	**750**	**1000**
Resistant variety					
Bengal	10.0 ± 2.0 c	1.3 ± 0.5 bc	0.5 ± 0.3 c	0.2 ± 0.2 c	0.1 ± 0.1 e
Jupiter	1.9 ± 0.8 ab	0.1 ± 0.1 d	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 e
Pirogue	13.4 ± 1.6 ab	16.2 ± 2.4 ab	9.7 ± 1.3 b	7.7 ± 1.3 ab	8.7 ± 1.1 bc
Wells	8.8 ± 1.2 b	2.4 ± 1.1bc	0.4 ± 0.4 c	0.5 ± 0.3 c	0.5 ± 0.4 e
Susceptible variety	22.9 ± 4.4 a	22.4 ± 3.8 a	25.3 ± 1.1 a	20.4 ± 1.9 a	18.6 ± 2.3 a
Akita	19.1 ±2.6 ab	17.1 ± 3.4 ab	14.7 ± 1.0 ab	12.8 ± 4.8ab	11.3 ± 0.5 ab
Cocodrie	19.1 ± 2.2 ab	13.9 ± 3.6 ab	11.2 ± 3.3 b	8.7 ± 1.0 ab	3.3 ± 0.5 d
M-205 Rico	9.7 ± 1.0 ab	8.1 ± 1.3 bc	6.8 ± 0.8 b	7.6 ± 2.9 b	5.1 ± 1.4 cd

Means ± within the same DE concentration followed by different letters are significant at P<0.05 (Bonferroni (Dunn) *t-test,* (Chanbang et al., 2008).

3.1.2. Diatomaceous earth

Diatomaceous earth is used in a number of countries for stored-product protection. The admixture of finely ground silica-based dusts with quartz as the active ingredient for the control of stored-product insect pests is an ancient practice because during the 1930s and 40s, commercial products such as 'Naaki' and 'Neosyl' were marketed in Germany and England, respectively as grain protectants (Jenkins, 1940; Parkin 1944). The commercial DE formulations currently available are predominantly made up of amorphous silica and contain little or no crystalline silica (Subramanyam and Roesli, 2000). Diatomaceous earth is the fossilised siliceous remains of diatoms that were deposited during the Cenozoic era. Diatoms are microscopic unicellular aquatic plants closely related to brown algae that have a fine shell made of silica ($SO_2 + H_2O$). The main constituent of these deposits is therefore silica (SO_2), although there are small amount of oxides of other minerals such as aluminum, iron, lime, magnesium and sodium. As particle aggregates, DE is used as an industrial absorbent and as non-toxic insecticide to control stored-product and household pests.

3.1.3. Mineral (non-silica) dusts

Mineral (non-silica) dusts have been tested for their efficacy as grain protectants. Several workers have reported the use of different types of mineral dusts for the control stored-product insect beetle and moth pests (Davis et al., 1984; Davis and Boczek, 1987). Typical mineral dusts include calcium carbonate (lime), rock phosphate, zinc oxide, magnesium oxide and dolomite.

3.1.4. Silica gels

Silica gels are made up of 99.5% silicon dioxide. Silica gels are dusts that contain extremely small particles of less than 3 micrometers with a bulk density in the range of 72-450 g/L and specific surface in the range of 200-850 m^2/g (Quarles, 1992). Silica gels are capable of absorbing about 1.9-3 g of linseed oil per gram gel and this makes them more effective than DE dusts (Subramanyam and Roesli, 2000). There are three main types of silica gels namely, precipitated silica gel, silica aerogel and fumed silica. In general silica gels could become suitable alternatives for grain protection in most developing countries where access to quality insecticides is limited or where local expertise on the use of synthetic insecticides is lacking. Currently, some commercial DE products such as Dryacide and Protect-It contain silica gels to improve their effectiveness (Fields and Korunic, 2000).

3.2. Mode of action of inert dusts

Inert dusts cause desiccation in insects by destroying the wax layer in the cuticle. Insects die of desiccation when they lose 60% of their water i.e. 30% total body weight (Ebeling, 1971). Inert dusts such as silica aerogels can absorb as much as three times their own weight in oils. As insects move through the grains the inert dusts absorb waxes from the insect cuticle. Because storage insects live in very dry environments with limited access to free water, water retention is crucial to their survival. Also by their small size insects have a large surface area in relation to their body weight and therefore have greater problems retaining water than large animals. The wax layer in the insect cuticle consists of an ordered monolayer of lipid, and determines the permeability characteristics of the cuticle. Indeed, the wax layer that inert dusts destroy is one of the main mechanisms insects use to maintain water balance.

The presence of powdered dusts between grains also interferes with the movement and respiration of insects. Dusts may also affect the oviposition behavior and sensory perception in insects. In summary, the modes of action of inert dusts include the following: (inert dusts block insect spiracles and insects die from asphyxiation, inert dusts lodging between cuticular segments increase water loss through abrasion of the cuticle, inert dusts absorb water from the insect's cuticle (d) insects may die from ingesting the dust particles, and inert dusts absorb the epicuticular lipids of insects leading to excessive loss from the cuticle (Subramanyam and Roesli, 2000).

3.3. Susceptibility of storage pests to inert dusts

Insects differ in their susceptibility to inert dusts. In general, *Tribolium* species are the most resistant and *Cryptolestes* species are the least resistant. The capacity of insects to survive dry conditions is correlated with resistance to inert dusts. The effectiveness of inert dusts may be determined by such factors as greater capacity of insects to gain water from their food, greater water re-absorption during excretion, less water loss through the cuticle, type of cuticular wax or amount of movement through grain. The effectiveness of inert dusts may also depend on the size of the particles, the finer the particle size, the more active they are. Generally, DEs are more effective against insects at higher temperatures and lower grain moisture contents (Fields and Korunic, 2000). The condition of the grain may affect efficacy of inert dusts. Most DE dusts are more effective on clean grain than in cracked grain (McGaughey, 1972).

3.4. Commercially available inert dusts

Commercially, a number of inert dusts especially diatomaceous earth products are registered as residual grain protectants and for use in crack and crevice treatment and disinfestation of storage structures before new grain is stored in a few countries, especially in the USA. Several products have been registered by the US Environmental Protection Agency. Australia has made considerable progress in integrating DE with aeration and fumigation (Bridgeman, 2000). In Germany, SilicoSec has been a registered diatomaceous earth since 1997. It is a natural silica powder based on fossilized diatom algae and contains 96% inert amorphous SO_2 with particle size between 13-15 microns. SilicoSec controls all stored grain insect pests including weevils, beetles, borers and moths. Even species resistant to chemical insecticides are controlled. The sharp-edged silica particles destroy the wax layers of the insect cuticle and quickly absorb lipids and body fluids leading to desiccation and death.

In the UK, two products of diatomaceous earth are commercially available for use in stored-product protection. These are Protect-It and Dryacide. These products have been found to be effective in protecting grain against insect pest damage for small-scale on-farm storage systems in Zimbabwe. The two products have been evaluated in a community-wide basis in Tanzania and the treated products include maize, sorghum and beans. However, field tests in Malawi using different rates of Dryacide, Protect-It and a precipitated silica gel (Gasil 23D) failed to provide long term protection of shelled or cob maize against infestation by *Tribolium castaneum* (Herbst) and *Sitotroga cerealella* (Olivier) (Gudrups et al., 2000). Other examples of commercial inert dust products include Perma-guard (DE), Dri-Die (DE and silica aerogel), Sipernat (silica aerogel) and SG-67 (silica aerogel).

In India during the 1960, about 70% of grain was treated with activated Kaolin clay. Egypt also used rock phosphate as grain protectant. Some local farmers in West Africa use ashes, lime and fine sand dusts as grain protectants.

3.5. Shortcomings of inert dusts

Inert dusts decrease the bulk density and flowability of grain. They adhere to the surface of grain kernels and increase the friction between the grains so that grains do not flow as easily thereby increasing angles of repose and decreasing bulk density. They also affect the appearance of the treated product and are dusty to apply. There have also been some concerns that inert dusts will increase wear on machinery.

3.6. Research needs

Compatibility of inert dusts with other control techniques will increase their practical utilization for stored-product protection. To enable more countries to explore ways of making inert dusts especially DE a part of the pest management strategy, research is needed to develop appropriate technologies for integrating inert dusts with other techniques such as conventional insecticides, botanicals, aeration and heat treatment. More research is also needed to evaluate the effectiveness of inert dusts under different range of field conditions and treatment techniques to empty storage facilities and on grain. Information on the effect of sanitation on the performance of inert dusts such as DE and silica gels in food-handling facilities will facilitate their utilization in such establishments. At the 7th International Working Conference on Stored-Product Protection (IWCSPP) held in China in 1998, a working group was formed comprising scientists from Australia, Brazil, Canada, Germany, UK and the USA to develop standardized techniques for evaluating DE dusts. This group published a standard protocol at the subsequent IWCSPP (Fields et al., 2002). The development of such standardized procedures will ensure that consistency is achieved from results obtained from different laboratories for comparison.

4. Current status of the use of botanicals by small-scale farmers

Botanicals are plant-derived compounds with different modes of action (Weaver and Subramanyam, 2000). The use of locally available plant materials for stored-product protection is a common practice, and has more potential in subsistence and traditional farm storage conditions, in developing and underdeveloped countries (Golob and Webley, 1980; Obeng-Ofori, 2007). Resource-poor farmers in different countries in Asia and Africa have utilized plant materials to protect durable stored products against insect infestation for a long period of time (Golob et al., 1999; Obeng-Ofori, 2007). Many of these plants are widely used in traditional medicine by local communities for the treatment of several ailments. The local farmers also admix leaves and powders with various cereals and pulses as protectants in different parts of the world, particularly India, China and most sub-Saharan African countries for the control of mostly insect pests. The practical advantage of using locally available material to protect stored products destined for household and small-scale use remains compelling (Weaver and Subramanyam, 2000). A number of excellent publications provide useful information regarding the types of plants used in different parts of the world for stored-product protection (Golob and Webley, 1980; Golob et al., 1999; Weaver and Subramanyam, 2000; Obeng-Ofori, 2007). It is, however, the transfer of such technology to other environments or the extension of the use of these methods to other communities within the same areas that have not been feasible to date.

There is therefore the need for more systematic studies to determine how farmers utilize plant protectants, the methods employed and their effectiveness in the field. For this to be achieved, a good understanding of the farming and sociological systems operating in the target communities is required. The introduction of rapid rural appraisal (RRA) and participatory rural appraisal (PRA) techniques in recent years has facilitated the collection of this type of information (Golob et al., 1999). In a survey in Benin, West Africa, out of the 33 plants collected and tested, the powders of *Nicotiana tabacum, Tephrosia vogelii* and *Securidaca longepedunculata* significantly reduced progeny production of *Callosobruchus maculatus* (F.) in stored cowpea while *Clausena anisata, Dracaena arborea, T. vogelii, Momordica charantia* and *Blumea aurita* were repellent to the beetle (Boeke et al., 2004). In a similar survey of plants used as traditional insecticides in 12 districts in forest areas of the Ashanti Region in Ghana involving about 500 farmers, 26 different plant species were found to be used as grain storage protectants (Cobbina et al., 1999). The most common were *Chromoleana odorata* (Siam weed), *Azadirachta indica* (neem) and *Capsicum annum* (chili pepper). Smoking maize stores was the most common method of control in most districts of the region.

In another PRA survey in the northern, semi-arid regions of Ghana only 16 plants were identified as being used as grain protectants. Apart from neem, none of the plants were used as stored-product

protectant in the survey carried out in the Ashanti Region. Two of the plants, *Chamaecrista nigricans* and *C. kirkii* (both known locally as 'lodel'), and said to be the most effective, have not been recorded any where in the world and have not been studied to evaluate their potential as grain protectants. Another plant found to be commonly used by subsistence farmers as dry powder and admixed with grains in northern Ghana to protect stored cowpea, bambara groundnut, millet, sorghum and maize was *Cassia* species (Belmain et al., 1999; 2001). These plants, like many of the others used in Northern Ghana, are weeds and serve no other useful purpose. Based on the above studies, the Ghanaian Ministry of Food and Agriculture (MoFA) has identified 16 different plant species used by farmers for stored-product protection (Table 6).

Table 6 Plant species used by farmers to protect food stuffs against pest infestation in Ghana.

Azadiracta indica	*Ocimum americana*
Capsicum annuum	*Pleiocapa mutica*
Cassia sophera	*Pterocarpus erinaceus*
Chamaecrista nigricens	*Securidaca longepedunculata*
Citrus sinensis, Combretum sp.	*Synedrella nodiflora*
Cymbopogon schoenanthus	*Chromolaena odorata*
Khaya senegalensis	*Vitellaria paradoxa*
Lippia multiflora	*Mitragyna inermis*

Plant materials from several families including Annonaceae, Piperaceae and Rutaceae are used for the protection of stored products against insect pests in Nigeria (Okonkwo, 2004). Of the several families identified to have insecticidal properties only a few in the genera *Azadirachta, Citrus, Dennettia* and *Piper* have been sufficiently tested in the laboratory to provide an indication of their potential usefulness as stored-product protectants.

Botanical products are an essential part of post-harvest pest control in East Africa (Kokwaro, 1976; Weaver et al., 1994). The commonly used grain protectants in East Africa particularly Kenya and Tanzania include *Ocimum* spp., *Eugenia aromatica, Bascia* spp., *Tagetes* spp., *Tephrosia vogelis*, *Azadirachta indica*, *Eucalyptus* spp., and *Lantana camara.* Over 450 botanical derivatives are used in traditional agricultural systems in India with neem as one of the well-documented trees, in which almost all the parts of the tree have been found to have insecticidal value. The use of neem leaves and powdered kernels in managing pests of stored grains is an ancient practice in India. Turmeric, garlic, *Vitex negundo*, glyricidia, castor, Aristolochia, ginger, Agave Americana, custard apple, Datura, Calotropis, Ipomoea and coriander are some of the other widely used botanicals to control and repel crop pests (Saxena et al., 1992).

Clearly, there is adequate information on the use of plants by farmers in Asia and sub-Saharan Africa for stored-product protection. Undoubtedly, there are many plants used as grain protectants by rural communities, which are yet to be identified and characterised. To encourage local production of plant protectants it is essential that farm practices are recorded and more information acquired on the exact application procedures and formulations used by farmers. Currently, little or no information is available on how farmers apply plant protectants to protect their stored produce against pest infestation. This is because farmers are either unable to describe the procedures with sufficient accuracy or their accounts vary considerably from one farmer to another. Thus, research projects which pursue optimal methods of using plant protectants on grain must focus on the development of the most cost-effective procedures for application as well as identifying the biologically active components involved.

4.1. Application of plant materials at farm level

A number of surveys have been conducted in some countries in Africa to determine the methods and procedures by which small-scale farmers treat stored grains with plant materials (Hassanali et al., 1990; Belmain et al., 1999; Cobbina et al., 1999; Boeke et al., 2004). In most cases the dry whole leaves are placed as layers when undehusked maize cobs are stacked in cribs or other locally constructed storage structures. Milled dry leaves in the form of powder are also admixed with grains in storage. In addition, many rural farmers in Africa, particularly West Africa admix wood ashes of various plants with grains as a physical control treatment against infestation by storage pests. Rural farmers in some African countries also commonly use smoke from burning plant materials to protect on-farm stored cereal grains against

pest infestation. Thus, farmers have different ways of preparing the botanicals as storage protectants. Some farmers prepare hot water extract of the plant, which they pour over their commodity or use as grain dip. Experiments in Northern Ghana have shown that, dipping durable produce into a hot water extract appeared to be more effective than admixing powdered plant materials (Belmain et al., 2001).

Currently, currently no standardized procedures are used by farmers with regards to methods and dosages of the plant materials that are applied. The precise strategy used by different communities varies from place to place and appears to depend partly on the type and perceived efficacy of suitable materials available in different localities. In some communities, the usage of plant materials is ethnically and culturally biased and this could be a constraint to the availability of indigenous knowledge systems. The efficacy or otherwise of the plant materials are also not determined by the farmers, nevertheless most rural farmers claim these materials are effective. Detailed information on the reliability and efficacy of botanicals with respect to where and when the plant materials are collected is also lacking. Plant secondary metabolites are well-known to vary according to climatic, seasonal, geographical location and genetic effects. For example, in Ghana materials derived from neem collected from Upper East Region were generally found to be more potent than those from Northern Region (Belmain et al., 1999).

4.2. Vegetable oils as storage protectants

The use of plant oils including, vegetable oils, essential oils and mineral oils by rural farmers in sub-Saharan Africa for the control of durable stored-product pests is an ancient practice (Obeng-Ofori, 1995). Examples of the commonly used plant oils by small-scale farmers include coconut, palm oil, groundnut oil, cotton seed oil and soybean oil. Others are sunflower oil, castor oil, sesame oil and mustard oil. Plant oils are usually mixed with grains such as maize, rice, wheat and cowpea. The mode of action of plant oils is not clearly understood. However, the protection of grains by oils could be due to both physical and chemical factors. The oils may kill the embryos of unhatched eggs or block the trachea of the insects and thus interfere with respiration. Plant oils could also act as antifeedant or modify the storage micro-environment, thereby discouraging insect penetration and feeding (Don-Pedro, 1989).

Plant oils are harmless to humans, are easily obtained and can be integrated with other control methods. Vegetable oils are the most commonly used cooking oils in Africa and are generally available in rural communities. The practical utilization of plant oils and botanicals as grain protectants is limited by the high rates of oil required to disinfect grain and the low persistence in grain. It had been demonstrated that pirimiphos-methyl and botanical insecticides can be used at reduced rates if combined with lower dosages of plant oils to control the infestation of stored-product beetle pests (Obeng-Ofori and Amiteye, 2000). Combination of lower doses of pirimiphos-methyl and neem oil provided adequate protection of maize stored in traditional cribs in Ghana against pest infestation (Table 7). Plant oils can also act as potentiation agents for botanicals by increasing their potency and persistence in grain (Obeng-Ofori and Reichmuth, 1999).

Table 7 Grain damage caused by *S. zeamais* and *P. truncatus* in maize treated with mixtures of neem oil and pirimiphos-methyl and stored in traditional cribs for 3 months in Ghana.

Pirimiphos-methyl (mg/kg)	Neem oil (mg/kg)	Weight loss (%)
2.0	0	0.1
0.5	1.0	0.6
0.5	2.0	0.5
1.0	1.0	0.2
1.0	2.0	0.1
0	5.0	2.1
0	0	7.5
LSD (P<0.05)	-	0.3

4.3. Challenges to the utilization of botanicals pesticides

Many plant species contain secondary metabolites that are potent against several pest species. Not only are some of the plants (e.g. the neem trees) of major interest as sources of phytochemicals for more environmentally sound crop protection, they can also play important role as instruments in the arena of global climatic changes. Some of the tropical trees are excellent carbon dioxide sinks. They are fast

growing, tolerant to high temperatures and drought and can thrive well in degraded, eroded and acid soils. They can, therefore be useful in mitigating the process of desertification by serving as windbreaks, producing leaf-litter and fuel wood to poor rural communities where fuel is becoming a scarce resource (Obeng-Ofori, 2007). The tress can also help restore fertility to highly exhausted soils. Thus, these plants are valuable natural resource, which could be harnessed to advance sustainable economic development in the least developed countries of Asia and sub-Saharan Africa. Phytochemical products can provide environmentally sound pesticides, increase incomes of rural farmers and promote safety and quality of food and life in general.

There is no doubt that the successful utilization of botanicals can help to control many of the world's destructive pests and diseases, as well as reduce erosion, desertification, deforestation, and perhaps even control human population due to the anti-fertility action of some of them such as the neem (Obeng-Ofori, 2007). Although the possibilities of using botanical pesticides seem almost endless, so many details remain to be clarified. Many obstacles must be overcome and many uncertainties clarified before their potential can be fully realized. These limitations seem surmountable; however, they present exciting challenges to the scientific and economic development communities. Solving the following obstacles and uncertainties may well bring a major new resource which will benefit much of the world. These obstacles include:

- Lack of experience and appreciation of the efficacy of botanicals for pest control. There are still doubts as to the effectiveness of plant-derived products (both 'home-made' and commercial products) due to their slow action and lack of rapid knock-down effect
- Genetic variability of plant species in different localities
- Difficulty of registration and patenting of natural products and lack of standardization of botanical pesticide products
- Economic uncertainties occasioned by seasonal supply of seeds, perennial nature of most botanical trees and change in potency with location and time with respect to geographical limitations
- Handling difficulties as there is no method for mechanizing the process of collecting, storing or handling the seeds from some of the perennial trees
- Instability of the active ingredients when exposed to direct sunlight
- The usage of botanicals is still not held in high social esteem in many countries
- Competition with synthetic pesticides through aggressive advertising by commercial pesticides dealers and commercial formulated botanicals are more expensive than synthetic insecticides and are not as widely available
- Possible health hazards when seeds used in the preparation of the products are infected with the *Fungus aspergillus flavus* which produces aflatoxins, which is one of the most potent carcinogens known in the world.
- Rapid degradation, although desirable in some respects, creates the need for more precise timing or more frequent applications.
- Data on the effectiveness and long-term (chronic) mammalian toxicity are unavailable for some botanicals, and tolerances for some have not been established.

4.4. Field-based trials using botanicals

Active research is going on in several countries to determine the efficacy and practical utilization of locally available plants for controlling insect pests. These countries include India, Bangladesh, Pakistan, the Philippines, Japan, Rwanda, Nigeria, Ghana, Senegal, Benin, Kenya, Egypt, Israel, Germany, the Netherlands, UK and USA. However, the bulk of the trials are laboratory-based and usually of very short duration. They therefore do not reflect real farm conditions in the field. A few trials have been undertaken in the field in some developing countries which partially simulate on-farm conditions. The use of jute bags impregnated with 10% concentration of aqueous extracts from *Chenopodium ambrosiodes* and *Lantana camara,* to reduce infestation to cowpea and broad bean seeds by *C. maculatus* and *Acanthoscelides obtectus* (Say) was compared with direct seed treatment using plant powders at 4% (w/w) (Koona et al., 2007). After 6 months storage, the jute bags impregnated with plant extracts were found to be more effective than seed treatment with plant powders in terms of reduction in seed damage. Application of *A. indica* (neem) seed extract at 8% by weight to wheat in jute bags in the Sind, Pakistan was considered to be as effective as 5 mg kg^{-1} pirimiphos-methyl, reducing insect populations after six

months storage by 80% (Golob et al., 1999). Field trials conducted to control the larger grain borer, *P. truncatus* using extracts of neem leaves were found to be relatively unsuccessful in simulated storage experiments in both Tanzania and Ghana (Golob and Hanks, 1990). Treatment of maize with a commercial neem product (Calneem oil) and stored in on-farm cribs in Ghana protected the grain against insect pest infestation for two to five months (Tables 8 & 9)

Table 8 Protectant effect of Calneem oil and Actellic on damage caused by *E. cautella* to maize stored in the crib for 5 to 20 weeks in Ghana.

Treatment (mL/L)	**Weight loss ± SE (%)**			
	Time (weeks)			
Calneem oil	**5**	**10**	**15**	**20**
0.0	3.03±0.40	3.39±0.32	3.61±0.47	4.55±0.18
5.0	0.24±0.02	1.15±0.20	1.65±0.11	1.69±0.26
Actellic 2.0	0.18±0.01	1.20±0.15	1.15±0.03	1.23±0.15
LSD (P<0.05)	0.85	0.82	0.65	0.75

Table 9 Percent dry weight loss of maize caused by *E. cautella* after 60 days of storage using the count and weigh method.

Dosage (ml/L)	**Dry weight loss ± SE (%)**
Control	2.5±0.0
Hocklicombi	0.4±0.0
Novaluron	0.6±0.0
Neem oil	0.6±0.0
LSD (P<0.05)	0.9

4.5. Commercial development and use of botanicals for grain protection

Little information is available on the evaluation of a large number of plant species from a wide range of families for their potential as grain protectants. It had been suggested that the most promising botanicals were to be found in the families Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae and Canellaceae. In a comprehensive review references to trials using plant species from over 50 families were cited (Golob et al., 1999). The most numerous were in the families Compositae, Fabaceae, Labiatae, Leguminoceae, Solanaceae and Umbelliferae. From the evidence available to date the most promising candidate plant materials for consideration as future grain protectants are *Azadirachta*, *Acorus, Chenopodium, Eucalyptus, Mentha, Ocimum, Piper* and *Tetradenia* together with plant oils from various sources.

However, so far only products from four plant species have found widespread use as insecticides. Rotenone is obtained from *Derris* and *Lonchocarpus* species and was widely used during the early part of the century in England as an insecticide. *Derris elliptica* and *D. malaccensis* occur quite commonly in East Africa and China where the roots have been used as fish poison. Pyrethrum has been produced commercially for more than 150 years, with over 90% of the world's production coming from Kenya, Tanzania, Equador, Rwanda and Japan mainly for export. Neem is the only other plant from which several commercial products have been developed in different countries. Neem products are broad spectrum in activity and potent against over 300 species of insects, mites and some micro-organisms. They also have insecticidal, repellent, antifeedant, sterilizing and growth inhibition effects. Neem extracts have been found to contain several active ingredients including azadirachtin, memaliantriol, selamin, nimibin and nimbidin, which act in different ways under different circumstances (Schmutterrer, 1990).

Over 100 neem-based products are marketed in India alone. Commercial neem-based products are marketed in a few African countries including Kenya, Benin, Nigeria, Senegal and Ghana. Currently a number of commercial neem products are also registered and marketed in some developed countries such as the USA, Germany, Australia, Italy, Switzerland, Sweden, Denmark, Austria, Spain and Israel (Foster and Moser, 2000). The only other plant to be exploited commercially is *Acorus calamus*. A preparation containing 70 percent β -asarone, is marketed by Alrich of Germany. It must be emphasized that before

any of the botanicals can be commercialised even for local production and consumption, it must be shown to be safe.

4.6. Research needs

Clearly, there is enough information on the use of plants by farmers in Asia and Africa for stored-product protection. There are also undoubtedly, many plants used as grain protectants by rural farmers, which are yet to be identified and characterized. To encourage local production of plant protectants it is essential that farm practices are recorded and more information acquired on the exact application procedures and formulations used by farmers. There is often an erroneous assumption that plant compounds because of their natural source are innately safer. Some botanicals such as nicotine are as toxic to mammals as some synthetic pesticides (Weaver and Subramanyam, 2000). A largely neglected area has been the safety of botanicals from the point of environmental contamination. It must be emphasized, however, that for practical utilization of plant materials for stored-product protection further information is required on the residual effects of plant materials over a longer duration period of 6-12 months or more against key insect species, toxicity of the materials to non-target organisms, and local availability of appropriate extraction and application techniques. Of important research priority is well designed on-farm trials to validate the efficacy of plant materials under real farm conditions. Agricultural extension officers must also be trained in the practical utilization of plant materials to enable them transfer the technology to farmers. The development of appropriate infrastructure at the community level for the introduction of plant materials to be used as protectants is also necessary.

Botanical pesticide research is undertaken to find solutions to economic problems. Research efforts should therefore not focus only on their efficacy but also cost implications relating to processing, storage, extraction, formulation, product stability and application. Donor agencies must give priority attention to botanicals and support research projects which pursue optimal methods of using plant protectants on durable products at the farm level with the focus on the development of the most cost-effective procedures for application as well as identifying the biologically active components and establishing appropriate safety standards.

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Residual efficacy of aerosols to control *Tribolium castaneum* and *Tribolium confusum*

Arthur, F.H.*#
USDA-ARS Center for Grain and Animal Health Research, 1515 College Avenue. Manhattan, KS, USA, 66502, Tel.: 785-776 2783, Fax: 785-539 5584, Email: frank.arthur@ars.usda.gov

* Corresponding author
# Presenting author

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Abstract

Aerosol insecticides can be important components of insect management plans for mills, food warehouses, and processing plants. In the United States, synergized pyrethrin is used alone or combined with an insect growth regulator (IGR), either methoprene or hydroprene. The presence of food material can result in increased survival of adult *Tribolium castaneum* (Herbst) or *Tribolium confusum* Jacquelin du Val exposed to synergized pyrethrins, but larvae appear to be more susceptible than adults. Results of field trials involving methoprene and pyriproxyfen indicate residual persistence of the IGRs. *Tribolium castaneum* is more susceptible than *T. confusum* to IGRs, but combination of pyrethrin with the IGR may produce an additive effect on *T. confusum.*

Keywords: Aerosol, Insecticide, Insect growth regulator, *Tribolium castaneum*, *Tribolium confusum*

1. Introduction

Aerosol insecticides [also known as ultra low volume (ULV), fogging, and space sprays], can be used as part of management programs for flour mills, processing facilities, and food warehouses in the United States (Peckman and Arthur, 2006). Several recent field studies have examined distribution and efficacy of pyreithrin or a pyrethroid applied alone or combined with the insect growth regulator (IGR) methoprene (Arthur et al., 2009; Jenson et al., 2010a, b), but there is little research regarding the residual activity and toxicity of an aerosol application. Methoprene and also the IGR pyriproxifen have residual efficacy when applied to different surfaces (Arthur et al., 2009; Jenson et al., 2009), therefore it is assumed that an IGR applied as an aerosol would also provide some level of residual control.

Insect populations inside a mill or food warehouse could be directly exposed to an aerosol or be to the residual deposits resulting from an application. Efficacy can therefore be measured by direct and indirect methods of exposure. However, it is often difficult to bring live insects of any life stage inside a commercial facility, and indirect methods of evaluation may be required to evaluate residual efficacy. The objectives of this study were to determine residual efficacy of pyrethrin combined with either methoprene or pyriproxyfen aerosol, through direct and indirect methods of exposure.

2. Materials and methods

2.1. Aerosol Field Trial 1

A field trial was conducted in a flour mill with an installed aerosol/ultra low volume (ULV) system that dispensed either a 1% pyrethrin (Entech Fog 10®)+ methoprene (Diacon II®) mixture, or a 3% pyrethrin (Entech Fog 30® + methoprene mixture, as specified on the product label for these formulations. Treatment arenas were constructed by filling the bottom portion of a Petri dish, which measured about 62cm^2, with slurry of a driveway patching material (Rockkite®) to create a smooth concrete surface. After these arenas were prepared in the laboratory, they were sent to the mill for direct exposure studies.

Trials were first done with the 1% pyrethrin + methoprene mixture. For each of 5 replicates, 20 arenas were sent to the mill, and exposed to the aerosol/ULV application, which was generally done on a Saturday. These arenas were placed on the fourth floor of the mill, directly on the floor so that there were no obstructions to the movement or drifting of the aerosol/ULV particles. Upon completion of the application, the area was vented for several hours according to label instructions, and the arenas were collected, boxed, and shipped to the Center for Grain and Animal Health Research (CGHAR) in Manhattan, KS. Upon arrival at the CGHAR, which was considered to be time 0, four arenas were randomly selected. Approximately 300 mg of flour media was placed on each arena. In one arena 10 eggs of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), the red flour beetle, were placed on

the flour, on a second arena 10 3-week old *T. castaneum* larvae were placed on the flour, on a third arena 10 4-week-old *T. castaneum* larvae placed on the flour, and on the fourth arena 10 *T. castaneum* pupae were placed on the flour. In another set of four arenas that were not exposed to the aerosol/ULV, 300 mg of flour was put in each one, and they were set up as described above with each of the four life stages as untreated controls. All arenas containing the life stages were placed in an incubator at 27°C and 60% r.h., and held until adult emergence was completed in the untreated controls. This required approximately 2, 3, 4, and 7 weeks for the pupae, 4-week-old larvae, 3-week-old larvae, and eggs, respectively. The emergence of adults that appeared to be normal with no morphological deformities was the criterion used for assessment, with main effects of concentration, residual time, and life stage. Data were analyzed using the General Linear Models (GLM) Procedure of the Statistical Analysis System (SAS, 2007).

2.2. Aerosol Field Trial 2

This field trial was conducted during summer and autumn of 2007 by first creating concrete exposure as described for the first aerosol field trial. For each replicate trial in this test, twenty four of these arenas exposed to an aerosol applications of pyrethrin + the IGR pyriproxyfen (Nygard®), at the label rate for both products, by placing six concrete arenas at each of the following positions: low and open on the floor, low and obstructed on the floor (underneath a pallet), and at similar positions called "high", which was about 4 m from the ground.

The aerosol was usually applied late Friday afternoon, the dishes were picked up on Saturday morning, and the next week shipped back to the CGHAR. At the time the dishes were received (week "0"), one dish from each position was removed and about 300 milligrams of flour was placed in each dish. Ten 4-week-old *T. castaneum* larvae were placed in each dish. Companion dishes of untreated controls were set up as well. The remaining dishes were held and then assessed at residual intervals of 2, 4, 6, 8, and 10 weeks after they were originally exposed to the aerosol. This process was repeated for three separate spray replicates. In another series of three replications, the tests were repeated using the procedures described above, and conducting residual bioassays with larvae of *Tribolium confusum* Jacqueline du Val (Coleoptera: Tenebrionidae), the confused flour beetle. Data were analyzed using the GLM Procedure of SAS with residual time, exposure position, and species as main effects.

2.3. Aerosol Field Trial 3

This trial was conducted during spring and summer of 2008 using different methods of efficacy assessment. Treatment arenas were constructed and sent to the field site, where they were placed at the same positions as described for the 2007 study. In this trial, only *T. castaneum* was used as the test species. When the arenas exposed to the aerosol arrived in at the CGHAR, one arena from each of the four exposure positions was selected. Three hundred mg of flour was placed in each of the arenas, along with ten mixed-sex 1 to 2-week old adult *T. castaneum*. Untreated controls are also set up as well following the same procedure. The adults are allowed to remain on the arenas for one week at 27°C and 60% r.h. inside a Percival® incubator, and then removed. The arenas containing the flour were then returned to the incubator and held for an additional period of six weeks to record progeny from the exposed parental adults. At this time all of the adults in the untreated controls had emerged, so they were counted and the arenas discarded. In the treatment dishes, the beetles had advanced only to the larval stage. Therefore, an additional 300 mg of flour was added to each of the arenas and the arenas returned to the incubator for an additional period of four weeks. After that time, the arenas were removed from the arenas, the emerged adults were tabulated, and the arenas discarded. Data were analyzed using the GLM Procedure of SAS with residual time and exposure position as main effects.

3. Results

3.1. Aerosol Field Trial 1

Main effects treatment, concentration, and life stage were all significant at $P < 0.01$ ($F = 787.5$, df = 1, 228; $F = 16.7$, df = 1, 228; $F = 48.7$, df = 3, 228), but week post-treatment was not significant ($F = 0.3$, df = 4, 228, $P = 0.85$), indicating equal effectiveness of the aerosol residues at all weekly exposure intervals. The only interactions that were significant ($P < 0.05$) were concentration by treatment, life stage by treatment, and concentration by life stage by treatment (all others $P \geq 0.05$). Data were further analyzed by combining the data for post-exposure week.

In all comparisons, the percentage of emerged adults was less in the treatments than in the controls ($P < 0.01$), and for life stages except pupae, adult emergence was lower in the arenas exposed to the 3% pyrethrin + methoprene ULV than in the 1% pyrethrin + methoprene ULV ($P < 0.01$) (Table 1). Except for pupae, adult emergence was zero for all life states in the treatment arenas exposed to the 3% pyrethrin + methoprene ULV. Adult emergence from pupae in the treatment arenas was the same at both insecticide concentrations.

Table 1 Percentage of adult emergence (mean ± SE) of eggs, 3-week-old larvae, 4-week-old larvae, and pupae of *Tribolium castaneum* in concrete arenas exposed to 1% pyrethrin + methoprene and 3% pyrethrin + methoprene, at 0-4 weeks post-treatment (data combined for the post-treatment bioassays). Means within columns for each concentration followed by different letters indicate significant differences in adult emergence among exposed life stages ($P < 0.05$, Waller-Duncan *k*-ratio *t*-test)[a].

Concentration	Life Stage	Treatment	Control
1% pyrethrin+ methoprene	Pupae	62.5 ± 6.2a	96.5 ± 1.3a
	4-week-old larvae	30.0 ± 7.9b	96.0 ± 8.7a
	3-week-old larvae	20.0 ± 8.7b	91.3 ± 2.6a
	Eggs	22.0 ± 6.9b	70.5 ± 4.2b
3% pyrethrin+ methoprene	Pupae	63.6 ± 5.7a	95.5 ± 1.9a
	4-week-old larvae	0.0 ± 0.0b	93.5 ± 1.7a
	3-week-old larvae	0.0 ± 0.0b	86.5 ± 2.5b
	Eggs	0.0 ± 0.0b	81.0 ± 3.2b

[a] Adults emergence always lower in treatments versus controls, and for each life stage except pupae emergence was lower for 3% pyrethrin + methoprene versus 1% pyrethrin + methoprene.

3.2. Aerosol Field Trial 2

Main effects treatment and species were both significant ($F = 2{,}710.4$, df = 1, 176, $P < 0.01$; $F = 5.0$, df = 1, 176, $P < 0.02$) for adult emergence from larvae in the arenas exposed to the aerosol, but neither the position at which the arenas were originally exposed (open or obstructed, on the floor versus 4 m off the floor) or the residual bioassay week were significant ($F = .01$, df = 3, 176, $P = 0.93$; $F = .04$, df = 3, 176, $P = 0.81$). The week by species and the treatment by species interactions were significant ($P < 0.01$) but the rest of the interactions were not significant ($P \geq 0.05$). *T. castaneum* was the more susceptible species, with adult emergence of 4.1 ±1.1% compared to 10.1 ± 2.2 % for *T. confusum* in arenas exposed to the aerosol. Adult emergence of larvae in the untreated controls was 92.1 ± 1.2% and 93.4 ± 2.1% for *T. castaneum* and *T. confusum*, respectively. The residues from the aerosol application appeared to be effective for 10 weeks, assessed by the methodology employed in this experiment.

3.3. Aerosol Field Trial 3

In the arenas that were exposed to the aerosol, there were very few adults, and no difference regarding where the arenas were placed when exposed to the aerosol (on the floor, off the floor, open versus obstructed positions, $F = 0.6$, df =3, 168; $P = 0.64$) or the residual bioassay time in which the adults were first put on the arenas exposed to the aerosol ($F = 0.4$, df =5, 168; $P = 0.84$). For the entire test, the average number of adults in the control arenas was 26.0 ± 1.2 compared to 0.7 ± 0.3 in the arenas exposed to the aerosol. In all of the arenas exposed to the aerosol, only 8 adults were found during the entire experiment, compared to more than 2,700 in the equal number of untreated control arenas.

4. Discussion

Results of these field trials show that the pyrethrin+IGR mixtures seem to have residual activity against larvae of *T. castaneum* and *T. confusum*. Recent field studies have also shown that regular applications of a pyrethrin+ methoprene mixture can suppress resident populations of *T. castaneum* (Jenson et al., 2010a). However, more detailed tests should be conducted to make an accurate assessment of the level of efficacy, especially because earlier studies have shown that *T. castaneum* is the more susceptible of the two species to pyrethrin aerosol (Arthur, 2008) and the IGRs hydroprene and pyriproxifen (Arthur and Hoernemann, 2004; Arthur et al., 2009). Although each individual mill or warehouse has its own characteristics that will affect insect control, certain common themes are present in all locations.

In the field trial with pyrethrin + methoprene, increasing the application rate of pyrethrin from 1% AI to 3% AI produced an apparent corresponding increase in residual efficacy even though the concentration of methoprene remained the same. When adult beetles were placed on these arenas at the same time as the immature stages there was no adult mortality, hence the assumption that it was the methoprene component that was providing the residual control. However, immature stages of *T. castaneum* and also *T. confusum* may be more susceptible to pyrethirn than the adults (Arthur, 2008), therefore any residual efficacy relating to the pyrethrin component would be reflected in the reduced adult emergence from exposure of eggs and larvae. Perhaps there was also an additive effect for the pyrethirn and the IGR.

The indirect methods of exposure employed for the field trials with pyrethrin and pyriproxifen, whereby the concrete arenas themselves were exposed to the aerosol, could be used for expanded field trials with other insecticides or application system. The flour apparently absorbed some of the residues from the exposed surface, and larvae encountered these residues through contact toxicity and feeding on the flour. Utilizing this procedure for evaluation of residual activity of aerosols would alleviate risks associated with bringing live insects inside an active commercial facility.

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This paper reports the results of research only. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

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Management of the yam moth, *Dasyses rugosella* Stainton, a pest of stored yam tubers (*Dioscorea* spp.) using plant products

Ashamo, M.O.*#
Department of Biology, Federal University of Technology, P.M.B 704, Akure, Nigeria.
Email: oashamo@yahoo.com

* Corresponding author
# Presenting author

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Abstract

Yams are members of the genus *Dioscorea*, which produce bulbils, tubers or rhizomes that are of economic importance. West Africa accounts for 90-95% of world production, Nigeria being the major producer. In 2004, the total world production of yam was about 47 million metric tonnes (MT), with 96% of this coming from Africa. Nigeria accounts for about 70% of world production. In spite of the great economic importance of this food item, 20-30% (about 9.4-14.1 million tonnes) is lost during storage. Storage losses of the order of 10-15% after the first three months and approaching 50% after six months have been observed. Yam tubers in storage are attacked by several moth and beetle pests. The moth pests include *Dasyses rugosella* Stainton, *Euzopherodes vapidella* Mann and *Decadarchis minuscula* Walsingham. *Dioscorea alata* L (water yam) was found to be the most susceptible species of yam to infestation by these moths. The plant powders tested for their efficacy against *D. rugosella* included *Capsicum frutescens* L. (fruit), *C. annum* Miller (fruit), *Piper guineense* Schum and Thonn (seed), *Aframomum melegueta* Schum (seed), *Allium cepa* L. (scale), *A. sativum* L. (scale), *Citrus sinensis* Osbeck (peel) and *Azadirachta indica* A. Juss (leaf). In another experiment, the oil extracts of the following plants were tested against *D. rugosella*, *Jatropha gossypifolia* L. (fruits), *Arachis hypogaea* L. (seeds), *Citrus sinensis* Osbeck (seeds), *Elaeis guineensis* Jacq (kernel), *Piper guineense* Schum and Thonn (seeds), *Aframomum melegueta* Schum (seeds) and *Adansonia digitata* L. (fruits). Results showed that powders of *C. annum* and *C. frutescens* were effective against the adult moth producing 100% mortality within 24hrs of application of powder. In addition, *P. guineense*, *A. cepa* and *A. sativum* were effective against *D. rugosella* within 3 days of application of plant powder. However, *C. annum* and *C. frutescens* were able to effectively persist for 14days after application of plant powders. There was no fecundity of the moth in sample treated with *C. annum* and *C. frutescens* while fecundity was reduced in others. The survival of the moth from eggs to adults when treated with the plant powders showed that there was significantly ($P<0.05$) more adult emergence in the control (73.3%) compared to others. Oil extracts of *Arachis hypogaea* and *E. guineensis* were effective in preventing adult emergence. This study showed that some plant products (powders of *C. annum* and *C. frutescens* and oils of *A. hypogaea, P. guineense* and *E. guineensis*) were toxic or very effective against the yam moth, *D. rugosella* and the powders can be applied on cut or damaged surfaces of yam tubers to prevent hatching of the eggs of the moth thereby helping in their management and also minimize rotting.

Keywords: Toxicity, *Dasyses rugosella*, Management, Mortality, Powder extract

1. Introduction

Yams are members of the genus *Dioscorea*, which produce bulbils, tubers or rhizomes that are of economic importance. Yams are staple food for millions of people in the tropical regions of the world, as it is the second most important tropical root crop in West Africa after cassava. West Africa accounts for 90-95% of world production, Nigeria being the major producer (Osunde, 2008). In 2004, the total world production of yam was about 47 million metric tonnes (MT), with 96% of this coming from Africa. Nigeria accounts for about 70% of world production. In spite of the great economic importance of this food item, 20-30% (about 9.4-14.1 million tonnes) is lost during storage. Storage losses reach 10-15% after the first three months and approach 50% after six months (Osunde, 2008). The heavy post harvest losses and quality deterioration caused by storage pests are major problems of agriculture in developing countries, such as Nigeria. Several viruses, bacteria, nematodes, mammals and insects frequently attack the stored yam tubers. The most important insects that attack stored yam tubers are the beetles and

moths. The moths include *Dasyses rugosella* Stainton, *Euzopherodes vapidella* Mann and *Decadarchis minuscula* Walsingham.

Several attempts have been made to resolve the problem of food production confronting the tropical countries by placing emphasis on increasing production of grain crops with lesser attention given to tubers such as yams (Osagie, 1992). Chemical method still remains the most effective means of controlling both field and storage pests. Despite the success in the control of insect pests using synthetic insecticides, there are several drawbacks such as high mammalian toxicity, high level of persistence in the environment, health hazards, toxic residues on food, adverse effects on non-target organisms and pest resistance (Sighamony et al., 1986). These parameters have necessitated the use of other control measures which have little or no negative impact on the environment and are not toxic to mammals. One solution to these problems would be to replace synthetic chemicals with compounds, which occur naturally in plants (Olaifa et al., 1987). Vegetable oils, plant powders and extracts have been used to reduce post harvest losses of cereals and grain legumes (Lale, 1992; Odeyemi, 1998; Adedire and Lajide, 1999; Ofuya et al., 2007; Nwaubani and Fasoranti, 2008). So far, many reports on deterrent activity of plant products on stored product insects have been focused on beetle pests (Lale, 1992) and very few reports exist on the efficacy against moth pests. This work investigated the control of *D. rugosella* using plant products at 28±3°C and 75±5% r.h.

2. Materials and methods

2.1. Rearing of Dasyses rugosella

The infested water yam tubers, *Dioscorea alata* that formed the initial source of culture were collected from farms and market stores around Akure, Nigeria, and were brought to the laboratory. The infested tubers were kept in 2-lt jars. Signs of moth infestation of tubers included presence of black granules of larval faecal matter held together by silken threads and the presence of empty pupal cases on the surface of the tubers. The openings of the jars were covered with muslin cloth placed with rubber bands to prevent the escape of emerged adult moths. The jars were in turn kept inside insect breeding wire mesh cages (75x60x50cm^3). The four stands of the cages were placed in Petri dishes filled with water to which a few drops of kerosene were added to prevent access of predatory ants to the cultures. The Petri dishes were refilled with water whenever they were likely to dry up. A culture of *D. rugosella* was set up and maintained with healthy and fresh tubers as old infested tubers deteriorated. The culture and experiment were maintained at 28 ± 2°C and 75 ± 5% r.h.

2.2. Preparation of plant powders and extracts

Some of the plant parts used was purchased from the local markets in Akure and some were obtained from farms located within the Federal University of Technology, Akure, Nigeria. The main criterion for selecting the plants was that they are edible and form an important part of the diet of Nigerians. The plants included *Allium cepa* L., *A. sativum* L., *Citrus sinensis* Osbeck, *Capsicum annum* Miller, *C. frutescens* L., *Azadirachta indica* A. Juss, *Piper guineense* Schum and Thonn and *Aframomum melegueta* Schum. The characteristics of the plants evaluated are shown in Table 1. The plant parts were rinsed in clear water to remove sand and other impurities, cut into smaller pieces; sun dried and pulverized using an electric blender, then sieved to pass through 1mm mesh size and kept in specimen bottles until needed.

Table 1 Plants evaluated for their insecticidal activity.

Name of Plant	Common name	Family	Parts used
Citrus sinensis Osbeck	Sweet orange	Rutaceae	Seed/peel
Allium cepa L.	Onion	Liliaceae	Bulb
Allium sativum L.	Garlic	Liliaceae	Bulb
Capsicum annum Miller	Pepper	Solanaceae	Fruit
Capsicum frutescens L.	Chilly pepper	Solanaceae	Fruit
Piper guineense Schum & Thonn	Black pepper	Piperaceae	Seed
Aframomum melegueta Schum	Alligator pepper	Zingiberaceae	Seed
Arachis hypogaea L.	Ground nut	Papilionaceae	Seed
Jatropha gossypiofoila L.	Wild cassada	Euphobiaceae	Seed
Elaeis guineensis Jacq	Oil palm kernel	Palmae	Seed
Adansonia digitata L.	Baobab tree	Bombacaceae	Seed
Azadirachta indica A. Juss	Neem	Meliaceae	Leaf

Oil was extracted from the seeds of *Adansonia digitata* L., *Arachis hypogaea* L., *Aframomum melegueta* Schum, *Citrus sinensis* Osbeck, *Elaeis guineensis* Jacq, *Jatropha gossypifolia* L. and *Piper guineense* Schum & Thonn, using soxhlet extractor; these oils were tested as insecticides against the eggs of the yam moth. The different seeds were cleaned, sun dried and pulverized into fine powder using electric blender. One hundred grams of each powdered materials was weighed into a thimble and extracted with petroleum ether in a soxhlet extractor. The extraction was carried out for about four hours. Thereafter, the thimble was removed from the units and the petroleum ether was recovered by re-distilling the content of the soxhlet extractor at 40-60^{o}C. The resulting extract was air dried in order to remove traces of the solvent.

2.3. Experimental set up

Cut tubers of yam (*D. alata*) were kept in plastic jars after drying. Plant powders weighing 0.15 and 0.25g per species were measured using a sensitive weighing balance (Mettler E200). The different plant powders were applied evenly to the cut surfaces of the tubers measuring 7cm diameter and about 10cm long. The top of the jars was covered with muslin cloth. The bottoms of the plastic jars were lined with filter papers. Ten (0-24hr old) adult *D. rugosella* (4 males, 6 females) were placed in the jars containing treated tubers of yam. This was done for all the plant materials and the treatment replicated six times. The jars were arranged in a completely randomized manner in insect breeding cages. The mortality of the adults was recorded at 1, 2 and 3 d after the application of the powder. Mortality was also recorded after reintroduction of adults at 7, 14 and 21d after the application of the powder. At the end of three days, the adults, dead or alive were removed from the jars and the fecundity of adults in treated samples and the control were determined. Similarly, twenty (0-24 h old) eggs of *D. rugosella* were introduced on top of treated tubers using the above concentrations. The jars were observed daily and the number of adults emerging from each treatment was recorded.

In another experiment, 1 mL of each oil extract was spread on 9 cm diameter filter paper inside Petri dish and allowed to stay for a few minutes. Twenty (0-24 h old) eggs of *D. rugosella* were introduced on top of the filter papers and replicated three times. Percentage hatchability was recorded. The hatched eggs were then introduced on incisions made on small yam tubers and the number of adults emerging was recorded. All data obtained were subjected to analysis of variance and where significant differences existed, treatment means were separated using the Tukey's test.

3. Results

The effect of the various plant powders on mortality of *D. rugosella* at different periods after treatment is presented in Tables 2 and 3. In each treatment, the mortality of *D. rugosella* increased gradually with time of exposure. At both treatment levels, 100% mortality was obtained in those tubers treated with *Capsicum annum* and *C. frutescens* at 1 d after application. However after 3 d of application of powders, 100% mortality was produced in those tubers treated with *C. annum*, *C. frutscens*, *A. cepa*, *A. sativum* and *P. guineense*. There was no significant difference (P>0.05) in the mortality produced by *C. sinensis*, *A. indica* and *A. melegueta*. *C. annum* and *C. frutescens* powders were still effective after 14 days of application of powders.

Table 2 Mean percentage mortality of *Dasyses rugosella* adult treated with various powders (0.15g/16g of yam).

	Post treatment period (d), mean ± SE					
Powder	**1**	**2**	**3**	**7**	**14**	**21**
Allium cepa	66.7±3.8c	100.0±0.0d	100.0±0.0c	40.0±6.7b	20.0±8.2a	13.3±6.7ab
Allium sativum	40.0±7.8c	86.7±6.7c	100.0±00c	13.3±6.7a	13.3±6.7a	13.3±6.7ab
Citrus sinensis	0.0±0.0a	26.7±6.7a	60.0±6.7b	13.3±6.7a	13.3±6.7a	13.3±6.7ab
Capsicum frutescens	100.0±0.0d	100.0±00d	100.0±0.0c	100.0±0.0d	53.3±6.7b	26.7±6.7b
Capsicum annum	100.0±0.0d	100.0±00d	100.0±0.0c	86.7±6.7c	40.0±3.8b	20.0±6.7b
Azadirachta indica	0.0±0.0a	40.0±11.5b	73.3±11.5b	13.3±6.7a	13.3±6.7a	0.0±0.0a
Piper guineense	40.0±6.7c	80.0±6.7c	100.0±0.0c	33.3±6.7b	33.3±6.7ab	13.3±6.7ab
Aframomum melegueta	13.3±1.8b	20.0±6.5a	66.7±6.7b	33.3±6.7b	13.3±6.7a	0.0±0.0a
Control	0.0±0.0a	10.0±0.0a	10.0±0.0a	10.0±0.0a	10.0±6.7a	10.0±0.0a

* Means followed by the same letter in the same column are not significantly different at 5% significance limit using Tukey's test.

Table 3 Mean percentage mortality of *Dasyses rugosella* adult treated with various plant powders (0.25g/16g of yam).

	Post treatment period (days) mean ± SE					
Powder	**1**	**2**	**3**	**7**	**14**	**21**
Allium cepa	80.0 ± 11.5c	100.0±0.0d	100.0±0.0c	66.7±11.5c	20.0±6.7b	20.0±11.5b
Allium sativum	40.0 ± 11.5b	100.0±0.0d	100.0±0.0c	33.3±6.7b	13.3±6.7a	13.3±6.8ab
Citrus sinensis	26.7 ± 6.7b	66.7±6.7c	80.0±0.0b	33.3±6.7b	13.3±6.7a	0.0±0.0a
Capsicum frutescens	100.0 ± 0.0d	100.0±0.0d	100.0±0.0c	100.0±0.0d	73.3±6.7c	26.7±6.7b
Capsicum annum	100.0 ± 0.0d	100.0±0.0d	100.0±0.0c	100.0±0.0d	53.3±6.7bc	13.3±6.7b
Azadirachta indica	0.0 ± 0.0a	60.0±0.0c	80.0±0.0b	33.3±6.7b	13.3±6.1a	10.0±0.0a
Piper guineense	66.7 ± 6.7c	100.0±0.0d	100.0±0.0c	46.7±6.7b	40.0±11.0b	13.3±6.7ab
Aframomum melegueta	20.0±11.5ab	33.3±6.7b	86.7±6.7b	40.0±0.0b	13.3±6.7a	13.3±6.7ab
Control	0.0±0.0a	10.0±0.0a	10.0±0.0a	10.0±0.0a	10.0±6.7a	10.0±0.0a

*Means followed by the same letter in the same column are not significantly different at 5% significance limit using Tukey's test.

The fecundity of *D. rugosella* showed that no eggs were laid on samples treated with *C. annum* and *C. frutescens* at both treatment levels (Table 4). When eggs were introduced on treated tubers of yam, significantly ($P<0.05$) more adult emergence was recorded in the control than treated samples (Table 5), although the value was lowest in samples treated with *C. frutescens*.

Table 4 Fecundity of *Dasyses rugosella* treated with various plant powders.

	0.15g/16g of yam		**0.25g/16g of yam**	
Plant	**Mean total no of eggs laid ± SE**	**No of eggs per individual**	**Mean total no of eggs laid ± SE**	**No of eggs per individual**
Allium cepa	40.0 ± 1.8b	6.7	0.0 ± 0.0a	0.0
Allium sativum	60.0 ± 3.2b	10.0	23.4 ± 1.7b	3.8
Citrus sinensis	160.2 ± 5.1c	26.7	62.6 ± 4.7c	10.4
Capsicum frutescens	0.0 ± 0.0a	0.0	0.0 ± 0.0a	0.0
Capsicum annum	0.0 ± 0.0a	0.0	0.0 ± 0.0a	0.0
Azadirachta indica	200.0 ± 11.1c	33.3	119.4 ± 5.2d	19.9
Piper guineense	60.0 ± 5.0b	10.0	55.4 ± 4.3c	9.2
Aframomum melegueta	170.0 ± 7.2c	28.3	72.0 ± 3.5c	12.0
Control	437.4±46.5d	72.9	437.4 ± 46.5e	72.9

*Means followed by the same letter in the same column are not significantly different at 5% significance level using Tukey's test.

Table 5 Survival of *Dasyses rugosella* eggs treated with various powders.

Powder	**No of eggs incubated**	**0.15g/16g of yam % adult emergence**	**0.25g/16g of yam % adult emergence**
Allium cepa	20	26.7 ± 1.6c	25.0 ± 2.5c
Allium sativum	20	30.0 ± 2.1b	30.0 ± 1.9b
Citrus sinensis	20	43.3 ± 1.9b	41.7 ± 2.6b
Capsicum frutescens	20	13.3 ± 2.0d	10.0 ± 1.9d
Capsicum annum	20	15.0 ± 2.0d	15.0 ± 1.9d
Azadirachta indica	20	36.7 ± 3.5b	35.0 ± 3.5b
Piper guineense	20	25.0 ± 1.9c	21.7 ± 2.3c
Aframomum melegueta	20	33.0 ± 2.1b	35.0 ± 3.8b
Control	20	73.3 ± 1.6a	73.3 ± 1.6a

*Means followed by the same letter in the same column are not significantly different at 5% significance level using Tukey's test.

The bioassays also showed that some plant powders were effective in controlling *D. rugosella*. Also oils from seeds of *Arachis hypogaea*, *Elaeis guineensis, P. guineense* and *Jatropha gossypifolia* were effective in controlling the eggs of *D. rugosella* (Table 6).

Table 6 Effect of oil extract on egg of *Dasyses rugosella.*

Name of extract	% Hatchability	% Adult emergence
Jatropha gossypiofolia	50.0 ± 8.7d	46.7 ± 3.6d
Arachis hypogaea	3.3 ± 0.7a	0.0 ± 0.0a
Citrus sinensis	53.3 ± 1.7d	48.7 ± 6.7d
Elaeis guineensis	16.7 ± 6.7b	10.0 ± 2.3b
Piper guineense	16.7 ± 4.7b	6.7 ± 1.3b
Aframomum melegueta	40.0 ± 3.6c	33.3 ± 6.7c
Adansonia digitata	53.3 ± 2.5d	46.7 ± 2.7d
Control	73.0 ± 7.2e	53.3 ± 3.3e

*Means followed by the same letter in the same column are not significantly different at 5% significance level using Tukey's test.

4. Discussion

The two *Capsicum* species, *C. annum* and *C. frutescens* powders were very effective against *D. rugosella* causing 100% mortality of adults within 1 d of application. They also reduced oviposition and suppressed adult emergence showing that they probably have oviposition-deterrent and ovicidal properties. The observed activity may be due to the "pepperish" nature and pungency of the *Capsicum* species. The pungency of *Capsicum* species was attributed to capsacin (Miyakado et al., 1979). The powders of *A. cepa*, *A. sativum* and *P. guineense* were also slightly effective against the moths from this study producing 100% mortality of adults at 3 d after application. These powders probably acted through contact and fumigant mode of action. The biological activity of *P. guineense* was ascribed to the presence of chavicin and piperine, an unsaturated amide (Lale, 1992).

Plant products have been extensively used against Coleopteran pests such as *Callosobruchus maculatus* (F.) and *Sitophilus zeamais* Motschulsky (Ashamo and Odeyemi, 2001; Maina and Lale, 2005; Oparaeke and Dike, 2005; Kachhwaha et al., 2006; Ofuya et al., 2007). However there is paucity of information on the control of moth pests of yam tubers using plant products. Ofuya et al. (2007) observed that powders from dry flower buds of *Eugenia aromatica* Baill. and dry fruits of *P. guineense* were effective against *C. maculatus*.

The oil of *A. hypogaea* was very effective in suppressing adult emergence in *D. rugosella* with no adult emergence while the oil from *P. guineense* and *E. guineensis* were also effective against the eggs since adult emergence was significantly lower than that in the control. Many vegetable oils have been screened for use in preventing post-harvest losses due to insects (Golob and Webly, 1980; Don Pedro, 1989). For example, Don Pedro (1989) showed that some vegetable oils (ground nut, traditional coconut, industrial coconut, palm, and shark silver oil) were able to kill eggs of *Dermestes maculatus* Degeer on dried fish. The oil probably blocked the minute perforations on the surface of the eggs causing suffocation due to lack of oxygen.

Since adult moth do not feed on food commodities but only visit to deposit their eggs the use of oviposition inhibitors would be advantageous for the management of lepidopteran pests. The powders of these plants could be applied to cuts or bruises on the surface of yam tubers before storage. Local farmers have been known to apply neem powder to cuts on yam tubers. All the plants whose powders were used are edible and many are spices and form an important component in the diet of tropical people and are therefore likely to be safe for human consumption at least at the rates applied in this study. Further research is being carried out on the *Capsicum* species to determine their bioactive compounds.

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Effect of four powdered spices as repellents against adults of *Rhyzopertha dominica* (F.), *Sitophilus granarius* (L.) and *Tribolium castaneum* (Herbst) in laboratory conditions

Shayesteh, N.*#[1], Ashouri, S.[2]
[1] Department of Plant Protection, Agricultural and Natural Resources Faculty, Islamic Azad University, Branch of Mahabad, Iran. Email: profshayesteh@yahoo.com
[2] Department of Entomology, Faculty of Agriculture, Urmia University, Urmia, Iran.

* Corresponding author
# Presenting author

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Abstract

Studies were conducted to test the repellency of four powdered spices, black pepper (*Piper nigrum.*), chili pepper (*Capsicum annuum*), cinnamon (*Cinnamomum aromaticum*) and turmeric (*Curcuma longa*), against three stored-product insects, the lesser grain borer, *Rhyzopertha dominica*, the granary weevil, *Sitophilus granarius* and the red flour beetle, *Tribolium castaneum*. The cup bioassay technique was used, to determine the response of insects to potential repellents by measuring their movement from treated grain. The device is made of galvanized screening with 2 mm perforations shaped into a cylinder of 6 cm diameter and 15 cm high, with a mesh bottom, and is placed in the centre portion of plastic container of 15 cm diameter and 15 cm high. The powdered spices were poured into 200 g of wheat mass by a long-stemmed funnel at concentrations of 0, 0.25, 0.75, 1.5 and 2.5% on (w/w) basis. Twenty adults of three species are released into the centre of the grain mass in the container through a long-stemmed funnel. The experiments were conducted at room conditions. The number of trapped insects was determined at 3 different intervals after the introduction of the insects. Results showed that all tested plant powders had repellent activity against the three stored-product insects. Adults of *S. granarius* repelled faster, followed by *T. castaneum* and *R. dominica*. At the highest concentrations and intervals, wheat grains treated with cinnamon powder were the most repellent to adults of *S. granarius* (up to 92.5% after 1 h), followed by chili pepper treatment for *T. castaneum* (up to 72.5% after 6 h) and black pepper treatment for *R. dominica* (up to 58.75% after 24 h).

Keywords: Repellency, Spices, Rhyzopertha dominica, Sitophilus granarius, Tribolium castaneum

1. Introduction

Insects are one of the basic problems of stored grains throughout the world, due to the quantitative and qualitative losses they cause (Fields, 2006). The efficient control of stored grain pests has long been the aim of entomologists throughout the world. Synthetic chemical pesticides have been used for many years to control stored grain pests (Salem et al., 2007). However, the potential hazards for mammals from synthetic insecticides, increased concern by consumers over insecticide residues in processed cereal products, the occurrence of insecticide-resistant insect strains, the ecological consequences, the increased of the cost of application and the precautions necessary to work with traditional chemical insecticides, call for new approaches to control stored-product insect pests (Aslam et al., 2002; Udo, 2005; Fields, 2006; Salem et al., 2007; Mahdi and Rahman, 2008). Therefore, there is a need to look for alternative organic sources that are readily available, affordable, less toxic to mammals and less detrimental to the environment (Udo, 2005).

The use of plant materials as traditional protectants of stored products is an old practice used all over the world (Aslam et al., 2002). The protection of stored products generally involves mixing grains with plant-based protectants (Tapondjou et al*.,* 2002).

In fact, management of stored product pests using materials of natural origin is nowadays the subject which received much attention, because of their little environmental hazards and low mammalian toxicity (Nadra, 2006). Previous research indicated that some plant powders, oils and extracts have strong effects on stored grain insects such as high toxicity and the inhibition of reproduction (Emeasor et al*.*, 2005; Nadra, 2006). In addition to high toxicity to insects, many natural products are also repellent or attractive (Mohan and Fields, 2002). Peasant farmers and researchers often claim successful use of material of plant origin in insect pest control including spices and powders of plant parts (Akinneye et al., 2006).

The mode of action of powders vary, but with low to moderate dosages, the effect is repellent or toxic, never mechanical (Rajapakse, 2006).

Spices are dried seed, fruit, root, bark or vegetative substance used in nutritionally insignificant quantities as a food additive for flavoring. Many of these substances have other uses, e.g. food preservation, as medicine, in religious rituals, as cosmetics, in perfumery or as vegetables (Mahdi and Rahman, 2008). The use of spices is less costly, easily available for the developing world, safer and do not cause hazards in the commodity (Aslam et al., 2002; Mahdi and Rahman, 2008). Many repellents have been tested using laboratory bioassays; however, these tests do not mimic field conditions or require large amounts of grain to be treated. In the present study, we used a simple, rapid and reliable technique to determine if specific plant products are repellent to stored-product insects and exploits the oriented movement of insects away from or towards the product (Mohan and Fields, 2002).

2. Materials and methods

The trials were conducted at the laboratory of the Department of Entomology, University of Urmia, Iran, during 2008-2009.

2.1. Preparation of spices and stored products

Black pepper (*Piper nigrum* L.) (Piperaceae) seed, chili pepper (*Capsicum annuum* L.) (Solanaceae) fruit, cinnamon (*Cinnamomum aromaticum* Ness.) (Lauraceae) bark and turmeric (*Curcuma longa* L.) (Zingiberaceae) rhizome powders were used in this investigation. They were selected based on the assumption of absence of mammalian toxicity owing to their use as popular spices in several diets. The spices and wheat kernels were purchased from a local market in Urmia, Iran. The spices were bought dry and brought to the laboratory where they were passed through a 40-mesh sieve to obtain a fine dust before application to the grains. The powders were carefully placed inside airtight containers and kept until the beginning of the experiments. Wheat grains were disinfested by keeping them in a freezer at a temperature of –18°C for 24 hours, and then conditioned to room temperature before being used for experimental purposes.

2.2. Rearing of experimental insects

Local strains of three important stored-product pests were obtained from wheat flour factories, Urmia, Iran. The granary weevil (*Sitophilus granarius* (L.)) (Curculionidae) and the lesser grain borer (*Rhizopertha dominica* (F.)) (Bostrychidae) were reared on uninfected whole kernels of wheat, and the red flour beetle (*Tribolium castaneum* (Herbst)) (Tenebrionidae) was cultured on wheat flour mixed with yeast (10:1 w/w). Insects were released at the rate of 200 adults in 1 L jars containing 400 kg of wheat grains or flour. The jars were covered with muslin cloth and tied with a rubber band and kept in an incubator maintained at a temperature of 28 ± 1°C and 70 ± 5% r.h. After two weeks of oviposition, the parent insects were separated and egg laid materials were maintained and re-cultured to produce newly emerged adults of same generation. For this purpose, the insects emerged after four weeks were removed. One-14 day old adults were used in the experiments.

2.3. Repellency tests

The cup bioassay technique (Mohan and Fields, 2000) determines the response of insects to potential repellents by measuring their movement from treated grain (Pretheep Kumar et al., 2004). The device is made of galvanized screening with 2 mm perforations, which allows only insects and not grain to pass through, shaped into a cylinder of 6 cm diameter and 15 cm high, with a mesh bottom, and is placed in the centre portion of plastic container of 15 cm diameter and 15 cm high. This plastic cup collects the insects that left through the sides and bottom. The powdered spices were poured into 200 g of wheat mass by a long-stemmed funnel at concentrations of 0.25, 0.75, 1.5 and 2.5% on the weight of plant material/weight of grain (w/w) basis. Controls without treatment were maintained to record natural movement. Twenty adults of three species are released into the centre of the grain mass in the container through a long-stemmed funnel. The container was covered by a muslin cloth to prevent the escape of flying insects. All experiments were conducted at a room conditions. The repellency was measured in terms of speed of response shown by the insects in their movement away from the treated source or grain. The number of trapped insects was determined at 3 different intervals after the introduction of the

insects, 1, 6 and 24 h for *R. dominica*, 15, 30 and 60 min for *S. granarius* and 1, 3 and 6 h for *T. castaneum*, respectively. There were four replicates per treatment.

2.4. Statistical analysis

Data were transformed with an arcsine (percentages) method before ANOVA because the percentage data ranged from 0 to 100%. All counts were submitted to a two-way ANOVA ($P<0.05$), by using the MSTATC statistical package. The experimental design was completely randomized design. Means of the four replicates of treatments were compared using Tukey's multiple comparison tests for significance of their differences.

3. Results

Results showed that all tested powdered spices possess repellent activity against the three stored-product insects. The repellency of these powders increased with the increase in dosage as well as the increase in the period of exposure to the plant powders. Adults of *S. granarius* were repelled most quickly followed by *T. castaneum* and *R. dominica*, after 1, 6 and 24 h, respectively. At the highest concentrations and time periods, wheat grains treated with cinnamon powder were the most repellent to adults of *S. granarius* (up to 92.5% after 1 h), followed by chili pepper treatment for *T. castaneum* (up to 72.5% after 6 h) and black pepper treatment for *R. dominica* (up to 58.7% after 24 h). The order of repellency effects of four powdered spices at the highest dosages and period of time on *R. dominica* were black pepper>cinnamon>chili pepper>turmeric, on *S. granarius* were cinnamon>black pepper>chili pepper>turmeric and on *T. castaneum* were chili pepper>black pepper>cinnamon>turmeric.

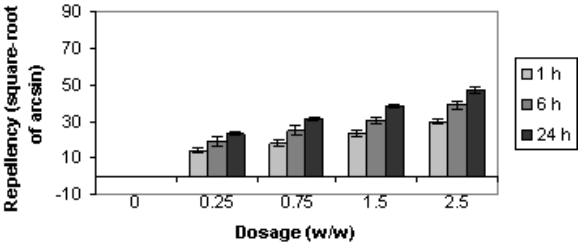

Figure 1 Mean repellency of cinnamon powder to *Rhyzopertha dominica* adults.

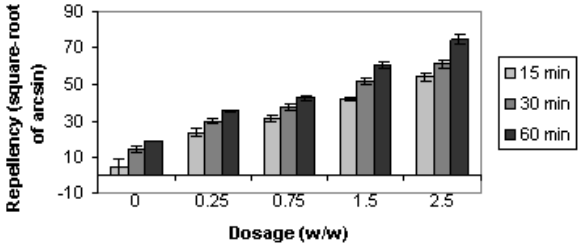

Figure 2 Mean repellency of cinnamon powder to *Sitophilus granarius* adults.

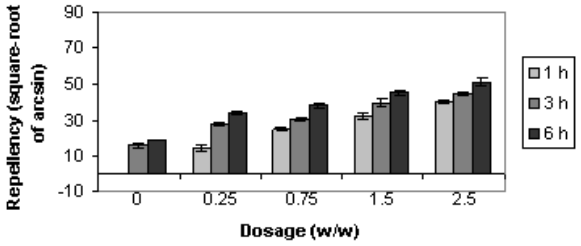

Figure 3 Mean repellency of cinnamon powder to *Tribolium castaneum* adults.

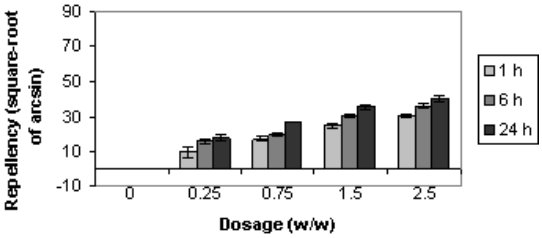

Figure 4 Mean repellency of turmeric powder to *Rhyzopertha dominica* adults.

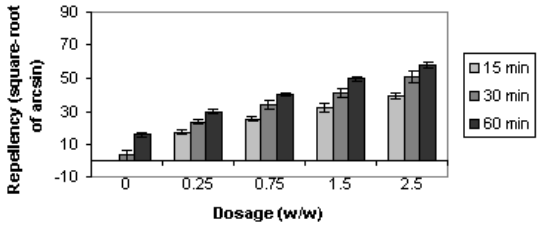

Figure 5 Mean repellency of turmeric powder to *Sitophilus granarius* adults.

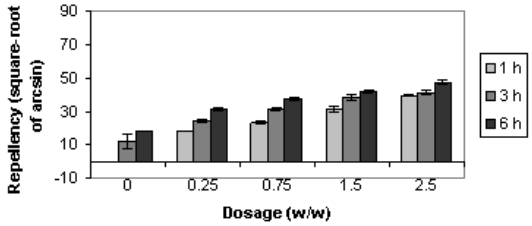

Figure 6 Mean repellency of turmeric powder to *Tribolium castaneum* adults.

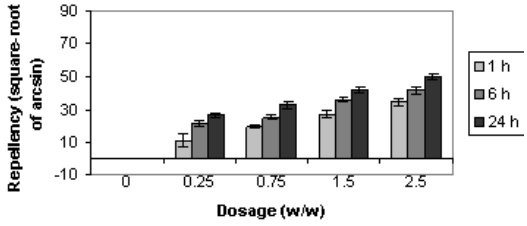

Figure 7 Mean repellency of black pepper powder to *Rhyzopertha dominica* adults.

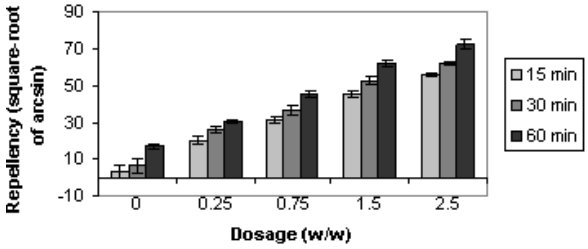

Figure 8 Mean repellency of black pepper powder to *Sitophilus granarius* adults.

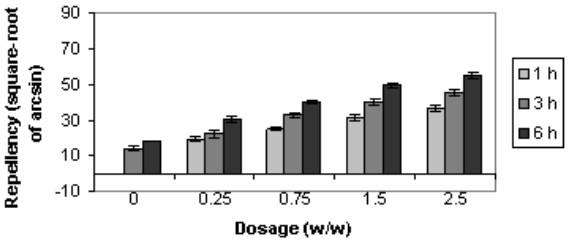

Figure 9 Mean repellency of black pepper powder to *Tribolium castaneum* adults.

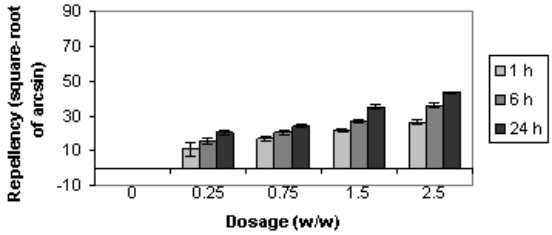

Figure 10 Mean repellency of red pepper powder to *Rhyzopertha dominica* adults.

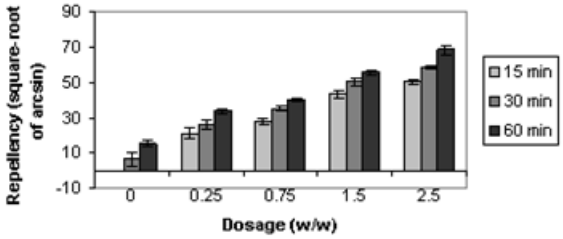

Figure 11 Mean repellency of red pepper powder to *Sitophilus granarius* adults.

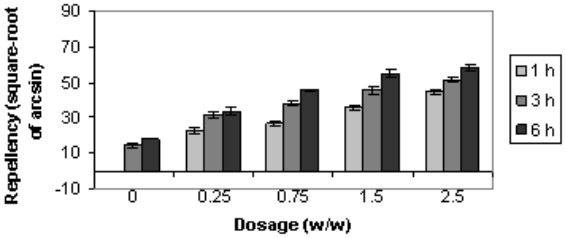

Figure 12 Mean repellency of red pepper powder to *Tribolium castaneum* adults.

4. Discussion

The cup bioassay technique used to test the repellency of plant products against the stored product insects closely mimics the grain storage conditions. The main advantage of the cup bioassay technique is that it is in the grain medium and actual storage conditions are taken into consideration. The cup bioassay technique can be used for the preliminary screening of plant products for their repellency against stored-product insects. This will help to save time in identifying the insecticidal property of plant products (Pretheep Kumar et al., 2004).

A good level of repellency was achieved with powders of these four spices, especially on the *S. granarius* adults, which indicates that would be highly effective in preventing the infestation by *S. granarius*. Cinnamon powder was found to be the highest repellent agent in comparison to all other powders on *S. granarius*. In addition, the repellency effects of cinnamon powder on adults of *R. dominica* and *T. castaneum* indicate that it caused noticeable repellent effects. The repellency of black pepper and chili pepper also showed the effective result to the insects at 2.5%. Turmeric powder produced the least effect to the three species examined.

Unfortunately, there is still inadequate information regarding the effects of these powders on *R. dominica, S. granarius* and *T. castaneum*. Nevertheless, the findings are similar with the observation of Udo (2005) who reported that powder of *P. guineense* had the highest repellent effect of 80% on maize weevil adults, *Sitophilus zeamais* (Mots.) (Curculionidae), among five different spices. It also corresponds to the studies of Salvadores et al. (2007) who showed that the powders of *P. nigrum*, *C. annuum* and *Cinnamomum zeylanicum* Blume had a repellent effect on *S. zeamais*.

Based on the present findings, it could be concluded that plant powders pose potential in protecting wheat against three species of tested insects. Regarding the side effects of synthetic pesticides, the study demonstrates that these plant powders can play an important role in protection of wheat from insect invasion during storage. This technology is cheap, safe, environmentally friendly and easy to adopt by small-scale farmers.

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Efficacy of insecticides for control of stored-product psocids

Athanassiou, C.G.[1,2,3,*#], Arthur, F.H.[1], Throne, J.E.[1], Opit, G.P.[1,4], Hasan, M.M.[5], Aikins, M.J.[5], Phillips, T.W.[5], Kavallieratos, N.G.[6]
[1] Center for Grain and Animal Health Research, USDA-ARS, 1515 College Avenue, Manhattan, Kansas, 66502, USA Email: athanassiou@agr.uth.gr
[2] Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, 75 Iera Odos str., 11855, Athens, Greece
[3] Current address: Laboratory of Entomology and Applied Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Phytokou st., Nea Ionia, Magnissia, 38446, Greece
[4] Department of Entomology and Plant Pathology, Oklahoma State University, 127 Noble Research Center, Stillwater, Oklahoma, 74078-3033, USA
[5] Department of Entomology, Kansas State University, 123 Waters Hall, 66506-4004, Manhattan, Kansas, USA
[6] Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta str., Kifissia, 14561, Attica, Greece

* Corresponding author
# Presenting author

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Abstract

A series of experiments were carried out between 2007 and 2009 to test the efficacy of selected insecticides against several stored-product psocids. Three series of experiments were conducted against *Liposcelis* spp. (Psocoptera: Liposcelididae) and *Lepinotus reticulatus* (Psocoptera: Trogiidae). In the first series of tests, contact insecticides were evaluated in the laboratory as grain protectants. Among these insecticides, diatomaceous earth (DE), natural pyrethrum, and the insect growth regulator methoprene were unable to control psocid populations on wheat, rice, and maize. For the same commodities, spinosad was effective against *L. reticulatus*, but was effective for *Liposcelis entomophila* only on maize; spinosad was not effective against *Liposcelis bostrychophila* and *Liposcelis paeta*. Chlorpyriphos-methyl + deltamethin and pirimiphos-methyl were very effective for all species tested. In the second series of tests, sulfuryl fluoride (SF) was tested against *L. paeta* eggs, nymphs, and adults, and *Liposcelis decolor* eggs and adults. Nymphs and adults were very susceptible; for most species mortality was 100%, after 48 h of exposure to SF doses ranging between 4 and 8 g of SF/m^3. In contrast, eggs were less susceptible to SF, and 100% mortality after 48 h of exposure was recorded only at doses ranging between 24 and 96 g of SF/m^3. In the third series of experiments, several contact insecticides were evaluated as surface treatments on concrete. In these tests, pyriproxifen and esfenvalerate provided poor control of psocids. The results of the above tests indicate that *Liposcelis* spp. and *L. reticulatus* were generally less susceptible than other major stored-product insect species to several insecticides, and susceptibility level is determined by the target species, the insecticide, and the commodity.

Keywords: Psocoptera, Stored grains, Grain protectants, Sulfuryl fluoride

1. Introduction

Currently, stored-product psocids are emerging pests in stored grains and related amylaceous products (Throne et al., 2006; Nayak, 2006). Psocids are generally considered as secondary pests, unable to infest sound grains. However, recent studies indicate that psocids can infest sound kernels (Nayak et al., 2005; Athanassiou et al., 2009b), and can develop resident populations (Kučerová, 2002; Athanassiou et al., 2010). Throne et al. (2006) recorded several psocid species in steel bins containing wheat and in empty bins. Opit et al. (2009) found that the peak of psocid presence was during autumn, especially in the center of the grain mass, where temperature and moisture content were higher in comparison with the peripheral layers of the grain bulks. In the laboratory, Kučerová (2002) found that *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae) could cause about 10% grain loss after three months of infestation. Apart from the quantitative losses, several psocid species are responsible for the development of allergies (Turner and Ali, 1996), the transfer of fungal spores and other microorganisms, and qualitative degradations (Obr, 1978).

Fumigants and grain protectants are used to control psocids, however, several psocid species are resistant. For example, *L. bostrychophila* is resistant to phosphine, especially in the egg stage (Nayak et

al., 1998, 2002; Dou et al., 2006). Nayak et al. (1998) found that *Liposcelis entomophila* (Enderlein) and *Liposcelis paeta* Pearman were tolerant to the organophosphate (OP) chlorpyriphos-methyl. Similarly, *L. bostrychophila*, *L. entomophila*, and *L. paeta* were tolerant to the pyrethoids deltamethrin and bioresmethrin (Nayak et al., 1998) and the carbamate carbaryl (Nayak et al., 2002), while *L. bostrychophila* was resistant to the OP dichlorvos (Dou et al., 2006). In the case of insect growth regulators (IGRs), *L. bostrychophila* was tolerant to fenoxycarb (Bucci, 1994), while *L. entomophila*, *L. paeta*, and *L. bostrychophila* were tolerant to methoprene (Nayak et al., 1998). Spinosad, which is a bacterial-based insecticide, provided moderate control against *L. bostrychophila*, *L. paeta*, and *Liposcelis decolor* (Pearman) (Psocoptera: Liposcelididae) (Nayak et al., 2005). Hence, it is evident that psocid control in stored-product facilities is problematic and requires additional investigation. Between 2007 and 2009, an extensive series of laboratory bioassays were done at the USDA-ARS Center for Grain and Animal Health Research (CGAHR), and Department of Entomology at Kansas State University, both in Manhattan, KS, USA, to evaluate several insecticides against the most common stored-product psocid species. An extensive summary of these results is presented in this paper.

2. Materials and methods

2. 1. Insects

The psocid species used in the bioassays were *L. bostrychophila*, *L. entomophila*, *L. paeta*, *L. decolor*, and *Lepinotus reticulatus* Enderlein (Psocoptera: Trogiidae). All insects were reared on a mixture of 97% cracked wheat kernels, 2% rice krispies (Kellogg Company, Battle Creek, MI), and 1% wheat germ at 30°C and 75% r.h. Adults used in bioassays were <4 wk-old and obtained following procedures described by Opit and Throne (2008b).

2.2. Efficacy of neurotoxic grain protectants

Four insecticides were applied at the US labeled rates: pirimiphos-methyl (Actellic 5E, Agriliance, USA), chlorpyriphos-methyl + deltamethrin (Storicide II, Bayer Crop Science, USA), spinosad (NAF 313, Dow Agrosciences, USA), and natural pyrethrum (PyGanic Pro SC, MGK Co, USA) to untreated, clean, and infestation-free wheat, rice, or maize (13.5% mc). Pirimiphos-methyl was used only on maize at 8 ppm, Storicide II was used only on wheat and rice at 3 ppm chlorpyriphos-methyl and 0.5 ppm deltamethrin, spinosad was used at 1 ppm in all three grains, and pyrethrum was used at 1.6 ppm on wheat and maize and 2.2 ppm on rice. The psocid species used in these tests were *L. reticulatus*, *L. entomophila*, *L. bostrychophila*, and *L. paeta*, and the bioassays were carried out at 30°C, 70% r.h. and continuous darkness. Ten g samples were taken from treated lots of grain and placed in a small cylindrical plastic vials (3 cm diameter by 8 cm in height). For each psocid species, grain, and pesticide combination, ten adult females were placed in a vial. There were three replications. Mortality was checked after 14 d of exposure, while progeny production was checked after 30 d (Athanassiou et al., 2009a). For each species and commodity, data were submitted to one-way ANOVA and means separated using Tukey-Kramer (HSD) test (alpha = 0.05).

2.3. Efficacy of diatomaceous earths (DEs)

DE formulations Insecto (Insecto Natural Products Inc., USA), Protect-It (Hedley Technologies Ltd., Canada), and Dryacide (Entosol Ltd., Australia) were tested at 500, 400 and 1000 ppm, respectively on wheat, rice, and maize against *L. entomophila*, *L. reticulatus*, and *L. decolor*. Psocids obtained and tested as described above, and grains treated as described in Athanassiou et al. (2009b). Mortality was corrected for control mortality by using Abbott's formula (Abbott, 1925). Mortality and progeny production were analysed by species using two-way ANOVA, commodity and DE as main effects, and HSD test.

2.4. Efficacy of methoprene

Efficacy of methoprene (Diacon II, Wellmark International, USA) against *L. bostrychophila*, *L. entomophila*, and *L. reticulatus* on wheat, rice, and maize was evaluated as described above except three dose rates (1, 5, and 10 ppm) were tested, grain was held at 27.5°C and 75% r.h., and adult progeny production was evaluated after 40 d. The data were analyzed by species using two-way ANOVA, commodity and application rate as main effects, and HSD test.

2.5. Efficacy of pyriproxifen and esfenvalerate

Pyriproxifen (Archer, Syngenta, USA) efficacy against *L. bostrychophila, L. paeta,* and *L. decolor* was evaluated in Petri dishes with concrete bottoms (2.3 mg of active ingredient per m^2). Ten 10 young nymphs obtained as described above, were added to dishes and treated as follows: a) dishes sprayed before introduction of psocids, b) dishes sprayed after introduction of psocids, c) dishes containing ten cracked wheat kernels sprayed before introduction of psocids, and d) dishes containing ten cracked wheat kernels sprayed after introduction of psocids. Dishes were then held as described in previous experiment, and number of live adults recorded 28 d after spraying. Data were analyzed by species by using one-way ANOVA, and HSD test. Efficacy of aerosol applications of esfenvalerate (Conquer, Paragon Professional Pest Control Products, USA) against *L. bostrychophila, L. decolor,* and *L. paeta* was evaluated by exposed them on concrete dishes placed in sheds, and treated at a rate of 29.6 mL / 28.3 m^3 of airspace for a period of two hours. Control dishes were held in a separate untreated shed. Characteristics of the sheds can be found Toews et al. (2009). A replicate consisted of 20 adults in each of four dishes. The dishes containing the psocid species were examined immediately after treatment, and mortality recorded. Approximately 5 g of cracked wheat was then added to each dish, and then dishes were held at 27°C and 60% r.h. for 48 h and mortality assessed again. Data were analyzed using the General Linear Models (GLM) procedure of SAS (SAS Institute), with treatment and species as main effects.

2.6. Efficacy of sulfuryl fluoride (SF)

Efficacy of the fumigant SF (ProFume, Dow AgroSciences, USA) at doses of 2, 4, 6, 24, 48, 72, and 96 g/m^3, was evaluated against *L. paeta* eggs, nymphs, and adults and *L. decolor* eggs and adults. Large jars were used as the experimental chambers, 10 adults, nymphs, or eggs in small cylindrical glass vials were placed in each jar. Mortality of nymphs and adults was assessed 48 h later. In the case of eggs, vials were transferred to the laboratory at 30°C and 75% r.h., and egg hatch observed daily.

3. Results

3.1. Efficacy of neurotoxic grain protectants

For all four psocid species, mortality was approximately 100% with little or no progeny production when exposed to chlorpyriphos-methyl + deltamethrin on wheat, and pirimiphos-methyl or spinosad on maize (Table 1). Conversely, pyrethrum efficacy and impact on progeny production was low on all commodities. Spinosad efficacy varied among insecticides, psocid species and commodity. Within a psocid species, progeny production in controls varied considerably among the different commodities.

3.2. Efficacy of Des

In general Des did nor provide the levels of mortality obtained with some of the protectants reported above; ranging from 37.5 to 92.6% mortality (Table 2). The three Des did not differ in psocid mortality, except for *L. entomophila* on treated wheat, where Dryacide had significantly higher mortality than Protect-It, and *L. reticulatus* on treated maize, where Dryacide had greater mortality than the other two Des. *L. reticulatus* was most susceptible to DE of the species tested, mortality exceeded 75% in all cases and *L. decolor* was the most tolerant, given that mortality did not reach 50% for any of the DE-commodity combinations tested. Generally, the presence of DEs significantly reduced progeny production in comparison with the control, for all species tested (Table 2). However, progeny production in the treated grains was still high, and no significant differences were noted among DEs.

3.3. Efficacy of methoprene

Generally, for *L. bostrychophila*, with the exception of rice at 1 ppm, no significant differences were noted in adult progeny among methoprene doses for the three grains examined (Table 3). For *L. entomophila*, with the exception of maize, an increase in dose significantly reduced progeny production. Progeny production was generally higher on rice than in the other two commodities. For *L. paeta*, methoprene dose significantly affected progeny production on treated rice, but there was no difference in progeny production between 5 and 10 ppm. In contrast, progeny production was not affected by methoprene application rate on wheat and maize.

Table 1 Mean ± SE mortality (%) of psocid adults after 14 d of exposure, and mean progeny production (live individuals/vial ± SE) after 30 d of exposure in the treated and untreated commodities (within each column and commodity, means followed by the same letter are not significantly different; Tukey-Kramer (HSD) at $P < 0.05$).

Commodity	Treatment	*L. reticulatus*		*L. entomophila*		*L. bostrychophila*		*L. paeta*	
		14 d	30 d	14 d	30 d	14 d	30 d	14 d	30 d
Wheat	Chlorpyriphos-methyl + deltamethrin	100.0 ± 0.0a	0.2 ± 0.2a	100.0 ± 0.0a	0.2 ± 0.1a	98.9 ± 1.1a	0.7 ± 0.6a	100.0 ± 0.0a	0.2 ± 0.1a
	Spinosad	96.7 ± 2.4a	0.0 ± 0.0a	84.4 ± 7.8b	7.7 ± 5.9ab	75.6 ± 10.2b	42.3 ± 18.0b	55.6 ± 11.5b	40.1 ± 12.2b
	Pyrethrum	65.7 ± 13.5b	11.6 ± 6.3b	55.6 ± 11.8c	26.4 ± 9.0b	30.0 ± 11.1c	52.0 ± 21.0b	14.4 ± 4.7c	11.6 ± 2.1c
	Control	21.1 ± 7.4c	60.8 ± 11.7c	13.3 ± 4.7d	84.0 ± 21.5c	6.8 ± 3.3d	161.8 ± 21.1c	5.6 ± 1.8c	93.1 ± 15.2d
Rice	Chlorpyriphos-methyl + deltamethrin	100.0 ± 0.0a	1.6 ± 1.0a	100.0 ± 0.0a	0.0 ± 0.0a	100.0 ± 0.0a	0.3 ± 0.3a	100.0 ± 0.0a	0.8 ± 0.6a
	Spinosad	71.1 ± 13.0b	3.3 ± 1.0a	52.3 ± 4.6b	6.1 ± 1.9b	45.7 ± 10.1b	106.9 ± 14.3b	18.8 ± 6.4b	35.0 ± 4.4b
	Pyrethrum	14.3 ± 8.8c	91.1 ± 13.9b	16.7 ± 5.8c	82.1 ± 17.6c	26.5 ± 9.6bc	134.5 ± 19.3b	31.1 ± 12.5b	41.3 ± 7.2b
	Control	15.5 ± 4.1c	115.6 ± 13.5b	10.0 ± 4.7c	81.9 ± 9.5c	9.7 ± 2.4c	298.5 ± 49.0c	3.4 ± 1.6c	93.7 ± 12.9c
Maize	Pirimiphos-methyl	100.0 ± 0.0a	0.0 ± 0.0a	100.0 ± 0.0a	0.0 ± 0.0a	100.0 ± 0.0a	0.0 ± 0.0a	100.0 ± 0.0a	0.0 ± 0.0a
	Spinosad	100.0 ± 0.0a	0.1 ± 0.1a	98.9 ± 1.1a	0.3 ± 0.2a	76.4 ± 7.7b	10.9 ± 6.8b	37.8 ± 11.4bc	8.2 ± 1.8bc
	Pyrethrum	86.7 ± 5.8b	0.2 ± 0.2a	60.0 ± 12.5b	8.5 ± 1.6b	23.3 ± 7.8c	1.9 ± 0.4ab	18.9 ± 5.9cd	2.7 ± 1.8ab
	Control	61.1 ± 11.9c	29.7 ± 8.3b	14.4 ± 4.1c	65.5 ± 14.1c	13.5 ± 5.3c	82.7 ± 16.0c	14.1 ± 4.1d	18.1 ± 6.4c

Table 2 Mean ± SE mortality (%) after 14 d of exposure and mean progeny production (number of individuals/vial ± SE) for three psocid species on three commodities treated with three DE formulations (means for a species within each column followed by the same letter are not significantly different; where no letters exist, no significant differences were noted; Tukey-Kramer (HSD) at $P < 0.05$).

Species	Treatment	Wheat		Rice		Maize	
		Mortality	Progeny	Mortality	Progeny	Mortality	Progeny
L. entomophila	Control	-	153.7 ± 36.3a	-	201.1 ± 28.4a	-	117.2 ± 20.2a
	Protect-It	63.6 ± 4.8b	37.3 ± 4.6b	73.5 ± 4.0	54.4 ± 12.8b	60.5 ± 7.5	40.0 ± 5.2b
	Dryacide	79.0 ± 3.6a	43.5 ± 6.9b	69.8 ± 6.9	42.6 ± 7.4b	70.4 ± 5.7	32.3 ± 4.1b
	Insecto	71.6 ± 5.7ab	49.1 ± 9.0b	64.8 ± 5.3	38.8 ± 7.7b	74.1 ± 4.9	29.4 ± 4.4b
L. reticulatus	Control	-	128.0 ± 21.2a	-	172.4 ± 39.0a	-	92.9 ± 17.7a
	Protect-It	85.2 ± 3.8	34.9 ± 4.1b	80.6 ± 6.2	28.0 ± 6.3b	76.0 ± 5.8b	27.7 ± 3.3b
	Dryacide	89.4 ± 4.1	30.1 ± 3.9b	89.4 ± 3.7	37.4 ± 6.8b	92.6 ± 2.1a	36.3 ± 4.5b
	Insecto	84.0 ± 5.0	41.9 ± 8.8b	82.9 ± 4.9	34.6 ± 5.6b	84.3 ± 3.0ab	32.4 ± 4.0b
L. decolor	Control	-	194.9 ± 41.5a	-	217.5 ± 34.7a	-	181.0 ± 28.5a
	Protect-It	37.7 ± 6.7	60.3 ± 12.3b	38.4 ± 6.3	79.3 ± 18.4b	37.5 ± 5.0	53.4 ± 9.5b
	Dryacide	49.5 ± 4.7	76.2 ± 17.3b	38.9 ± 4.5	49.2 ± 13.2b	43.1 ± 4.3	59.4 ± 12.4b
	Insecto	46.3 ± 4.5	61.3 ± 10.2b	47.2 ± 6.1	64.3 ± 16.7b	46.3 ± 5.9	42.0 ± 8.7b

Table 3 Number of adults of three psocid species per vial (mean ± SE) 40 days after the introduction of 10 parental individuals on untreated commodities (0 ppm) or commodities treated with methoprene at three dose rates (within each row, means followed by the same uppercase letter are not significantly different; within each column, means followed by the same lowercase letter are not significantly different; where no letters exist, no significant differences were noted; in all cases for rows df=2,24, for columns df = 3, 32; Tukey-Kramer (HSD) at $P < 0.05$).

	L. bostrychophila			*L. entomophila*			*L. paeta*		
Dose (ppm)	Wheat	Rice	Maize	Wheat	Rice	Maize	Wheat	Rice	Maize
0	102.6 ± 18.0a	75.8 ± 13.0a	48.2 ± 14.6a	155.3 ± 10.8Aa	132.1 ± 10.0Aa	66.9 ± 14.6Ba	151.2 ± 7.2Aa	87.8 ± 15.4Ba	45.1 ± 5.7Ca
1	6.3 ± 1.4Ab	66.7 ± 15.3Ba	4.2 ± 0.9Ab	40.0 ± 15.8Ab	97.0 ± 15.4Ba	17.8 ± 9.6Ab	14.7 ± 5.0Ab	93.1 ± 11.5Ba	12.3 ± 5.5Ab
5	4.2 ± 0.7b	5.8 ± 1.4b	3.1 ± 0.8b	8.3 ± 0.8Ac	15.2 ± 2.7Bb	6.1 ± 0.6Ab	6.9 ± 1.8b	11.0 ± 1.8b	5.8 ± 0.9b
10	4.5 ± 0.5b	5.8 ± 0.9b	2.8 ± 0.7b	7.5 ± 1.1c	8.8 ± 0.5b	7.2 ± 0.5b	6.1 ± 0.8b	6.0 ± 1.3b	6.1 ± 1.2b

3.4. Efficacy of pyriproxifen and esfenvalerate

Pyriproxifen efficacy was affected by the presence of psocids before spraying, as well as by the presence of food, but the pattern was not similar among psocid species (Table 4). In the case of *L. bostrychophila* and *L. paeta*, the placement of food and psocids after spraying generally increased adult emergence in comparison with the other treatments, but significant differences were not recorded in all combinations. The reverse was true for *L. decolor*. For the esfenvalerate experiments, survival in untreated controls ranged from 93 to 100% (data not included). Survival of psocids exposed directly to esfenvalerate aerosol ranged from 44 to 62%, with variation among species, and was generally less than the corresponding control survival. Moreover, psocid mortality did not increase 48 h after for any of the species tested (Table 5).

Table 4 Number of adults per dish, 28 d after spraying, for each species tested for efficacy of pyriproxifen as a surface treatment; Tukey-Kramer (HSD) test at $P < 0.05$).

Dish containment before spraying	***L. bostrychophila***	***L. decolor***	***L. paeta***
No psocids, no food	2.0 ± 0.8a	0.8 ± 0.3ab	4.1 ± 1.2a
Psocids and food	0.4 ± 0.4b	0.3 ± 0.2b	2.9 ± 0.6ab
Food but no psocids	0.0 ± 0.0b	2.1 ± 0.8a	1.1 ± 0.6bc
Psocids but no food	0.0 ± 0.0b	0.2 ± 0.1b	0.0 ± 0.0c

Table 5 Mean ± SE mortality (%) of L. bostrychophila, L. decolor, and L. paeta immediately after exposure to esfenvalerate application and mortality after 2 d.

Species	**Day**	**Mortality (%)**
L. bostrychophila	0	58.7 ± 13.4
	2	61.7± 12.9
L. decolor	0	46.3 ± 13.1
	2	44.6 ± 13.0
L. paeta	0	45.8 ± 13.6
	2	45.4 ± 13.3

3.5. Efficacy of SF

Complete mortality of *L. paeta* adults and nymphs was recorded at 6 g/m^3 (Fig. 1). However, eggs were much more tolerant than the other life stages, given that mortality reached 100% only at the maximum dose tested (96 g/m^3). *Liposcelis decolor* adults were the most tolerant in comparison with the adults of the other species examined, but they were more susceptible than eggs, since 100% mortality was noted at 72 g/m^3 (Fig. 2).

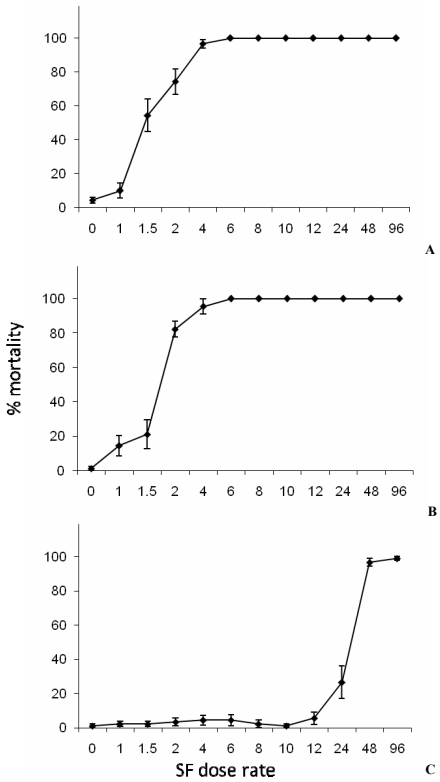

Figure 1 Mean (%) mortality (± SE) of *L. paeta* adults (A), nymphs (B), and eggs (C) after 48 h of exposure to various doses of SF (in g/m^3).

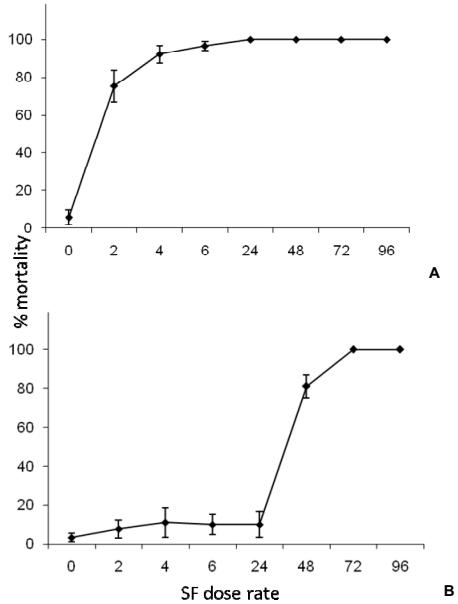

Figure 2 Mean (%) mortality (± SE) of *L. decolor* adults (A) and eggs (B) after 48 h of exposure on various doses of SF (in g/m^3).

4. Discussion

The four psocid species tested varied in their susceptibility among insecticides and commodities. Commodity is an important variable that affects insect development, as well as the insecticidal effect of grain protectants (Chanbang et al., 2008; Athanassiou et al., 2008b; 2009a). Opit and Throne (2008a) found that *L. reticulatus* and *L. entomophila* have different developmental rates on different diets. Based on the present results, rice may be the most suitable grain commodity for survival, development and reproduction of psocids, especially for *L. reticulatus* and *L. bostrychophila.* In light of our findings, OP insecticides result in higher mortality levels than spinosad or pyrethrum, despite variation among species and commodities. Hence, chlorpyriphos-methyl + deltamethrin was the most effective protectant on wheat and rice, and pirimiphos-methyl on maize, for all species tested. Nayak et al. (2003) found that chlorpyriphos-methyl on concrete was very effective against *L. bostrychophila*, but moderately effective against *L. entomophila* and *L. paeta.* Daglish et al. (2003) reported that chlorpyriphos-methyl with synergized bifenthrin was effective against *L. decolor*, *L. bostrychophila*, and *L. paeta* but not *L. entomophila.* The combination of OPs with pyrethroids are also effective against other stored-product insect species (Daglish, 1998; Nayak et al., 1998; Huang and Subramanyam, 2004). Generally, the current results clearly indicate that a single formulation that contains chlorpyriphos-methyl and deltamethrin is effective against all psocids species tested. However, Nayak et al. (1998) found that pirimiphos-methyl was unable to control *L. entomophila* and *L. paeta*, but it was effective against *L. bostrychophila*. Previously, there were no data available on protectant efficacy against psocids on maize; our results indicate that pirimiphos-methyl, at the label rate, can be used successfully, at least for the species range examined here.

Diatomaceous earth was not effective against psocids, despite variations among species, commodities, and formulations. Generally, DEs are considered to be less effective on maize than on wheat or rice against stored-grain beetle species (Subramanyam and Roesli, 2000; Athanassiou et al., 2003), probably because of reduced retention of DE particles on maize kernels (Kavallieratos et al., 2005). The combination of DEs with other, reduced-risk insecticides may be a solution to this problem. Athanassiou et al. (2008a) found that adults of the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae), were very susceptible to an abamectin-enhanced DE. Similar combinations should be further evaluated for control of psocids (Athanassiou et al., 2009b).

Methoprene reduced the numbers of adult progeny in all commodities and application rates, with the exception of 1 ppm. Bucci (1994) noted that high application rates of methoprene (47.5-190 ppm) reduced population growth of *L. bostrychophila*. Nevertheless, methoprene, even at 10 ppm, did not suppress adult progeny, in any of the commodities evaluated. Consequently, >10 ppm are needed for complete progeny suppression. Nayak et al. (1998; 2002) also noted that methoprene was unable to control *L. bostrychophila*, *L. entomophila*, *L. paeta*, and *L. decolor*, while a survey of field populations of *L. bostrychophila* and *L. entomophila* indicated that this tolerance is a natural phenomenon and not due to methoprene resistance. Moreover, methoprene efficacy was different among commodities. Samson et al. (1990) found that the efficacy of three IGRs against *R. dominica* differed between paddy rice and maize. In our study, adult numbers were continuously higher on rice than on wheat and maize which is in accordance with the observations obtained from the previous experiments.

There are many studies available for the efficacy of contact insecticides on different surfaces against psocids. Guedes et al. (2008) in laboratory bioassays found that beta-cyfluthrin and chlorfenapyr were very effective against *L. bostrychophila* and *L. entomophila* on concrete. Collins et al. (2000) also reported that chlorpyriphos-methyl was not able to provide long-term protection on concrete against *L. entomophila* and *L. paeta*. However, there was no data available on the efficacy of pyriproxifen or esfenvalerate against psocids on concrete. The results of the present study indicate that pyriproxifen efficacy varied with psocid species, presence of psocids before or after the application, as well as the presence of food (kernels). Survival on esfenvalerate-treated surfaces was generally high for the tested psocid species, but, longer exposure intervals on the treated surfaces need to be evaluated in order to examine if, and to what extent, delayed mortality can gradually eliminate the population.

Sulfuryl fluoride is perhaps the most promising alternative fumigant to methyl bromide, as it combines high efficacy and good penetration characteristics (Bell and Savvidou, 1999; Small, 2007; Baltaci et al., 2009). Sulfuryl floride has been evaluated with success against a wide range of stored-product insect

species, but there is still inadequate information on its efficacy against psocids. Our results show that psocids are generally susceptible as nymphs or adults to low doses of SF. In contrast, eggs are very tolerant to SF and can survive at doses that are usually lethal for the control of other major insect species. High egg tolerance to SF has been reported for some stored-product pests (e.g. Phillips et al. 2008), but there are exceptions (e.g. Baltaci et al., 2009). Additional experimentation is required with more psocid species, commodities, doses, and exposures, in order to evaluate the factors that could contribute to higher SF efficacy against psocid eggs.

In conclusion, this paper has briefly discussed the results of a series of experiments aimed at assessing the efficacy of registered pesticides against psocids. For the entire treatment of the grain mass, chlorpyriphos-methyl + deltamethrin and pirimiphos-methyl were more effective than spinosad, pyrethrum, DEs, or methoprene. For concrete surfaces, neither pyriproxifen nor esfenvalerate were able to completely control psocids, and based on previous publications, OPs may be more effective in this case as well. Finally, SF is effective against psocids, but higher doses and/or longer exposures are needed to obtain 100% egg kill. All the above observations underline the need for a more integrated approach for psocid management in storage facilities.

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Efficacy of dust formulations of spinosad for controlling insects infesting stored wheat

Bonjour, E.L.*#, Opit, G.P.
Dept. of Entomology and Plant Pathology, 127 Noble Research Center, Stillwater, Oklahoma 74078-3033, U.S.A..
Email, edmond.bonjour@okstate.edu

* Corresponding author
# Presenting author

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Abstract

Laboratory experiments were conducted to compare the efficacy of three new dust formulations (B1, C3, and D1) of spinosad as grain protectants on stored wheat. Evaluations were conducted on grain that was held for 1 d and 12, 24, 39, and 52 wk after insecticide treatments were applied. Bioassays for adult mortality and progeny production were conducted at 28°C and about 65% r.h. Dust formulations B1 and C3 effectively controlled adult *Rhyzopertha dominica* and prevented progeny development for 52 wk while formulation D1 was less effective. Only formulation B1 controlled *Sitophilus oryzae* adults (> 91% parental mortality) but did not prevent progeny production. None of the dust formulations were effective against *Tribolium castaneum* adults but progeny production was lower on grain treated with formulations B1 and C3. Egg mortality of *Plodia interpunctella* was similar for all treatments although overall progeny production was less on grain treated with formulation B1. The type of dust formulation of spinosad is critical in controlling stored grain insects.

Keywords: Spinosad dust formulations, *Rhyzopertha dominica, Sitophilus oryzae, Tribolium castaneum, Plodia interpunctella*

1. Introduction

Alternative insecticides are needed for controlling stored-product pests, especially in raw grain. Spinosad is a broad-spectrum insecticide that is derived from two metabolites of a naturally occurring actinomycete soil bacterium *Saccharopolyspora spinosa* Mertz & Yao and is produced by fermentation (Mertz and Yao, 1990). It is registered by the U.S. Environmental Protection Agency as a reduced risk pest control product and the active ingredient is registered for use on more than 250 different crops.

Laboratory studies (Fang et al., 2002a) have shown that spinosad is highly effective as a grain protectant against several stored grain insect species. ang et al. (2002b) also demonstrated that spinosad residues on wheat placed in mesh pouches and exposed in farm bins for 12 mo degraded very little from the application rate.

The objective of our research was to determine the effectiveness of three spinosad dust formulations as grain protectants applied to wheat on three species of stored grain beetle pests, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), and one moth species, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), at five time periods after treatment up to 52 wk.

2. Materials and methods

A total of 2 kg of hard red winter wheat was placed in each of twenty-one 3.8 L glass jars. Seven treatments were tested during this experiment with each treatment having three replications:

a) Spinosad B1 (0.5% w/w; Bayer CropScience) applied at 1 ppm.
b) Spinosad C3 (0.5% w/w; Bayer CropScience) applied at 1 ppm.
c) Spinosad D1 (0.5% w/w; Bayer CropScience) applied at 1 ppm.
d) Secure™ dust (0.5% w/w; Bayer CropScience) applied at 1 ppm.
e) SpinTor® (22.8% w/w; Dow AgroSciences) applied at 1 ppm.
f) Storcide™ II (816.5 g chlorpyrifos-methyl and 140.6 g of deltamethrin per 3.8 L; Bayer CropScience) applied at 3 ppm chlorpyrifos-methyl and 0.5 ppm deltamethrin.
g) Each control received 1.39 g of water only.

Secure is an older dust formulation, Spintor is a liquid formulation of spinosad, and Storcide II served as a positive control.

After application of insecticides or water to the sides of the 3.8 L jars, 2 kg of wheat was added to each jar and the jars were turned end for end 10 times and then rotated a full revolution 10 times. Jars were left to sit on the lab bench for 2 h and then turned and rotated as before. Sealed jars of treated wheat were placed in an environmental chamber maintained at 28°C for storage during the experiment. The experiment had five time periods: 1 d, and 12, 24, 39, and 52 wk post-treatment. Prior to using grain for each period, 3.8 L jars were rotated end for end 10 times before removing wheat for the experiment.

The experimental unit for beetles used in the experiment was a 236.6 mL glass jar containing 100 g of diet. The diet for *R. dominica* and *S. oryzae* was 100 g of whole kernels and jar lids were fitted with a circular piece of US # 40 mesh copper screen sandwiched between two pieces of filter paper. For jars receiving *T. castaneum*, 95 g of whole kernels and 5 g of ground treated kernels were used, and jar lids were fitted with two pieces of filter paper. Ground kernels were obtained by grinding kernels for 30 sec using a laboratory blender. For *P. interpunctella*, the experimental unit was a 25-mL glass vial. Into each vial was placed 2.5 g of whole kernels and 2.5 g of ground kernels. Lids of vials were each fitted with a piece of paper toweling.

For each time period, a total of three replications for each species per treatment were set up. One replication came from the grain in each of 3.8 L jars for each treatment (e.g. three 3.8 L jars were treated with spinosad B1 and 100 g of grain were then taken from each of the three different 3.8 L jars for a given treatment and placed in a 236.6 mL jar). This was repeated for each beetle species. A total of 50 adult beetles were placed on the grain in each 236.6 mL jar. Beetles were approximately 2-3 mo old and obtained from laboratory colonies. A total of 20 *P. interpunctella* eggs that were less than 24 h old were placed on double-sticky tape attached to a strip of black filter paper. Filter paper strips were then placed on the grain in the vials. Three replications for each treatment were conducted. All experimental units were placed in an environmental chamber maintained at 28°C and about 65% r.h. with a photoperiod of 16:8 L:D.

In each treatment, adult beetles were removed from the jars and counted as live, moribund, or dead after 1 wk of being placed on the grain. Moribund and dead adults were placed in a 9-cm Petri dish containing a piece of filter paper moistened with 0.5 mL of water. These insects were then re-evaluated after 24 h for recovery. Jars were held in the environmental chamber for an additional 6 wk and then progeny were counted. There were two response variables for each treatment: mortality of adults after 1 wk and number of progeny after 6 wk.

Plodia interpunctella eggs in each treatment were evaluated for hatch after 2 wk. At the same time, larvae present were counted and then returned to the vial with the grain. After an additional 3 wk in the environmental chamber, progeny – larvae, pupae, and adults - were counted. There were three response variables for each treatment: mortality of eggs after 2 wk, number of larvae after 2 wk, and number of progeny after 5 wk.

Data were analyzed using the General Linear Models (GLM) Procedure of the Statistical Analysis System (SAS, 2007), with adult beetle mortality, beetle progeny production, moth egg mortality, and moth survivors as the response variable and treatment as the main effect. Percentage data for adult beetle mortality and moth egg mortality were transformed (arcsin sqrt [%/100]) before analysis.

3. Results

Spinosad dust formulations B1 and C3 effectively controlled adult *R. dominica* and prevented progeny development for 52 wk while formulation D1 was less effective (Tables 1 and 2). One hundred percent control was observed for adult *R. dominica* for treatments B1 and C3 for all post-treatment time periods except for grain treated with C3 at 24 wk post-treatment where the adult mortality was 99.3%. There were no *R. dominica* progeny produced on grain treated with B1, Secure, Spintor, or Storcide II at post-treatment periods of 12, 24, 39, and 52 wk (Table 2). Progeny production on grain treated with C3 was < 1.0 for the same time periods. On grain treated with D1, progeny production varied from 18.0 to 56.7 adults during these post treatment periods.

Table 1 Mean percent mortality (±SE) of adult beetles exposed for 7 d to various spinosad formulations at 52 wks post-treatment (n = 3).

Treatment	*T. castaneum*	*R. dominica*	*S. oryzae*
Control	1.4 ± 0.7 b	4.8 ± 3.8 c	0 c
Storcide II	6.9 ± 0.6 a	96.0 ± 1.2 b	91.8 ± 4.8 ab
Spintor	0 c	100 a	96.7 ± 2.4 a
Secure	1.4 ± 0.7 b	100 a	79.3 ± 9.7 b
Spinosad B1	0 c	100 a	97.3 ± 0.7 a
Spinosad C3	0 c	100 a	86.0 ± 8.1 ab
Spinosad D1	0 c	100 a	0 c

Means within a column followed by the same letter are not significantly different (P > 0.05; Fisher's least significant difference test). Percentage data were transformed (arcsin sqrt [%/100]) before analysis; untransformed values are presented.

Table 2 Mean progeny (±SE) per 236.6 ml jar of adult beetles exposed for 7 d to various spinosad formulations at 52 wks post-treatment (n = 3).

Treatment	*T. castaneum*	*R. dominica*	*S. oryzae*
Control	82.3 ± 8.1 a	207.7 ± 47.7 a	362.3 ± 19.9 ab
Storcide II	0 b	0 b	13.7 ± 8.1 c
Spintor	0 b	0 b	103.7 ± 59.9 c
Secure	0.3 ± 0.3 b	0 b	320.3 ± 129.4 ab
Spinosad B1	0 b	0 b	75.0 ± 21.0 c
Spinosad C3	1.7 ± 0.3 b	0 b	212.3 ± 86.8 bc
Spinosad D1	89.3 ± 9.8 b	39.0 ± 12.7 b	511.7 ± 60.9 a

Means within a column followed by the same letter are not significantly different (P > 0.05; Fisher's least significant difference test).

Only formulation B1 controlled *S. oryzae* adults above 91% but did not prevent progeny production which ranged from 75.0 to 769.0 during the post-treatment periods. All other spinosad formulations had high numbers of progeny produced at all time periods. Formulation D1 did not cause adult mortality at 24-, 39-, and 52-wk post-treatment. Formulation C3 only resulted in a range of 69.6 to 86.0 % mortality of adults for the duration of the experiment. Overall, Storcide II was the best product for reducing *S. oryzae* progeny production but only achieved 100% control for 1-d and 12-wk post-treatment. Progeny production reached a high of 13.7 adults at 52-wk post-treatment on grain treated with this product.

None of the dust formulations or the liquid formulation were effective against *T. castaneum* adults where mortality was $\leq$2% at all post-treatment periods. Progeny production was significantly lower on grain treated with formulations B1 and C3 than D1 at all post-treatment periods. Storcide II was the only treatment where no progeny were produced during the entire testing period.

Table 3 Mean percent mortality (±SE) of *Plodia interpunctella* eggs exposed for 7 d to various spinosad formulations at 39 wks post-treatment and mean number of survivors after 4 wks (n = 3).

Treatment	Egg Mortality	Survivors (total larvae, pupae, and adults)
Control	8.3 ± 6.0 a	11.3 ± 0.3 a
Storcide II	11.7 ± 1.7 a	0 c
Spintor	18.3 ± 8.8 a	0.3 ± 0.3 bc
Secure	6.7 ± 4.4 a	3.0 ± 1.5 b
Spinosad B1	11.7 ± 6.7 a	0.7 ± 0.7 bc
Spinosad C3	5.0 ± 2.9 a	5.3 ± 1.2 bc
Spinosad D1	11.7 ± 6.7 a	12.7 ± 1.5 a

Means within a column followed by the same letter are not significantly different (P > 0.05; Fisher's least significant difference test).

Percent egg mortality of *P. interpunctella* was similar for all treatments within a post-treatment period and varied from 3.3 to 36.6% during the study. Progeny production was much lower (≤1%) on grain treated with formulation B1 than formulations C3 and D1 for post-treatment periods 1-d, and 12-, 24-, and 39-wk (Table 3). Storcide II was the only treatment where no progeny were produced until the 52-wk post-treatment period.

4. Discussion

Formulation is a critical factor in the effectiveness of spinosad against stored grain insect pests. Overall, all formulations of spinosad tested were effective in controlling *R. dominica* adults which corresponds to previous studies (Fang et al., 2002a; Nayak et al., 2005). This species is one of the most important stored-product pests to control because it causes insect damaged kernels (IDK). IDK is a discount factor when selling grain so controlling pests that cause IDK is essential in a management strategy. Although not statistically significant, of the new formulations, B1 and C3 prevented progeny production by *R. dominica*. In a management strategy, a grain manager does not want even a few *R. dominica* in his grain so B1 would be the product of choice of the three new formulations.

Sitophilus oryzae was not effectively controlled by any of the spinosad products. However, of the three new dust formulations tested, B1 was the most effective. Athanassiou et al. (2008) also found that *S. oryzae* adults were less susceptible than *R. dominica* adults to spinosad dust containing 0.125% spinosad. *S. oryzae* also causes IDK that affects grain value. Progeny were not reduced in number by any of the spinosad products probably because eggs are laid inside kernels where larvae are protected from pesticide exposure.

Spinosad is not an effective product for controlling *T. castaneum* adults although formulation B1 was more effective than C3 and D1 in reducing the number of progeny. Even Storcide II lost its effectiveness against adults after the 1-d post-treatment exposure and steadily declined during the study. However, Storcide II prevented any progeny production at all post-treatment periods where the spinosad products did not, especially at 1-d, 12-wk, and 24-wk post-treatment periods (data not shown).

For *P. interpunctella*, formulation B1 was better at reducing progeny production than formulations C3 and D1 for the first four post-treatment periods. However, no spinosad formulation was 100% effective in controlling progeny production.

Overall, new dust formulation B1 was more effective than C3 and D1 but it still had its limitations. It was very effective against *R. dominica*, however, it has limited success against the other insects tested during this study. Therefore, an integrated approach is necessary to control a complex of stored grain insects infesting grain.

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Horizontal transfer of methoprene in *Tribolium castaneum*

Tucker, A.M.*[1], Campbell, J.#[2], Arthur, F.H.[2], Zhu, K.Y.[1]
[1] Kansas State University, Department of Entomology, 123 West Waters Hall, Manhattan, KS 66506 USA. Email address nos7147@ksu.edu
[2] United States Department of Agriculture, Agricultural Research Service, Center for Grain and Animal Health Research, 1515 College Ave., Manhattan, KS 66502, USA.

* Corresponding author
# Presenting author

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Abstract

Aerosol applications of reduced risk insecticides such as pyrethrins, pyrethroids, and insect growth regulators are becoming more commonly used to manage stored-product insects in food facilities. However, these applications have a limited ability to penetrate into hidden refugia, where the majority of the pest population is located. Horizontal transfer of insecticides could occur as individuals directly treated or exposed to treated surfaces move into hidden refugia and encounter untreated individuals. In this series of studies, the potential for horizontal transfer of methoprene from treated *Tribolium castaneum* , the red flour beetle, to untreated individuals was evaluated. Adding larvae, pupae, or adults treated with methoprene to flour patches with untreated *T. castaneum* larvae, resulted in increased pupa and adult deformities and higher numbers of dead focal individuals, which suggests the potential for this mechanism. The transfer mechanism might be flour substrate contamination, transfer during contact of individuals, and/or cannibalism of individuals exposed to insecticides. Experiments focused on isolating the impact of contact and cannibalism on horizontal transfer did not detect a significant increase in mortality. Experiments focused on flour substrate contamination resulted in decreased adult emergence as well as lower survival, and higher rates of deformities. These findings suggest that substrate contamination is the more likely mechanism for horizontal transfer, and although horizontal transfer can occur, the impact of this process on populations needs further evaluation.

Keywords: Red flour beetle, *Tribolium castaneum*, Methoprene, Horizontal transfer

1. Introduction

Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae), the red flour beetle, is a serious pest of stored-products in food facilities such as flour mills, rice mills, warehouses, and retail stores. They are difficult to manage because they can exploit both the stored commodity and locations within equipment and the building structure where food material accumulates. To control these hidden pest populations, the food industry has often relied on periodic structural treatments such as fumigation or heating, that have the ability to penetrate into these hidden refugia to eliminate or reduce infestations (Bell, 2000; Fields and White, 2002). Fumigation using methyl bromide has been widely used to manage *T. castaneum* populations in flour mills, but its use is being phased out under the Montreal protocol agreement (UNEP, 2000). A potential alternative to the use of methyl bromide is the use of aerosol insecticides, although these applications do not have the ability to penetrate into commodities or the building structure. A widely used aerosol insecticide formulation involves the combination of an insect growth regulator (IGR) such as methoprene with synergized pyrethrins (Arthur, 2008). Direct application of these compounds or exposure to previously treated surfaces has been shown to cause high mortality of *T. castaneum* (Arthur, 2008). However, since only a small percentage of the total *T. castaneum* population in a facility is in exposed areas where they may come in contact with the aerosol insecticide (Toews et. al., 2005), aerosol treatments at first appear to be unlikely to have a dramatic impact on *T. castaneum* populations. Although assigning a cause and effect relationship is difficult, there is evidence suggesting regular applications of aerosol formulations of methoprene and synergized pyrethrins as part of an IPM program can suppress *T. castaneum* populations (Campbell et. al., 2010).

There are several possible mechanisms for how aerosol applications might have a cumulative impact on the hidden pest population within a structure. Insecticides may move into hidden refugia exploited by insects through drift of aerosol, movement of treated flour, and/or movement of insects that have been

exposed to insecticide. Horizontal transfer of insecticides occurs when active members of the population ingest or come into contact with an insecticide and then return to a location where other members of the population come in contact with and/or consume the translocated insecticide (Buczkowsi and Schal, 2001). Horizontal transfer of insecticides has been demonstrated in several insect systems. Contact, cannibalism, and ingestion of fipronil residues are possible horizontal transfer mechanisms of fipronil in the German cockroach, *Blattella germanica* (L.) (Buczkowski and Schal, 2001). Red imported fire ants, *Solenopsis invicta* (Buren), transferred methoprene from S-methoprene baits by food sharing with adjacent colonies (Aubuchon et al., 2006).

Because of its good persistence, lack of activity against adult insects, and activity against immature stages at very low concentrations, methoprene may be the most likely insecticide for horizontal transfer in food facilities. Several behaviors and chemical ecology characteristics of *T. castaneum* may also facilitate horizontal transfer of methoprene. Firstly, *T. castaneum* is a cannibalistic species, with both adults and larvae consuming other life stages (Park et al., 1965; Godfrey and Hassell, 1997). Secondly, *T. castaneum* have a male produced aggregation pheromone, 4,8-dimethyldecanal, which attracts both sexes and can increase encounters among treated and untreated individuals (Suzuki, 1980). Lastly, *T. castaneum* adults readily move among flour patches (Campbell and Runnion, 2003), which means that they could be exposed to settling aerosol insecticide(s) or surfaces with residual insecticide(s) and carry it into refugia. The combination of these behaviors allows for the possibility of horizontal transfer of methoprene. In this paper, we summarize a series of experiments designed to determine if horizontal transfer of methoprene from treated individuals to untreated individuals can occur with *T. castaneum* and if so what mechanism(s) are likely involved in the process (e.g., physical contact with a treated individual, cannibalism of a treated individual, and transfer of insecticide to a substrate such as flour which is then contacted and/or eaten by an unexposed individual (substrate contamination)). Horizontal transfer was evaluated using a biological assay method where levels of mortality during the observation period (mortality), development to the adult stage (adult emergence), and presence of any type of physical abnormality (deformities) were used as measures of the amount of methoprene transfer.

2. Does horizontal transfer of methoprene occur with T. castaneum?

2.1. Materials and methods

To generate methoprene-treated developmental stages, larvae, pupae, and adults were collected from a *T. castaneum* laboratory culture. Selected individuals were placed in concrete exposure arenas consisting of a plastic Petri dish (62 cm^2 surface area) containing ~0.1 cm of concrete patch material (Rockkite®, Hartline Products, Co., Inc., Cleveland, OH, USA) in the bottom (Arthur, 2008). Methoprene (Diacon® II, Central Sciences International, West Schaumberg, IL, USA) or distilled water was sprayed evenly onto the arenas using an airbrush (Badger Air-Brush Company, Franklin Park, IL, USA) in order to evenly coat the surface and the insects. The labeled rate of methoprene for a surface treatment (3 mg/m^2) was applied to treatment dishes (0.25 ml/62 cm^2) and both treatments and controls received 0.132 ml of liquid. After application, the exposure arenas and insects were allowed to dry for ~15 min. Insects were transferred to new Petri dishes and placed in a freezer for 24 hrs. Insects were frozen to prevent degradation of methoprene (Henrick, 2007) and prevent movement in order to standardize amount of contact with treated individual in experiments.

Single late stage *T. castaneum* larvae (focal individuals), not treated with insecticide, were added to 18 g plastic vials with ~ 5 g of flour and then a dead larva, pupa or adult methoprene or water treated individual was placed in the vial. An additional control group, consisting of just flour and focal larvae in the vial, was also used. Vials were held at 32°C. Focal individuals were observed daily for 30 days and developmental stage, mortality, and presence of external deformities was recorded. The level of cannibalization of the dead individual in each vial was quantified using an index: 0 – no feeding observed; 1 – bites and/or scratches 2 – missing appendages; and 3 – complete consumption of the dead insect. Seven blocks with 3-11 replicates of each treatment group in each block were performed.

2.2. Results and discussion

For focal individuals, emergence as adults, mortality, and deformities were compared among treatments using Fisher's exact probability pair-wise comparisons. Comparisons of adult emergence and mortality were not significantly different between methoprene treatments and controls, with a couple exceptions.

There was a significant difference in adult emergence between larvae exposed to flour with methoprene-treated pupae (82%) and their respective water treated control (97%) (P=0.04). Larvae exposed to flour and methoprene treated larvae had significantly higher mortality (37%) compared to larvae exposed to water treated larvae (14%) (P=0.05). In general, deformities were more prevalent when larvae were exposed to methoprene treated individuals. More individuals displayed deformities when exposed to methoprene treated larvae and flour (46%) or methoprene treated pupae and flour (26%) compared to those exposed to flour alone (6%) (P<0.05). There was a similar pattern of deformities for comparisons between focal larvae exposed to water treated larvae (11%) and methoprene treated larvae (46%) (P<0.01) and water treated pupae (3%) and methoprene treated pupae (26%) (P<0.01). There were no significant effects on larval development when exposed to methoprene treated adults compared to controls. Some level of physical deformities in the control groups was not unexpected since these can occur normally during development.

These results indicate that some horizontal transfer of methoprene occurred, but typically not enough to cause a high rate of mortality and the degree of effect was influenced by the developmental stage initially exposed to methoprene. The limited mortality and adult emergence effects might be due to only a single individual being added to the flour. Adding methoprene treated adults did not have any detectable impact on larvae, which could be due to differences in the cuticle between adults and immatures and/or that there is less cannibalization of adults. Since the adults are the stage most likely to move between flour patches (Campbell and Runnion, 2003) and therefore most likely to be exposed to insecticide treatments, this could reduce the potential for horizontal transfer of methoprene in food facilities. Since this experimental design did not allow us to determine the relative contribution of the three mechanisms of horizontal transfer (contact, substrate contamination, and cannibalization), additional experiments were performed.

3. Does physical contact and/or cannibalization result in horizontal transfer of methoprene?

3.1. Materials and methods

The impact on *T. castaneum* development due to larvae direct contact with, and/or cannibalization of, methoprene treated individuals, without the potential for contamination of the flour substrate, was evaluated in this experiment. *T. castaneum* development stages were treated with methoprene or water, and frozen as described in the previous experiment. Five previously methoprene or water treated individuals were placed in an 18 g plastic vial with no flour and then one late-stage larva (focal individual) was added to each vial and its fate was observed for 30 days as described in the previous experiment. Two additional controls were also prepared: flour added to vial (flour control) and no food (no flour or dead insects) added (starvation control). Three blocks with 5 replicates of each treatment in each block were performed.

3.2. Results and discussion

There was 100% focal individual mortality and 0% adult emergence for larvae exposed to methoprene treated individuals and this was a significantly greater response than that in the controls (Fisher's exact probability pair-wise comparisons, P<0.05). Focal individual mortality when exposed to flour (20%) was lower compared to the starvation controls (79%) (P<0.01) or water treated adult controls (67%) (P=0.01). Observed deformities were also significantly higher in the larvae exposed to methoprene treatments as compared to their flour and water exposed counterparts. For example, 72% of larvae exposed to methoprene treated pupae had deformities compared to 13% of larvae exposed to just flour (P=0.01). Similarly, larvae exposed to methoprene treated pupae (72%) had significantly higher deformities as compared to larvae exposed to pupae treated with water (0%) (P<0.01). The high rate of mortality in the no food treatment group shows the effects of starvation, and these individuals typically died as adults and had very few deformities. The individuals exposed to water treated adults also had a high mortality rate and this is also likely from starvation due to the potential difficulties for larvae to cannibalize the dead adult.

Cannibalization rates were highly variable among individuals, as was found by Stevens (1989), but 100% mortality was obtained in all methoprene treatments. This suggests that physical contact is sufficient to generate these effects, with cannibalism being less important, but with this experimental design it is not possible to isolate the contact and cannibalization factors. Since larvae exposed to treated adults had high

mortality, and this is the stage most likely to be exposed and than to move into an untreated area, this suggests that horizontal transfer might occur in a food facility.

4. Does level of cannibalism affect horizontal transfer of methoprene?

4.1. Materials and methods

Because cannibalization rates varied so much among replicates in the previous experiment, in this experiment separate treatment categories were created based on the level of cannibalization. Methoprene and water treated *T. castaneum* pupae were generated as previously described. One dead methoprene or water treated pupa was placed in a 90 mm plastic Petri dish with one late-stage larva (focal individual). After 48 h, the dead pupa was observed to determine its level of cannibalization as previously described and the associated focal larvae were then sorted into one of four cannibalization rank groups. These larvae were then transferred individually to new Petri dishes with ~1 g of flour and their fate recorded as previously described. Larvae given flour or no flour for that initial 48 h period were used as controls. Two blocks, with 22 and 25 replicates per treatment group, respectively, were performed.

4.2. Results and discussion

There were no significant differences in adult emergence, mortality, or deformities among larvae exhibiting different levels of cannibalization of methoprene treated or water treated pupae (contingency table and χ^2 test, $P>0.05$). The only pair-wise comparisons that were significant ($P\leq0.05$) were between larvae exposed to flour and larvae exposed to no food, the latter having high mortality and low adult emergence. There was 79% mortality of the larvae that were exposed to no food compared to larvae that were exposed to flour only (0%) ($P<0.01$). These results suggest that cannibalization is not likely to be a primary mechanism for horizontal transfer of methoprene in this system. We are not aware of studies evaluating differences in methoprene efficacy resulting from consumption versus cuticle contact alone, but it could be that *per os* efficacy of methoprene is less than that due to contact with the cuticle.

5. Does physical contact alone result in horizontal transfer of methoprene?

5.1. Materials and methods

The impact on *T. castaneum* larvae of direct contact with methoprene treated adults, without the potential for contamination of the flour substrate or cannibalism was evaluated in this experiment. Methoprene or water treated adults were generated in the same way as previously described, however, these individuals were not killed. One treated adult and one late-stage larva (focal individual) was placed in a 90 mm plastic Petri dish containing a paper tent (2x1 cm), which served as a refuge area. After 24 hrs, the larva was moved into a new Petri dish with ~1g of flour and a new paper tent. The focal individuals were observed for 30 days as previously described. Larvae treated as above, but not exposed to an adult *T. castaneum*, and larvae added directly to flour were used as additional controls. There were three blocks of 10 replicates for each treatment per block.

5.2. Results and discussion

There were no significant differences in adult emergence, mortality, and deformities between larvae exposed to methoprene treated adults and control larvae exposed to just flour (contingency table and Fisher's exact probability pair-wise comparisons, all comparisons $P\geq0.05$). The same was true when comparing larvae between the methoprene and water treatments, except larvae exposed to methoprene treated adults had more deformities (26%) than larvae exposed to water treated adults (3%)($P=0.01$). These findings suggest that short term contact between individuals may not be an important mechanism for horizontal transfer of methoprene, although previous experiments suggested that transfer of methoprene from adults to larvae may be less than with other developmental stages. In these experiments the degree of contact was not quantified, and more physical contact between the individuals might further increase our ability to detect a response in adult emergence and mortality.

6. Does contamination of flour alone result in horizontal transfer of methoprene?

6.1. Materials and methods

Methoprene treated *T. castaneum* life-stages were generated as previously described. Five, 15, or 30 dead individuals of each life stage, respectively, were placed in 18 g plastic vials with ~5 g flour, and then agitated on a shaker table for 30 min to simulate individuals moving in flour. Individuals were sieved

from the flour and one late-stage focal larva was placed in the vial and observed as previously described. The experiment was performed in six blocks with a total of 12-15 replicates per a treatment.

6.2. Results and discussion

Focal individual adult emergence was less and mortality and deformities were greater for all comparisons between larvae exposed to flour conditioned with methoprene treated individuals and unconditioned flour, regardless of the number of treated individuals or developmental stage in the flour ($P \leq 0.05$) (contingency table and Fisher's exact probability pair-wise comparisons). The same pattern was observed for larvae exposed to flour conditioned with methoprene treated individuals and flour conditioned with water treated individuals ($P \leq 0.05$), except in five pair-wise comparisons. For example, 77% of larvae had deformities after exposure to flour conditioned with 15 methoprene treated larvae as compared with 0% of larvae having deformities after exposure to unconditioned ($P < 0.01$) or 23% of larvae having deformities after exposure to flour conditioned with 15 water treated larvae ($P < 0.01$). Deformities seen in the water treatments were generally minor, compared to those in the methoprene treatment, which frequently resulted in death of the focal individual. All comparisons of larvae exposed to unconditioned flour only were not significantly different from flour conditioned with water treated individuals, regardless of number or development stage ($P \geq 0.05$). These findings indicate that methoprene can be readily transferred from cuticle of treated individuals to the flour substrate and that contact and/or ingestion of this flour by larvae can have detrimental effects. In food storage facilities, this may be the more likely way methoprene can be transferred from treated adults to untreated developmental life-stages and therefore impact the population dynamics.

7. Conclusions

Our results indicate that horizontal transfer of methoprene between treated and untreated *T. castaneum* can occur under laboratory conditions, although the strength of the response varied considerably among experiments. Transfer of methoprene through contact with flour or direct contact appears to be a primary mechanism in our experiments. Strongest responses occurred when larvae were exposed to more than one treated individual. The fact that treated adults were able to transfer methoprene to the flour substrate indicates that horizontal treatment of methoprene could occur in flour mills since adults are primarily the individuals moving between hidden refugia. There is still a need to look at horizontal transfer under more realistic conditions and determine the potential population effects of horizontal transfer of methoprene.

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Deltamethrin residues through the food chain industries

Cognard, C.*#
Bayer SAS – Bayer Environmental Science - 16 rue Jean marie Leclair - CP 90106 - 69266 LYON Cedex 09 – France. Email: cecile.cognard@bayercropscience.com

* Corresponding author
# Presenting author

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Abstract

Deltamethrin is one of the authorized active ingredients for post-harvest use to protect stored grains in Europe. It has been used since many years alone or in combination with a synergist. It is included in the Annex 1 of the directive 91/414/CE since 2003. During the post-Annex I inclusion phase, a complete dossier has been submitted for deltamethrin-containing products (the K-Obiol range) including residue studies on numerous crops, and extensive data to support human and environmental risk assessment. The regulation 396/2005/CE gives European MRL of 2 mg/kg in cereals; the same value has been set by the CODEX Alimentarius, furthermore it fixes the value of 0.3 mg/kg as maximum residue level in flour. Nevertheless, commercial processors have sometimes been reluctant to include plant protection products on their lists of approved products for farm/supermarket protocols without supportive data concerning the effects of residues on the quality of processed foods. Consequently, a wide range of crops treated with deltamethrin have been tested over many years. Deltamethrin effects and residues levels have been followed through the processes leading to the manufacture of beer, bread and pasta (post-harvest treatment with K-Obiol products at a max 0.5 mg deltamethrin/kg).

Pasta processing industry (2002): Wheat, complying with the semolina and pasta quality standards, has been treated with deltamethrin (K-Obiol ULV6 at 84 or 42 mL/t). Treated wheat has been stored in cells for 1 month then production of semolina, milling derivatives and pasta has been performed. No residue of deltamethrin has been found in the semolina or in pasta made from semolina as shows in the Figure 1.

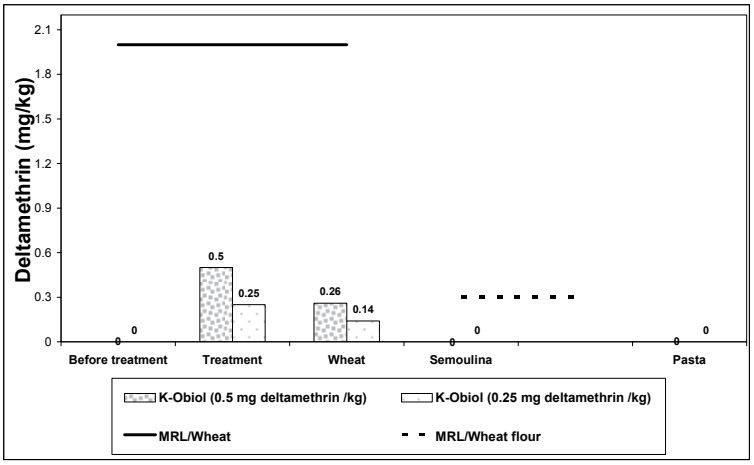

Figure 1 Residue of deltamethrin in cereals, semolina and pasta.

Beer processing industry (1999): Spring barley, complying with the brewery quality standards, has been treated with deltamethrin (K-Obiol ULV6 at 84 mL/t). Then malting and brewing have been performed one month after the treatment. There are no detectable residues in either wort or beer. The presence of deltamethrin in barley before malting (soaking and germination), and brewing (brewing and fermentation) does not influence these processes. No difference can be tasted between beer brewed with treated barley and reference (Fig. 2).

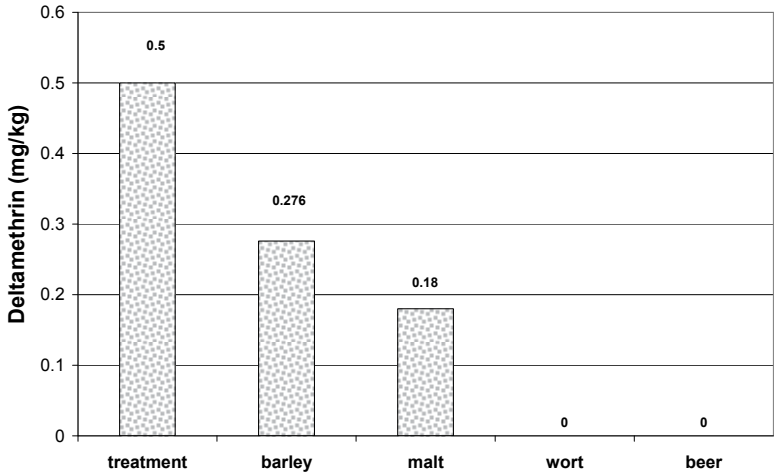

Figure 2 Residue of deltamethrin in cereals, wort, malt and beer.

Bread processing industry (2002-2003): Wheat has been treated with deltamethrin (K-Obiol ULV6 at 84 or 42 mL/t) after quality control of the grain then milling and bread making have been performed 15 days, 3, 6 and 12 months after the application. Protection of wheat during storage did not change the quality and characteristics of the dough (during kneading or fermentation) or those of the bread (appearance of the crust, crumb and texture) (Fig. 3).

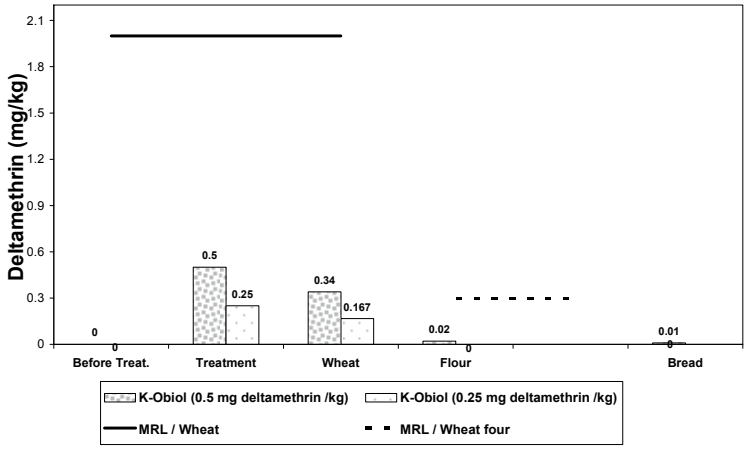

Figure 3 Residue of deltamethrin in cereals, flour and bread – 15 days after the application.

It has been demonstrated through the food chain that the treatment of stored grain with deltamethrin does not affect the main processing procedures. Furthermore, based on the current European MRL it has been concluded that the intended uses of deltamethrin do not cause any unacceptable risk to consumers due to chronic or acute exposure to residues through food. As the level of deltamethrin found in the manufactured products is very low or below the limit of quantification (below the European MRL), it can be confirmed that there is no unacceptable risk for the consumer. In conclusion, the use of deltamethrin to protect grain is effective, reliable and meets the requirements of the food industries.

Keywords: Deltamethrin, Residue, Food chain industries, Insecticide

Cross-contamination of oilseeds by insecticide residues during storage

Dauguet, S.*[1], Fleurat-Lessard, F.#[2], Loison, J.[1]
1 CETIOM, 11 rue Monge 33600 Pessac – France. Email: dauguet@cetiom.fr
2 INRA Research Unit 1264 (MycSA) 71, avenue Edouard Bourlaux – BP 81 33883 Villenave D'Ornon Cedex – France

* Corresponding author
# Presenting author

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Abstract

Pesticide residues are found in oilseeds (rapeseed and sunflower) and crude oils: they are mainly organophosphate insecticides (pirimiophos-methyl, malathion when authorized) used in empty storage facilities and for direct application to stored cereal grain. Even if some secondary pests are found in stored oilseeds, French regulations do not allow use of these insecticides on stored oilseeds. These residues arise from cross-contamination from storage bins and grain handling equipment of grain stores, and not from illegal use. This uptake of insecticide residues from their storage environment by oilseeds may lead to residue contents that exceed regulatory limits. A three-year investigation in grain storage companies allowed us to follow the course of sunflower batches and rapeseed batches during storage seasons 2006-2007, 2007-2008 and 2008-2009, from reception at the storage facility to outloading. Each of these batches was sampled at outloading, and was analyzed for insecticide residues. Traceability of oilseeds established by grain-store managers allowed us to identify cross-contamination sources. The insecticides that were most commonly detected were pirimiophos-methyl, malathion, and dichlorvos (in sunflower 2006-2007), plus chlorpyriphos-methyl and deltamethrin. Pirimiophos-methyl was the most commonly detected active substance, and caused the most cases of non-accordance with regulatory levels in rapeseed. Cross-contamination could have occurred when cereal grains were treated upon receipt, when rapeseed was also delivered, especially when treatments were done systematically to the cereal grains. For sunflower, the main cross-contamination hazard resulted from treatment of cereals at the period of receipt or at their outloading, just before sunflower seeds batches were outloaded. Another situation led to cross-contamination, but generally at a lower extent: oilseeds stored in bins that contained previously treated cereals, or loaded in empty bins with handling equipment treated before the receipt of oilseeds.

Keywords: Oilseed storage, Cross-contamination, Insecticide residues, Rapeseed, Sunflower

1. Introduction

Post-harvest insecticide residues can be sometimes found at low levels, however, on oilseeds stored in France no insecticides can be applied directly during storage, even if some secondary insect pests are found in the stored oilseeds (Dauguet et al., 2005). Consequently, maximum residue levels (MRLs) allowed by European regulations are very low (mostly at the lower limit of analytical determination): 0.05 $mg.kg^{-1}$ for pirimiophos-methyl, 0.05 $mg.kg^{-1}$ for chlorpyriphos-methyl on rapeseed and sunflower; 0.1 $mg.kg^{-1}$ for deltamethrin in rapeseed and 0.05 $mg.kg^{-1}$ for deltamethrin in sunflower. No MRL existed for malathion during this study, so it should not be found beyond the analytical limit of quantification (10 $\mu g.kg^{-1}$); but since September 2008 the MRL for malathion in oilseeds is 0.02 $mg.kg^{-1}$ (European Communities Commission (ECC) regulation n° 839/2008 of 31 July 2008).

These insecticide treatments are authorized on stored cereals and corn as grain protectants, and on empty storage and handling equipment as control agents for residual insect populations in empty granaries. Pirimiophos-methyl and malathion were the most common insecticides used during this study, except for the 2008-2009 storage season for when malathion was used. Dichlorvos and malathion, banned by the EU in May 2008, could be used only until 1 December 2008. As the MRL for dichlorvos was revised to be less than 0.01 $mg.kg^{-1}$ in cereals beginning in November 2006, this could no longer be used. The MRL of malathion was not lowered in cereals before withdrawal late in 2008, but it could be still be used until 2007-2008.

We hypothesized that a cross-contamination could result when various kinds of seeds, cereals and oilseeds, share the same grain handling equipment and storage systems. This cross-contamination occurred in Canada on rapeseed (Watters and Nowicki, 1982; White et al., 1983; White and Nowicki, 1985), when empty bins were treated with organophosphate insecticides (bromophos, malathion, fenitrothion). Canadian grain store managers were warned that treating cereal grain before storing rapeseed through the same handling equipment could lead to residues above the maximum allowable limits.

Uptake of pirimiophos-methyl by a single-layer of rapeseed or wheat on galvanized-steel surfaces was demonstrated in a laboratory study (Dauguet et al., 2007). It was shown that, for small bins (less than 50 tons), it could lead to residue contents above regulatory limits. In order to improve our knowledge about this post-harvest insecticide residues cross-contamination, especially in large elevators, an investigation was carried out with the collaboration of several French grain storage companies on sunflower seeds during the 2006-2007 storage season (Dauguet, 2007). second study was done on rapeseed from the 2007 harvest (Dauguet, 2009). Dichlorvos was not used during the storage season 2007-2008, because grain protection strategies changed in France. Rapeseed is harvested in June-July, about at the same period for cereal harvest (winter wheat and barley). A third study on rapeseed and sunflower was carried out during the 2008-2009 storage season.

2. Materials and methods

The process adopted for these three surveys on oilseeds was as follows.

First, we asked storage managers to trace oilseed lots from each step from delivery and receipt to outloading.

Second, we sampled each batch representative of oilseed delivered at the storage facilities ("first sample") and ensured safe storage of these samples. These samples were kept for long-term storage if we suspected that contamination by pesticide residues occurred before receipt at the surveyed grain store.

Third, we obtained a representative mean sample of outloaded oilseed, in order to constitute a "final sample", when the traced lot was downloaded for sale (storage time variable from one to 8 mo after harvesting). The residue content was determined in all these "final samples". The sampling method was done according to the standard for moving seeds, for contaminant quantification, with heterogeneous distribution determination, PR EN ISO 24333: 2006. For each grain bin, twenty five samples were done for each 500-Metric-tonnes-batch evenly distributed during the outloading of the grain bin (one sample each 20 t).

Fourth, we asked managers to fill out a questionnaire called "traceability" which recorded each step from receipt to outloading.

Determination of insecticide residues content in all "final samples" was done by an analytical laboratory ITERG (Pessac, 33, France) for samples taken during 2007 and 2008. In brief, the residue content in seeds was determined through the following protocol: 1) Soxhlet extraction of oil with hexane (NF EN ISO 659); 2/ pre-purification with acetonitril and freezing, purification with solid phase extraction C18 and Florisil cartridges; and 3/ analysis by gas chromatography with NPD detection (organophosphates) or ECD detection (pyrethroid). For 2009, the laboratory of CETIOM in Ardon (France) determined the residues using: 1/ a solid-liquid extraction with isooctane and liquid-liquid extraction with acetonitrile; 2/ purification with SPE C18 and Florisil cartridges for organochlorines and pyrethroids, and 3/ analysis by GC-NPD (organophosphorus) or GC-ECD (organochlorines and pyrethroids).

3. Results

3.1. Residues content in oilseeds and MRL

Twenty-eight samples of sunflower seeds in 2007, twenty-two samples of rapeseed in 2008, and thirty samples of sunflower and thirty-two samples of rapeseed in 2009 were analyzed (Table 1-4).The range of insecticide active substances that are used on cereals or for storage facilities treatment that were detected on rapeseed were pirimiophos-methyl, malathion, chlorpyriphos-methyl and deltamethrin. The most frequently detected substance was pirimiophos-methyl, which was detected in amounts over the lower limit in 55% of samples in 2008 and 37.5% in 2009. This substance also caused the most cases of non-compliance with the MRLs, (32 % of the samples in 2008 and 9.4% in 2009). The concentrations of

pirimiophos-methyl measured in rapeseed samples decreased in samples taken in 2008 compared to 2009. Pirimiophos-methyl was also the most commonly detected substance in sunflower seeds (more than the quantification lower limit in 39% of samples in 2007, and in 20% of samples in 2009). The mean concentration of pirimiophos-methyl in sunflower seeds was similar in 2007 and 2009, 19 µg/Kg^{-1} in 2007 and 25 µg/ Kg^{-1} , respectively.

Dichlorvos was detected in the samples from 2007-2008 because of the new regulations. Similarly, malathion was detected in amounts in excess of the MRL in samples 2007 and 2008 (18% of samples of sunflower seeds in 2007, and 18% of samples of rapeseed in 2008) but not in the samples from 2008-2009.

Table 1 Analytical results (expressed in µg kg^{-1}) on the 28 final samples of sunflower seeds (storage campaign 2006-2007).

	LQ	MRL	Mean	Median	Standard deviation	9th decile	Maxi	% samples ≥ LQ	% samples > MRL
Pirimiphos-methyl	10	50	19	5	55	29	295	39%	4%
Malathion	10	-	8	0	25	17	125	18%	18%
Chlorpyriphos-methyl	10	50	0	0	-	-	10	4%	0%
Deltamethrin	10	50	-	-	-	-	Und.	0%	0%
Dichlorvos	10	10	21	0	79	27	422	29%	21%

LQ: limit of quantification, MRL: maximum residues limit; Und.: undetected

Table 2 Aanalytical results (expressed in µg kg^{-1}) on the 22 final samples of rapeseed (storage campaign 2007-2008).

	LQ	MRL	Mean	Median	Standard deviation	9th decile	Maxi	% samples ≥ LQ	% samples > MRL
Pirimiphos-methyl	10	50	130	22	266	335	1117	55%	32%
Malathion	10	-	19	0	69	16	322	18%	18%
Chlorpyriphos-methyl	10	50	3	0	9	0	31	9%	0%
Deltamethrin	10	100	1	0	3	0	13	5%	0%
Dichlorvos	10	10	-	-	-	-	Und.	0%	0%

LQ: limit of quantification; MRL: maximum residues limits; Und.: undetected

Table 3 Analytical results (expressed in µg kg^{-1}) on the 32 final samples of rapeseed (storage campaign 2008-2009).

	LQ	LMR	Mean	Median	Standard deviation	9th decile	Max	% ≥ LQ	% > MRL
Pirimiphos-methyl	10	50	16	0	5	31	212	37,5%	9.4%
Malathion	10	20	0	0	2	0	13	3,1%	0%
Chlorpyriphos-methyl	10	50	1	0	40	0	24	6,3%	0%
Deltamethrin	10	100	3	0	2	5	64	6,3%	0%
Dichlorvos	10	10	-	-	-	-	Und.	0%	0%

LQ: limit of quantification, MRL: maximum residues limits; Und.: undetected

Table 4 Analytical results (expressed in µg kg^{-1}) on the 30 final samples of sunflower seeds (storage campaign 2008-2009).

	LQ	LMR	Mean	Median	Standard deviation	9th decile	Max	%>LQ	%>LMR
Pirimiphos-methyl	10	50	25	0	106	13	578	20%	6.7%
Malathion	10	20	-	-	-	-	Und.	0%	0%
Chlorpyriphos methyl	10	50	-	-	-	-	Und.	0%	0%
Deltamethrin	10	50	1	0	3	5	14	3,3%	0%
Dichlorvos	10	10	-	-	-	-	Und.	0%	0%

LQ: limit of quantification, MRL: maximum residues limits; Und.: undetected

3.2. Traceability of cross-contamination situations

Five cases leading to cross-contamination were identified:

1) K1, treatment of cereals at outloading, just before outloading oilseeds;
2) K2 outloading of cereals, treated at their receipt, just before outloading of oilseeds,
3) K3 storage of treated cereals in the same bin just before storage of oilseeds;
4) K4 treatment of empty bin and of handling equipment before receiving oilseeds;
5) K5 receipt of oilseeds at the same time that cereals treated at receipt (it concerned only rapeseed).

When no cross-contamination occurred it was classified as K0.

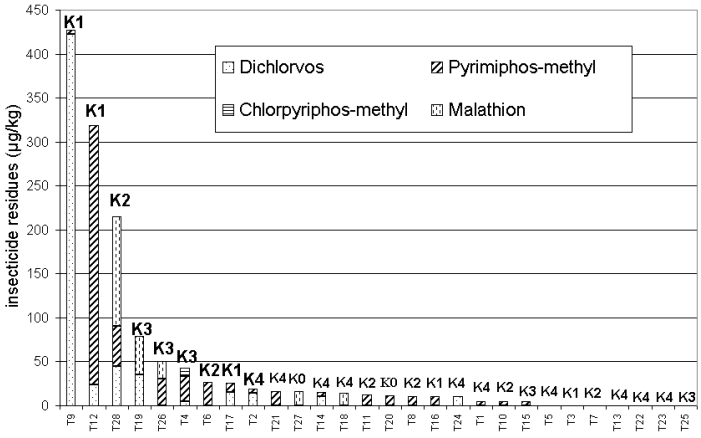

Figure 1 Distribution of the five cases of pesticide residues cross-contamination for each sunflower seeds batch (2006-2007).

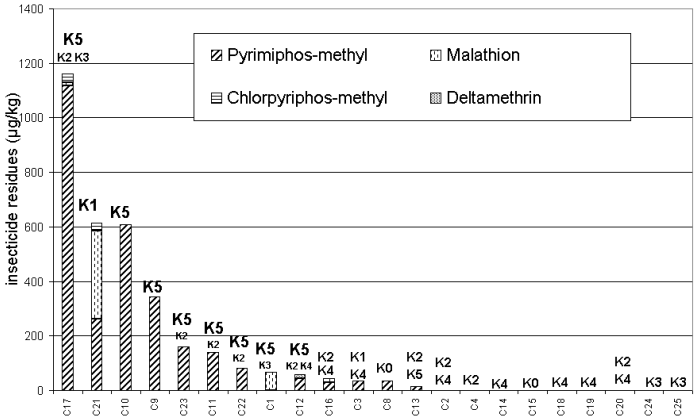

Figure 2 Distribution of the five cases of pesticide residues cross-contamination for each rapeseed batch (2007-2008).

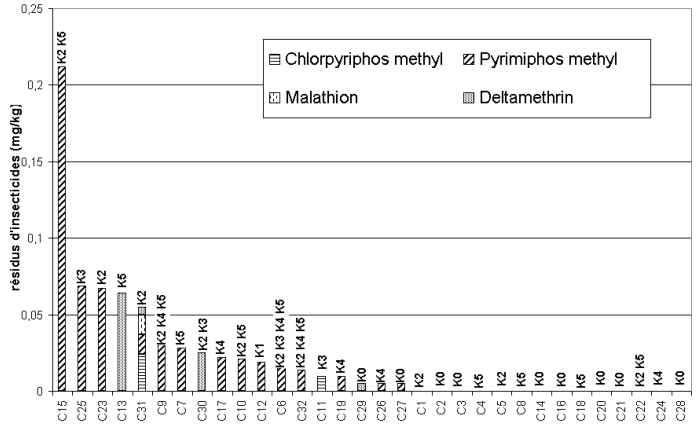

Figure 3 Distribution of the five cases of pesticide residues cross-contamination for each rapeseed batch (2008-2009).

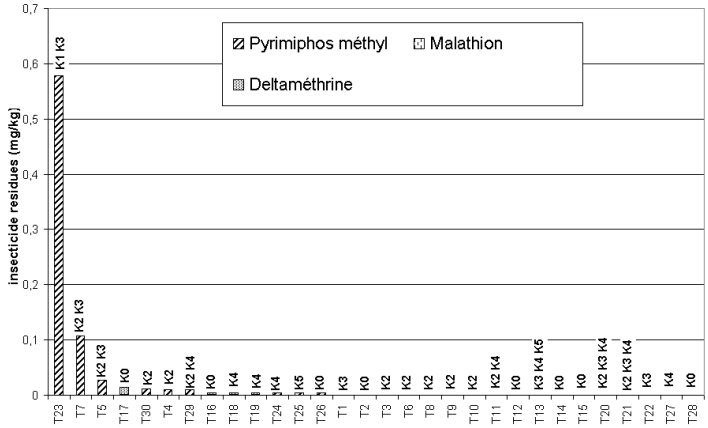

Figure 4 Distribution of the five cases of pesticide residues cross-contamination for each sunflower seeds batch (2008-2009).

4. Discussion

It appears that the highest cross-contamination on rapeseed occurred with situation K5 (Figs. 2 and 3). This one is characteristic of rapeseed, which is harvested at the same seasonal period than cereals (wheat, barley) during June-July. Most samples with pirimiophos-methyl above the MRL were from K5 (Fig. 2). Looking at each sample, we can observe that the highest contaminations occurred when treatments on cereals at receipt were systematically carried out. The occurrence of situations with a treatment of cereal batches immediately at their delivery increased in 2007-2008 because dichlorvos was already banned. Before this ban, dichlorvos treatment was more often used when a pest infestation was detected just before commercialization. Today, storage operators seem to prefer a preventive control of pests at the delivery before loading storage bins. In the 2007-2008 survey, it was observed that 81% of cereal deliveries were systematically or occasionally treated at their receipt. In the 2008-2009 investigation (on rapeseed and sunflower), it was observed that 75% of the delivered cereal grain batches were systematically or occasionally treated at their receipt at the silo. It was also shown that treatment of cereals at receipt could also lead to the cross-contamination situation K2 and K3, which were also frequent on rapeseed in 2008-2009 survey (Fig. 3).

The situation K5 can also be linked to a problem with insecticide application equipment: unsatisfactory proof for insecticide aerosol droplets release from insecticide treatment system, cereal treatment not stopped after the emptying of the handling circuit (leading to a direct accumulation of residues on empty

handling equipment), possible delay for switching off a cereal treatment before loading the handling system with a rapeseed batch received just after several cereal batches. These situations could neither be checked nor validated in our investigation.

The situation K1 was less frequent than the situation K5, but can also lead to cross-contaminations. It was this situation, as in the previous investigation on sunflower, that led to the highest contaminations when treatment of cereals was systematic at outloading (Fig. 1). It can also occur on rapeseed. In the case C21 (Fig. 2), malathion and chlorpyriphos-methyl were not used during the storage season 2007-2008, but was used during previous seasons. This silo was made of concrete; so we hypothesized this surface can retain residues more than a year. The situation K4 did cause few problems, except if it was associated to other risky situations. On sunflower (Figs.1 and 4), the highest contaminations were observed when treatment was systematic at outloading (K1) in 2006-2007. Even though dichlorvos had been banned, other practices led to classification of sunflower seeds as K2 and K3. This was most likely caused when silos treated the cereals upon receipt. Our study in real situations showed that cross-contaminations of oilseeds by post-harvest insecticide residues exist, and can sometimes lead to residue contents above regulatory tolerances. The highest risk of contamination for rapeseed appears to occur when cereals are systematically treated at receipt, at the same time that rapeseed is received, using the same conveyor circuits. Other identified cases can also lead to slighter contamination. We identified several situations within a storage facility that can increase the risk level for the cross-contamination. We did not identify all possible sources of residue contamination, but one more possibility for contamination is leakage of insecticide by the application equipment.

We noticed differences in cross-contaminations between sunflower and rapeseed, difference that we could relate to a very different harvest period for these two oilseeds. And also, we noticed a change between the three years of investigation: the withdrawal of dichlorvos had adverse indirect effects in leading to preventive strategies with more frequent treatments of organophosphates applied for preventative protection against storage insect pests.

To reduce cross-contaminations, we can advise storage managers to avoid sharing same receipt circuits when cereals are systematically treated, and to avoid accumulation of risky situations. It is also very important to periodically verify the proper use of insecticide treatment equipment. This investigation allowed us to make the storage companies aware about this issue, and to help them to understand how cross-contaminations can occur in their silos and how to avoid them, knowing that each silo is different than others.

Acknowledgments

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Toxicity of powder and extracts of *Zanthoxylum zanthoxyloides* Lam (Rutaceae) root bark from Nigeria to three storage beetles

Denloye, A.A.*[1], Makanjuola, W.A.[2], Ajelara, O.[1], Akinlaye, O.J.[1], Olowu, R.A.[3], Lawal, O.A.[3]
[1]Department of Zoology, Faculty of Science, Lagos State University, Lagos, Nigeria.
[1,2] Department of Zoology, Faculty of Science, University of Lagos, Nigeria.
[3]Department of Chemistry, Lagos State University, Lagos,

* Corresponding author
# Presenting author

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Abstract

The root of *Zanthoxylum zanthoxyloides* Lam is used as antibacterial toothbrush in southwestern Nigeria. The root bark was therefore screened as powder, aqueous and ethanolic extracts for toxicity to adult *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and the effects of the test extracts on oviposition and progeny development of *C. maculatus* in laboratory tests. A small scale field trial was also carried out to test the efficacy of test powder as a protectant of cowpea, *Vigna unguiculata* (L.) Walpers and maize, *Zea mays* L. grains against insect infestation. Results of the acute toxicity tests showed that all the formulations were toxic to the insects. The 48 h median lethal concentration (LC_{50}) values obtained for the test powder against *C. maculatus*, *S. zeamais* and *T. castaneum* are 0.05 g kg^{-1}, 0.01g kg^{-1} and 0.04 g kg^{-1}, respectively. For the aqueous extracts the LC_{50} values are 0.83 g L^{-1}, 0.34 g L^{-1} and 0.38 g L^{-1} against *C. maculatus*, *S. zeamais* and *T. castaneum*, respectively while the values are 0.02 g L^{-1}, 0.04 g L^{-1} and 0.09 g L^{-1}, respectively for ethanolic extract, indicating higher toxicity against the test insects relative to the water-based extract. The ethanolic extract demonstrated residual property, the toxicity to *C. maculatus* remaining fairly constant over a total post-treatment time of 336 h. Cowpea grain treatment with test plant ethanolic extract resulted in reduction of the number of eggs laid from 93.30 ± 3.46 in the control to 21.00 ± 4.57 in grain treated with 0.10 g L^{-1} extract without significant difference in the number of adult emergence from the treated grains. Field trials showed that cowpea and maize grains treated with test plant powder respectively were protected from insect infestation for 180 d. These results demonstrate the potentials of *Z. zanthoxyloides* for protecting cowpea and maize grains against storage insects.

Keywords: *Zanthoxylum zanthoxyloides, Callosobruchus maculatus, Sitophilus zeamais, Tribolium castaneum,* Toxicity

1. Introduction

Insects damage stored grains and also create conditions that allow secondary infestation by other pests and deterioration by microorganisms, primarily fungi (Agrawal et al., 1988; Oke and Muniru, 2001). Once an infestation is established insect pests generally cause gradual and progressive damage, leading to losses in nutritional, organoleptic and aesthetic quality as well as weight loss to stored grains. About 40 insect species can damage grains (Osuji, 1985; Sousa et al., 2005), including the cowpea weevil, *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and the red flour beetle, *Tribolium castaneum* (Herbst) (Colepotera: Tenebrionidae). While *C. maculatus* and *S. zeamais* are primary pests attacking intact cowpea, *Vigna unguiculata* Walpers (Fabaceae) seeds and maize, *Zea mays* L. (Poaceae) grains, *T. castaneum* infests grains and other products including groundnut, *Arachis hypogea* L. (Fabaceae) during post-harvest handling. These insects are responsible for up to 80 % of the infestations in infestation on cowpea, maize and groundnut during storage (Osuji, 1985; Jood et al., 1996), thus justifying control measures to protect these crops.

It is well established that many synthetic insecticides are effective in controlling insects in stored products. However, some of these insecticides can have deleterious side effects and the costs of application are excessive for many developing countries. These limitations necessitates search for new insecticides with novel mechanisms of action. In this regard, it the bioactivity of botanicals, particularly edible plant species, have been investigated as sources of insecticides that are safer to use (Golob and

Webley, 1980; Don-Pedro, 1984; Don-Pedro, 1985), and more easily and cheaply produced as crude or partially purified extracts (Rahman and Talukder, 2006), which would benefit subsistence level storage in developing countries. Thus, we conducted studies using *Zanthoxylum zanthoxyloides* Lam (Rutaceae). The root of this plant is commonly used as toothbrush because it has antimicrobial effect.

Traditionally in Africa, the storage time for grains is between three and six months before they are consumed, processed into livestock feed or used as seeds for the next planting season. More importantly, these grains are seasonal. Long-term studies on the insecticidal effect of plant species in protecting and controlling existing infestation of stored grains under ambient conditions in the field is therefore necessary.

The purpose of the present study is to determine the toxicity of powder, aqueous extract and ethanolic extract of *Z. zanthoxyloides* on adult *C. maculatus, S. zeamais* and *T. castaneum,* investigate the effect of the ethanol extract on oviposition and adult emergence, and assess the ability of the plant materials to protect stored cowpea and maize grains respectively from losses arising from insect infestation during field storage in traditional crib for six months.

2. Materials and methods

2.1. Plant materials and test insects

Test plant materials were used as powder, water and ethanol extracts against test insect species. Each of these formulations were prepared following the procedure used by Denloye et al. (2007). *Callosobruchus maculatus, S. zeamais* and *T. castaneum,* were obtained from cultures maintained at Nigerian Stored Product Research Institute (NSPRI), Abule-Oja, Lagos, Nigeria. Fresh experimental cultures were prepared from the original stocks as described by Denloye et al. (2007).

2.2. Bioassays

Powder, aqueous and ethanolic extracts of *Z. zanthoxyloides* were respectively screened using the method of Denloye et al. (2007) to detect bioactivity of the test materials against each of the insect species cultured for the present study. Twenty active 1 to 3-d-old *C. maculatus* adults (mixed sexes), 1 to 7-d-old *S. zeamais* (mixed sexes) or 1 to 7-d-old adult *T. castaneum* (mixed sexes) were separately exposed to grains treated with each formulation and mortality assessments made every 24 h after treatment for 2 d.

Test plant materials were retested against the test insect species in more elaborate bioassays to measure acute toxicity levels dependent on 48 h LC_{50} and LC_{95} values as described by Denloye et al. (2007). For these series of experiments, 20 unsexed adult insects, and same age ranges given earlier were exposed per replicate of each treatment and the controls. For the test powder against each insect species the admixture concentrations used were 0.125 to 8.00 g kg^{-1} grain. For aqueous extracts the grains were dipped in extracts of 0.10 to 1.60 g kg^{-1} concentrations and for ethanolic extract 0.01 to 0.032 g L^{-1} concentrations were used.

Forty undamaged cowpea grains were treated by dipping in concentrations of 0.01, 0.02, 0.04, 0.08 and 0.16 g L^{-1} ethanol extract in four replicates. Treated grains were allowed to drain on filter paper for 5 min before transferring into bioassay containers. Mortality of exposed *C. maculatus* was assessed every 24 h. Several sets of 40 cowpea seeds treated at these concentrations with untreated seeds as controls were prepared at the same time. For each set of treated seeds and controls, bioassays were started off by introducing 10 unsexed 1 to 3-d-old adult *C. maculatus* to 1-, 12, 24, 96, 168 and 136 h predetermined post-treatment time intervals after treatment. Each treatment and controls were replicated four times. Insect mortality was assessed every 24 h for 2 d.

Cowpea seeds were treated at two concentrations (0.025 g L^{-1} and 0.10 g L^{-1}) of *Z. zanthoxyloides* ethanolic extract by dipping. Four 0 to 3-d-old adult *C. maculatus* (2 ♂, 2 ♀) were then confined for seven days with 20 treated or untreated cowpea seeds in clean glass Petri dishes securely covered. All treatments including control seeds that were dipped in ethanol only were replicated five times. Adults that died within the 7-d exposure periods were removed and replaced with other insects of the same age and sex. At the end of the 7-d oviposition period, all adults were removed. The seeds were inspected for eggs and were counted under binocular microscope (x8 objective). The seeds bearing eggs were then kept in covered vials and monitored daily for adult emergence. Emerging adults were counted and

removed from each treatment daily for 14 d after the first emergence was observed to prevent overlap of generations.

Similar experiments as described above were carried out, however, in this case a series of grains were treated by dipping in 0.025 g L^{-1} and 0.20 g L^{-1} of ethanolic extracts of *Z. zanthoxyloides* and ethanol for control. Two 0 to 3-d-old adult *C. maculatus* (1 ♂ and 1 ♀) were then confined with each set of extract-treated or ethanol-treated cowpea grains for 24 h, after which the pair of *C. maculatus* was transferred onto another batch of treated cowpea using the same extract concentration or control. The process was repeated every 24 h for 7 d. Each treatment batch was replicated four times. The number of eggs laid per day in each replicate of the treatments and control grains were counted daily under a binocular microscope (x8 objective).

2.3. Protectant evaluation

Disinfested cowpea or maize grains (5.0 kg) were measured into jute bags and manually admixed with powdered *Z. zanthoxyloides* at 2.0 g kg^{-1} and untreated controls. Each jute bag with the treated or untreated grain was securely tied and stored in traditional crib with thatched roof in an open field for 180 d. There were four replicate bags of treated or untreated grain arranged randomly with one replicate of each treatment on each of the four layers per crib. The assumption was that bags of grain left in cribs in the field are liable to infestation by the appropriate storage insect pest over time. To evaluate the results of the series of experiment, 100-g samples of cowpea or maize were taken from each bag, once every 30 d and assessed for insect damage according to Odeyemi and Daramola (2000).

2.5. Data analyses

Toxicological dose-response data involving mortality of test insect were analyzed by probit analysis (Finney, 1971) after correcting for mortality in control based on a computer program to obtain the median lethal concentration (LC_{50}) and the corresponding LC_{95}. Analysis of variance (ANOVA) was used to compare treatment means where the design fitted the requirements dependent on Statistical Package for Social Sciences (SPSS) version 11.0 (SPSS, 2001). Post-hoc analysis was carried out only where there was a significant difference at the 5% ($P < 0.05$) level of significance by comparing pairs of means based on Least Significant Differences (LSD). Monthly weight loss in each treatment and control was determined from 100-g batches of grains in each jute bag after Odeyemi and Daramola (2000) as follows:

$$\text{Percent weight loss} = \frac{(W_u \times N_d) - (W_d \times N_u) \times 100}{W_u (N_d + N_u)}$$

Where W_u = Weight of undamaged grains

N_u = Number of undamaged grains

W_d = Weight of damaged grains

N_d = Number of damaged grains

3. Results

The results show all test formulations were toxic to each of the three insect species exposed on treated grains (Table 1). Based on 48 h LC_{50} values, more detailed bioassays showed that the powder was significantly more toxic to *S. zeamais* (0.012 g kg^{-1}) than to either *T. castaneum* (0.41 g kg^{-1}) or *C. maculatus* (0.50 g kg^{-1}) (Table 2). The LC_{50} values also shows that the ethanolic extract was significantly more toxic to each of the test insect species than the aqueous extract. The ethanolic extract was however significantly less toxic to *T. castaneum* than to either *C. maculatus* or *S. zeamais* respectively (Table 2). Tests also showed further that the toxicity of the ethanolic extract of *Z. zanthoxyloides* remained constant for 336 h, the LC_{50} values remaining fairly constant at about 0.010 g/kg^{1} (Figure 1).

Table 1 Mortality of test insects on grains treated with *Z. zanthoxyloides*

Formulation	Test Insect	Mortality		
Powder	**Species**	**0.00 g/kg**	**1.00 g/ kg**	**20.00 g/kg**
	C. maculatus	0.00 (0.71) a	83.35 (9.16) a	100.00 (10.02) b
	S. zeamais	0.00 (0.71) a	83.35 (9.16) a	93.30 (9.69) b
	T. casteneum	0.00 (0.71) a	71.65 (8.49) b	100.00 (10.02) b
Aqueous Extract		**0.00 g/L**	**1.00 g/L**	**10.00 g/L**
	C. maculatus	0.00 (0.71) a	6.65 (2.67) b	25.00 (5.05) b
	S. zeamais	0.00 (0.71) a	5.00 (2.35) b	20.00 (4.53) c
	T. casteneum	0.00 (0.71) a	0.00 (0.71) a	25.00 (5.05) b
Ethanolic Extract		**0.00 g/L**	**1.00 g/L**	**10.00 g/L**
	C. maculatus	0.00 (0.71) a	60.00 (7.78) b	90.00 (9.51) c
	S. zeamais	0.00 (0.71) a	63.35 (7.99) b	95.00 (9.77) c
	T. casteneum	0.00 (0.71) a	90.00 (9.51) b	100.00 (10.02) c

Each datum is a mean of three replicates. Values in parentheses are square root ($\sqrt{x} + 0.5$) transformed. Column transformed means for each test plant extract bearing the same superscripts are not significantly different by Least Significant Difference (LSD) following Analysis of Variance (ANOVA); $P < 0.05$.

Table 2 Acute toxicity of *Zanthoxyllum zanthoxyloides* to test insects

Formulation	Test Insect		95 % Confidence limits	Regression Equation	Degree of Freedom	Slope ± Standard Error
Powder	**Species**	**48 hr LC_{50} (g/kg)**				
	C. maculatus	0.050	0.007 – 0.228	Y = 2.77 + 2.124x	4	2.12 ± 0.75
	S. zeamais	0.012	0.0 – 0.055	Y = 1.54 + 0.803x	4	0.803 ± 0.042
	T. castaneum	0.041	0.007 – 0.111	Y = 1.806 + 1.303x	4	1.303 ± 0.088
Aqueous Extract		**48 hr LC_{50} (g/L)**				
	C. maculatus	0.834	0.633 – 1.042	Y = 0.127 + 1.605x	3	1.605± 0.034
	S. zeamais	0.334	0.26 – 0.427	Y = 0.586 + 1.232 x	3	1.232 ± 0.026
	T. castaneum	0.383	0.296 – 0.496	Y = 0.486 +1.168x	3	1.168 ± 0.025
Ethanolic Extract		**48 hr LC_{50} (g/L)**				
	C. maculatus	0.021	0.012 – 0.022	Y = 2.263 + 1.476x	3	1.476 ± 0.024
	S. zeamais	0.035	0.020 – 0.041	Y = 1.567 + 1.021 x	3	1.021 ± 0.021
	T. castaneum	0.085	0.029 – 0.096	Y = 0.486 +1.168x	3	1.021 ± 0.021

LC_{50} values with no overlap in their 95 % confidence limits are significantly different ($p < 0.05$).

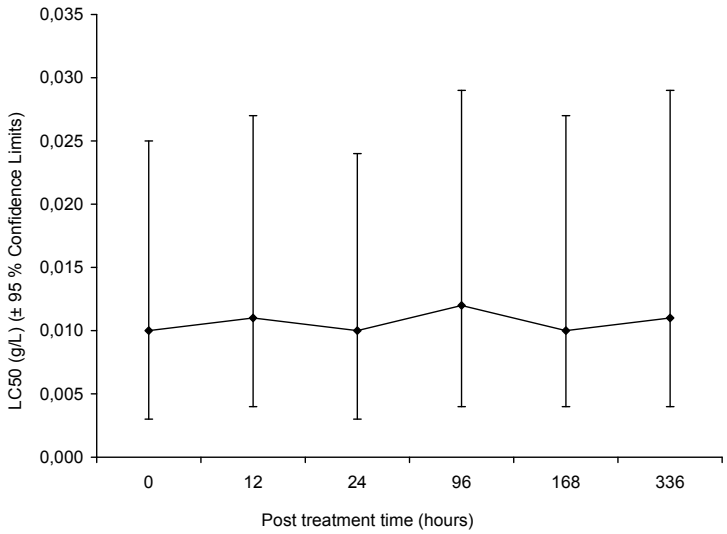

Figure 1 Persistence of *Z. zanthoxyloides* ethanol extract toxicity in treated cowpea grains

Treatment of cowpea grains with *Z. zanthoxyloides* ethanol extract caused significant reduction in the number of eggs laid by *C. maculatus,* but not a corresponding increase in adult emergence (Table 3a). Tests also showed that the test ethanolic extract treatment had no effect on the daily oviposition rate of *C. maculatus* at the low treatment (0.025 g kg^{1}) but it delayed the commencement of egg laying for three days at the high concentration with a significantly reduced number of eggs laid (Table 3b).The experiments on weight loss of treated grains showed that there was no weight loss for 180 d in treated cowpea and 150 d in treated maize, whereas the untreated grains had 3.12 g and 5.04 g for weight losses for maize and cowpea, respectively, after 180 d of storage (Table 4).

Table 3a Effect of *Zanthoxyllum zanthoxyloides* on oviposition and progeny production of *C. maculatus*

Treatment	(g/L)	Mean number of Eggs laid (± SE)	Mean adult emergence (± SE)	Mean percent adult emergence (± SE)
Z. zanthoxyloides	(0.00)	93.30 ± 3.46 a	41.00 ± 2.58	43.95 ± 4.76
	(0.025)	53.00 ± 1.63 b	18.00 ± 3.16	33.88 ± 5.28
	(0.10)	21.00 ± 4.57 c	7.00 ± 2.45	32.86 ± 4.99

Column means bearing same superscripts are not significantly different (P > 0.05) by Least Significant Difference (LSD) Test. SE = Standard Error

Table 3b Effect of *Z. zanthoxyloides* on daily oviposition by *C. maculatus* in treated cowpea seeds

Extracts	Concentration (g/L)	Oviposition days							
		1	2	3	4	5	6	7	Total
Z. zanthoxyloides	0.0	2	4	10	10	9	5	3	43 a
	0.025	1	5	7	10	10	2	2	37 a
	0.20	0	0	0	3	2	1	1	7 b

Each datum is a mean of four replicates. Means for total number of eggs laid on seeds treated with each test plant extract bearing the same superscripts are not significantly different by Least Significant Difference (LSD) following Analysis of Variance (ANOVA); P < 0.05.

Table 4 Weight loss of grains protected by treatment with *Z. zanthoxyloides*

	Weight loss (g) in treated grains			
Post-treatment	Maize		Cowpea	
Time (Days)	Control	Treated grains	Control	Treated grains
0	0.00 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.0 ± 0.00
30	0.00 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.0 ± 0.00
60	0.02 ± 0.00	0.0 ± 0.00	0.03 ± 0.001	0.0 ± 0.00
90	1.44 ± 0.35	0.0 ± 0.00	1.79 ± 0.23	0.0 ± 0.00
120	1.80 ± 0.35	0.0 ± 0.00	2.21 ± 0.42	0.0 ± 0.00
150	2.47 ± 0.29	0.0 ± 0.00	2.83 ± 0.19	0.0 ± 0.00
180	3.12 ± 0.44	0.05 ± 0.01	5.04 ± 1.15	0.0 ± 0.00

Each datum is a mean of four replications.

4. Discussion

The biological activity of *Z. zanthoxyloides* was investigated in laboratory bioassays and semi-field trials to evaluate the potentials of the plant as a source of insecticide for the protection of stored grains from attack by *C. maculatus, S. zeamais* and *T. castaneum*. On the basis of properties required in chemicals for controlling insects feeding on grains such as toxicity to adults and oviposition suppression the test plant materials have shown some appreciable potential under these parameters. In this study *Z. zanthoxyloides* powder and ethanolic extract demonstrated toxicity against the adults and the extract reduced oviposition by *C. maculatus*. These findings agree with a similar study by Ogunwolu and Odunlami (1996), where root bark powder of *Z. zanthoxyloides* was toxic to the adults and caused fewer eggs to be laid with a corresponding smaller number of emerged adults in cowpea seeds treated with the test powder compared with the control. In the present study, the ethanolic extract remained toxic in laboratory tests against *C. maculatus* for 336 h without losing its potency against the insects. The practicality of using the test plant

as grain protectant was demonstrated in this study when no weight loss of treated grains was recorded for at least 150 d (5 months).

Based on these results, the plant materials can similarly be used by subsistence farmers to protect stored cowpea and maize grains against *C. maculatus* and *S. zeamais* in small storage systems in Nigeria and other African countries such as Malawi and Benin Republic (Delobel and Malonga, 1987; Terpondju et al., 2002). The toxicity and oviposition suppression activity of the test plant products in this study were caused by the bioactivity of their chemical constituents and physical action of the formulations. While there is need to isolate and identify the chemical constituents of the test plant materials the physical actions may be explained. For instance the bioactive constituents of the plant materials may be more available in the ethanolic extract since and be responsible for the extract's higher toxicity. The powder formulations used in this study could have caused insect mortality due to their physical action on respiration through blockage of the spiracles of the test insects. Although there is no direct evidence of this in our test earlier studies have shown that there is a direct relationship between particle size of plant powders and insect mortality in treated grains (Ogunwolu and Odunlami, 1996; Ofuya and Dawodu, 2004). In addition, fine particle size such as the one used in this study helps to even the distribution of powders on the surface of seeds and the walls of the storage container, thus increasing their possibility of getting in contact with the insects and killing them. In addition, plant powders cause abrasion of insect cuticle and lead to water loss (Sousa et al., 2005), thus causing stress and eventual death. Our study shows *Z. zanthoxyloides* has high potential for use as protectant of maize and cowpea grains in small scale storage systems in Nigeria.

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A comparison of the effect of two diatomaceous earth formulations on *Plodia interpunctell*a (Hübner) and the effect of different commodities on diatomaceous earth efficacy

Timlick, B.[1]*, Fields, P.G.#[2]
[1] Canadian Grain Commission, 844 – 303 Main St. Winnipeg, Manitoba, Canada, R3C 3G8.
Email: blaine.timlick@grainscanada.gc.ca
[2] Cereal Research Centre, Agriculture and Agri-Food Canada, 195 Dafoe Road, Winnipeg, Manitoba, Canada, R3T 2M9.

* Corresponding author
# Presenting author

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Abstract

The efficacy of two diatomaceous earth formulations was tested on five commodities; maize, wheat, birdseed, sunflower and barley, against *Plodia interpunctella* (Lepidoptera: Pyralidae). Exposure to DE began at either the egg stage or 3rd instar stage and the reduction in emergence to adult was noted. There was no significant difference between the freshwater and marine (Protect-It) DE. When eggs were placed on treated diet, the LD_{90} was 135, 143 and 805 ppm for maize, wheat and birdseed, respectively. When 3rd instars were placed on diet, LD_{90} was 382, 444, and 1156 ppm for maize, sunflower and birdseed respectively.

Keywords: DE, Wheat, Maize, Birdseed, Sunflower, Indian meal moth

1. Introduction

Plodia interpuntella (Hübner) (Pyralidae: Lepidoptera), the Indian meal moth is a world-wide pest of many stored products including dried fruit and vegetables, nuts and cereals (Na and Ryoo, 2000; Barak and Harein, 1981; Harein and Subramanyam, 1990). In most cases, where raw durables are stored, *P. interpunctella* inhabits the top several centimeters of the stored bulk. Direct feeding on commodities is of concern, however, deposition of frass and webbing is a major cause for product rejection due to contamination (Carrillo et al., 2006).

One of the most particular aspects of *P. interpunctella*'s biology is how strongly correlated development and ultimately survival is with the commodity, lighting and temperature. Variation in the toughness of endosperm among varieties of the same class of grain has shown to affect development and mortality (Abdel-Rahman, 1968). The ability for *P. interpunctella* populations to quickly tolerate localized conditions is also cited as a mechanism for survival (Subramanyam and Hagstrum, 1993). Altering conditions such as photoperiod, temperature or diet will influence developmental time, food consumption, survival and fecundity. Therefore, disrupting any or all of these factors in an ongoing way may assist in maintaining the commodity quality.

Diatomaceous earth (DE) has been used as an alternative synthetic insecticides for many years, and as it does not break down rapidly, a single application can be used in an ongoing way to disrupt aspects of insect development as long as the commodity remains dry. The DEs are typically comprised of approximately 70 – 90% amorphous silicon dioxide with the balance comprised of inorganic oxides and salts. These products are mined from quarries, crushed, sieved to remove rocks, and for some formulations synergists are added. When processed into a dust, particle size distribution is between 0.5 – 100 microns, with the majority being in the range of between 10 – 50 microns (Korunic, 1998; Subramanyam and Roesli, 2000).

DE insecticides are known to protect commodities against stored-product pests in two ways. First, DE particles are picked up by the insect as they walk through the commodity. The body of adult stored-product insects is made up of an exoskeleton covered with waterproofing waxes and lipids. The wax layer on the insect's epicuticle is damaged, insects lose water through the cuticle and die from desiccation (Ebeling, 1971; Subramanyam and Roesli, 2000). In addition, another mode of action of DE is its ability to repel insects.

The objective of this study was to examine the effect of two diatomaceous earth formulations on *P. interpunctella* on wheat, barley, maize, birdseed and sunflower. A second objective was to determine if eggs or early instar larvae are more susceptible to DE on various commodities than 3rd instar larvae.

2. Materials and methods

Plodia interpunctella used in this study were from laboratory cultures. Cultures were reared on cracked wheat at 16% moisture content. All cultures, experiments and rearing were at 25°C and 70% r. h. with 16 hours light and 8 hours dark, as these conditions are considered optimal for pyralid moths production and to minimize induction of diapause. Insects were treated with one of two diatomaceous earth formulations; one from a marine deposit (P1, Protect-It®, Hedley Technologies Inc, Mississauga, Canada) and the other a freshwater deposit (P2). The formulations were both buff-colored with crystalline content of less than 1%. Both formulations were sieved using a #30 mesh sieve to remove clumps and both had 10% silica aerogel as part of the formulation. Application rates to the commodities described below were 0, 100, 200, 300, 500, 700 or 1000 ppm.

Experiments were conducted with five commodities; wheat, cv Katepwa (14% mc), barley, cv Robust (13% mc), cleaned feed maize, many varieties (13.5% mc for eggs experiments and 11.0% mc for larval experiments), Sunflower, cv Pioneer 6150 (4.5% mc) and birdseed, Kaytee brand (comprised of oilseed sunflower, millet, cracked maize, peanuts, safflower and calcium carbonate with undetermined moisture content).

Six hundred grams of each commodity were placed into a 4-L jar and were treated with one of the formulations at one of the seven doses. After application, jars were sealed and were shaken for one minute by hand, after which 200 g were placed in to each of three 1-L jars to serve as replicates. One hundred 1-d old *P. interpunctella* eggs were introduced into each treatment jar. Also, for each experiment, 100 eggs from the same cultures were placed onto moistened filter paper to determine percent hatch. Third instar larvae were also tested against the DE with sunflower, maize and birdseed to determine if there was an effect of early larval mortality on these commodities. Treatments were prepared as described and 50 early third instar larvae for each replicate were introduced. The total number of adults that emerged from each jar was observed daily once emergence began until emergence was complete. This was approximately six - eight weeks after the initial adults had emerged and until there were several observations of no emergence. Those that emerged were counted and recorded. The percentage of reduction of adult emergence was compared to that of the untreated control.

3. Results

Egg viability was very good, with hatch rates of 96% (maize), 93% (wheat), 98% (barley) and 92% (birdseed and sunflower). This would indicate that much of the mortality associated with the controls could be associated with the performance of the insect on the host food as adult emergence on the control food varied: 94% (maize), 69% (wheat), 0% barley, 85% (birdseed) and 80% (sunflower) (Table 1). Overall, the DE formulations could control *P. interpunctella*. In all cases, except for the P1 on birdseed, greater than 95% control was achieved at the higher dosages (Table 1). In addition, those that did emerge as adults on the various commodities at higher rates were noticeably smaller, and in many cases were somewhat deformed (mostly non functional wings).

Table 1 Adult emergence (± standard error of the mean) of *Plodia interpunctella,* 100 eggs were exposed to various levels of two diatomaceous earth formulations on three different commodities.

Commodity	DE	Dose (ppm)	Adult Emergence±SEM	Reduction Compared to Control (%)
Wheat	P1	Control	69.0±2.2	0
		100	19.0±2.5	68
		200	3.3±0.3	94
		300	0	100
Wheat	P2	0	59.0±2.2	0
		100	17.7±2.7	70
		200	2.3±0.3	96
		300	0	100
Maize	P1	0	95.0±1.7	0
		300	19.5±2.7	80

Commodity	DE	Dose (ppm)	Adult Emergence±SEM	Reduction Compared to Control (%)
		500	4.3±0.7	96
		700	0	100
Maize	P2	0	95.0±1.7	0
		300	14.0±3.0	86
		500	5.0±1.2	95
		700	1.0±0.1	99
Birdseed	P1	0	84.3±6.5	0
		100	62.3±7.1	27
		300	44.0±3.0	48
		500	21.0±3.1	75
		700	5.3±2.2	94
Birdseed	P2	0	84.7±6.5	0
		100	75.3±2.9	11
		300	55.3±3.3	35
		500	18.0±4.0	79
		700	3.0±1.5	97

There was no significant difference between the two formulations (Table 2). Wheat (LD_{90}; 143 ppm) and maize (135 ppm) required the least DE to control *P. interpunctella* from the early instar larvae, whereas birdseed required considerably more (860 ppm, Table 2).

When 3rd instars were exposed to on the treated commodities, complete control occurred at the highest doses (1000 ppm, Table 3) on maize and birdseed, but not for the sunflower seed, where only 85% reduction of adult emergence occurred. Birdseed required the least DE to control third instars (LD_{90}; 382 ppm), while maize required 444 ppm and sunflower required 1158 ppm (Table 4). On maize the LD_{90} for eggs was 380 ppm compared to 444 ppm for 3rd instars, in contrast, on birdseed more DE was required to control *P. interpunctella* exposed from the egg stage (860 ppm) then the 3rd instars (382 ppm).

Table 2 Probit analysis (LD_{50} and LD_{90}) (90% CI) of the mortality of *Plodia interpunctella* when exposed to three commodities treated with one of two diatomaceous earth formulations.

Commodity	Duration (d)	DE	LD_{50} (90% CI) (ppm)	LD_{90}(90% CI) (ppm)
Maize	59	P1	56 (41 – 68)	135 (122 – 150)
Wheat	57	P1	74 (61 – 84)	143 (131 – 160)
Birdseed	61	P1	251 (106 – 348)	806 (590 – 1744)
Maize	59	P2	36 (18 – 51)	129 (110 – 147)
Wheat	57	P2	68 (53 – 79)	139 (126 – 155)
Birdseed	61	P2	366 (318 – 404)	600 (540 – 698)

Table 3 Adult emergence (± standard error of the mean) of *Plodia interpunctella*, 50 3rd instar larvae are exposed to various levels of a P1, marine diatomaceous earth formulation on three different commodities.

Commodity	Dose (ppm)	Adult Emergence±SEM	Reduction Compared to Control (%)
Sunflower	0	40±1.0	0
	100	36±2.0	10
	300	18±2.0	55
	500	14±3.0	65
	1000	6±1.0	85
Birdseed	0	42±2.0	0
	100	34±2.0	19
	300	10±1.0	76
	500	1±1.0	98
	1000	0	100
Maize	0	38±1.0	0
	200	19±3.0	50

Commodity	Dose (ppm)	Adult Emergence±SEM	Reduction Compared to Control (%)
	400	8±3.0	79
	600	3±1.0	93
	800	1±1.0	98
	1000	0	100

Table 4 Probit analysis (LD_{50} and LD_{90}) (90% CI) of the mortality of *Plodia interpunctella* when exposed to three commodities treated with, P1, a marine diatomaceous earth formulation.

Commodity	Duration (d)	LD_{50} (90% CI) (ppm)	LD_{90} (90% CI) (ppm)
Maize	60	199 (172-223)	444 (400-503)
Sunflower	60	289 (239-338)	1158 (940-1545)
Birdseed	60	178 (159-204)	382 (340-436)

4. Discussion

Diatomaceous earth is an effective insecticide against many stored-product insect pests. There are several factors that determine the efficacy of DE, the main ones being: type of DE, target species, commodity treated and moisture content (McLaughlin, 1994; Korunic, 1998; Subramanyam and Roesli, 2000).

We only tested two DE formulations and found no difference between the two DE, however, caution should be exercised when extrapolating from our results to other DE, since there can be a four-fold difference in efficacy between DE (Korunic 1998). Snetsinger (1988) proposed that marine DE was less effective than freshwater DE, although a more recent study found just the opposite (Korunic, 1998)

There is very little information available on the efficacy of DE against pyralid moths (Wilbur et al., 1971; Nielsen, 1998, Arthur and Brown, 1994; Subramanyam et al., 1998; Mewis and Ulrichs, 2001). We found to control *P. interpunctella,* 200 to 1000 ppm were needed depending upon commodity. Arthur and Brown (1994) conducted field trials and found that 6,690 ppm Insecto on peanuts had no infestation over a six-month test, although there was only very low infestation in the untreated controls. Compared to other species *P. interpunctella* is relatively sensitive to DE (wheat, LD_{50} 74 ppm) compared to *Cryptolestes ferrugineus* (Stephens) (50 ppm), *Sitophlius oryzae* (L.) (300 ppm) and *Tribolium castaneum* (Herbst) (500 ppm) (Korunic 1998). In this study we exposed either 1st instars or the 3rd instars for as long as 61 d, whereas other studies used adult beetles exposed for a few days to a few weeks. When comparing larvae of *T. castaneum, Oryzaephilus surinamensis* (L.) and *P. interpunctella* all had very similar sensitivity to DE (Subramanyam et al., 1998).

Commodities have a large influence on DE rates. For Protect-It to control *C. ferrugineus* only 100 ppm is needed for wheat, but 150 is needed for barley, 500 ppm needed for oats and rye and 1000 ppm needed for maize (Canadian insecticide label, registration number 24259). Maize and sunflower seed is often cited as requiring more DE than wheat or barley (Subramanyam and Roesli 2000), but we saw no difference between wheat and maize for *P. interpunctella.* Sunflower and maize have a great deal more oil content then wheat, and therefore the DE may be rendered less active on these commodities.

The insect life stage also affects DE efficacy. Later instars of *P. interpunctella* are more resistant to DE than younger instars (Subramanyam et al., 1998). We found similar results for maize, but not for birdseed. In these tests, late instars were on the DE-treated diet for a less time than when placed on as eggs, so is it expected that the amount of DE required to control the insects would be less. The results with birdseed are unusual. Additional testing would be required to determine if these observations are repeatable, and if so why early instars require more DE than 3rd instars for complete kill.

In general, there was very high survival to adults on all diets except barley. No adults emerged from any of the treatments on barley. If *P. interpunctella* are to be successful when infesting barley, they likely require a proportion of the barley to be cracked or broken, thereby exposing endosperm that larvae could easily feed upon. It is also possible that barley requires higher moisture contents to be acceptable for *P. interpunctella* or it may be that barley has an attribute that causes *P. interpunctella* to perform poorly. Subramanyam and Harein (1989) found few *P. interpunctella* larvae in farm bins of barley. Finally, this

lot of barley may have been treated with insecticide. Further studies would be required to determine if barley is a poor host for *P. interpunctella.*

Further studies are required to determine if these rates are capable of protecting these commodities under field conditions. DE is repellant, so fewer eggs may be laid on the commodity. Larvae may move more because they are repelled by the DE. In addition to this, constant water stress may reduce the fecundity of any surviving adults. Finally, there are several parasitoids of pyralid moths, and these could be adversely affected by the DE (Perez-Mendoza et al., 1999).

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Altered proteolytic and amydolytic activity in insecticide-susceptible and -resistant strains of the maize weevil, *Sitophilus zeamais*

Silva, L.B.[1], Lopes, K.V.G.[2], Oliveira, M.G.A.[2], Guedes, R.N.C. *#[1]
[1] Setor de Entomologia, Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG 36571-000, Brazil. Email address: guedes@ufv.br
[2] Departamento de Bioquímica e Biologia Molecular, Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa, Viçosa, MG 36570-000, Brazil
[3] Instituto Nacional de Ciência e Tecnologia em Interações Planta-Praga (INCT-IPP), Universidade Federal de Viçosa, Viçosa, MG 36570-000, Brazil

* Corresponding author
# Presenting author

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Abstract

Fitness cost is usually associated with insecticide resistance and may be mitigated by increased energy accumulation and mobilization. Preliminarily evidence from tests with *Sitophilus zeamais* Motschulsky, the maize weevil (Coleoptera: Curculionidae) suggested possible involvement of proteinases and amylases in such a phenomenon. Therefore, trypsin-like serine-proteinases, cysteine-proteinases and α-amylases were purified and characterized from an insecticide-susceptible and two insecticide-resistant strains (one with associated fitness cost [resistant cost strain], and the other without it [resistant no-cost strain]). Trypsin-like serine-proteinases were purified by aprotinin-agarose affinity chromatography, while cysteine-proteinases were purified using thiol-sepharose affinity chromatography, and the main α-amylase of each strain was purified by glycogen precipitation and ion-exchange chromatography. The activity and inhibition profile differed among strains for each group of purified enzyme. The higher levels of activity observed for trypsin-like proteinases and amylase in the resistant no-cost strain, as well as their susceptibility to inhibition provide support for the hypothesis that enhanced trypsin-like protease and α-amylase activity may be playing a major role in mitigating fitness costs associated with insecticide resistance. In contrast, enhanced cysteine-proteinase activity is likely to play only a secondary role, if any, in mitigating the costs usually associated with insecticide resistance.

Keywords: Fitness cost mitigation, Insecticide resistance, Digestive enzymes, Amylases, Proteinases.

1. Introduction

Insecticide resistance is a frequent consequence of overreliance on insecticide use, which is particularly acute among stored product insects in warmer climates (Champ and Dyte, 1976; Badmin, 1999). Resistance can make a positive contribution to an individual's fitness under insecticide exposure, but also may place resistant individuals at a fitness disadvantage in the absence of the insecticide (Coustau et al., 2000; Oliveira et al., 2007). Although not universal, such fitness costs associated with insecticide resistance are commonly reported among insect pests and they are a frequent assumption in models of insecticide resistance evolution (Coustau et al., 2000; Guedes et al., 2006).

The expression of insecticide resistance mechanisms usually cause higher energy demands, consequently leading to higher metabolic rate. Therefore, increases in metabolic rate may be necessary for the maintenance of insecticide resistance mechanisms. Unless the energy metabolism in resistant insects is enhanced, energy relocation may deprive other basic physiological processes leading to the expression of fitness costs associated with insecticide resistance (Harak et al. 1999; Guedes et al., 2006; Araújo et al., 2008ab). Therefore, larger stores of energy reserve molecules may constitute an additional energy supply for the maintenance of insecticide resistance mechanisms, without compromising the energy demanded for the other physiological processes, thereby mitigating the costs associated with insecticide resistance (Guedes et al., 2006; Araújo et al., 2008ab).

Earlier studies with the maize weevil (*Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)) recognized pyrethroid-resistant strains with and without associated fitness costs (Guedes et al., 2006; Oliveira et al., 2007). The pyrethroid-resistant strain lacking fitness cost led to higher grain loss, and exhibited higher respiration rate, higher body mass, and larger energy reserve cells (i.e., trophocytes)

than the pyrethroid-susceptible and –resistant (with associated fitness cost) strains of *S. zeamais* (Guedes et al., 2006; Oliveira et al., 2007). Subsequent investigation surveying hydrolytic enzymes in these same strains associated high proteinase and particularly high amylase activity with the pyrethroid-resistant strain lacking associated fitness costs (Araújo et al., 2008ab). Such findings led to the hypothesis that high proteinase and high amylase activity may be underlying physiological mechanisms mitigating fitness costs associated with insecticide resistance in the maize weevil.

We reported here the purification and characterization of trypsin-like serine-proteinases, cysteine-proteinases and α-amylases from a susceptible and two insecticide-resistant strains *S. zeamais* (with and without associated fitness cost), which have been used as models for the study of mechanisms underlying the mitigation of fitness costs associated with insecticide resistance. Higher activity levels and distinct inhibition profile of the purified enzymes were expected in the pyrethroid-resistant strain without associated fitness costs, particularly in contrast with the pyrethroid-resistant strain with associated fitness costs, if these hydrolytic enzymes are indeed involved in the mitigation of fitness costs associated with insecticide resistance mechanisms, as previously suggested (Araújo et al., 2008ab).

2. Material and Methods

2.1. Insects

Three strains of *S. zeamais* were used. The susceptible strain, here referred as "susceptible", has been maintained for nearly 20 years without insecticide exposure; its insecticide susceptibility is periodically checked (Araújo et al., 2008a). Both the insecticide-resistant strains exhibit over 100-fold resistance to pyrethroids, which is also periodically checked (Araújo et al., 2008a). The strain here referred as "resistant cost" has reduced fitness in the absence of pyrethroid insecticides, while the strain here referred as "resistant no-cost" does not exhibit reduced fitness in the absence of pyrethroids (Guedes et al., 2006; Oliveira et al., 2007; Araújo et al., 2008a). All three insect strains were maintained in whole maize grains free of insecticides under controlled temperature (25 ± 2ºC), relative humidity (70 ± 5%) and photoperiod (12:12 L:D).

2.2. Chemicals

Enzymatic kits were used for determination of amylase activity (K003) and purchased from BIOCLIN (QUIBASA – Química Básica Ltda, Belo Horizonte, MG, Brazil). Glucose, potassium sodium tartarate, and potassium chloride were purchased from Merk S. A. Ind. Quím. (Porto Alegre, RS, Brazil), while the DEAE-Sephacel chromatographic resin was purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All of the remaining reagents used were purchased from Sigma-Aldrich Química Brazil (São Paulo, Brazil).

2.3. Preparation of crude insect extract

For purification of proteinases, frozen adult maize weevils (0.2 g mL^{-1}) were homogenized in 0.1 M Tris-HCl (pH 8.0) and used as enzyme source after cell lyses by a series of nitrogen freezing and thawing at 37 ºC water bath. Aliquots of 1 mL of crude extract were centrifuged at 100,000 *g* for 60 min at 4 ºC. The resulting supernatant was dialyzed against 100 volumes of 0.01 M Tris-HCl buffer (pH 7.5). The supernatant was subsequently recentrifuged at 100,000 *g* for 45 min at 4ºC and loaded onto a aprotinin-agarose affinity column (Sigma-Aldrich Química, São Paulo, Brazil) equilibrated with 0.01 M Tris-HCl and 5 mM $CaCl_2$ at a flow of 1 mL min^{-1} to retain serine-proteinases. The remaining sample (with serine-proteinases removed) was subsequently loaded onto a thiol sepharose 4 B column (Sigma-Aldrich Química, São Paulo, Brazil) equilibrated with 0.1 M Tris-HCl pH 7.5 containing 0.5 M $NaCl_2$ and 1 mM ethylenediaminetetraacetic acid (EDTA). After loading the sample, the medium was rinsed with binding buffer until the baseline was stable. Bound molecules were eluted with 0.1 M Tris-HCl pH 7.5 containing 25 mM dithiothreitol (DTT) and 1 mM EDTA. A flow rate of 0.5 mL/min was used, and 1 mL fractions were collected.

Non-sexed adult weevils (10.5 g for each strain) were used for α-amylase purification. The adult insects were nitrogen-frozen and homogenized in 20 mM sodium acetate buffer (pH 5.0) containing 20 mM NaCl and 0.1 mM $CaCl_2$ at a ratio of 3 mL buffer for 1 g of insect, following Baker and Woo (1985). The crude homogenates were filtered through glass-wool and centrifuged at 5,300 *g* for 30 min. Lipids were removed by collecting the supernatant and centrifuging it once more under the same conditions. The

resulting lipid-free supernatant was dialyzed in 20 mM sodium acetate buffer (pH 5.0) containing 20 mM NaCl and 0.1 mM $CaCl_2$ for 20 h at 4°C. The supernatant was subsequently recentrifuged at 5,300 *g* for 30 min. The soluble extract obtained was subjected to glycogen precipitation following Loyter and Schramm (1962), and the sample obtained was subjected to ion-exchange chromatography using a DEAE-Sephacel column (10 x 2 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM $CaCl_2$. The unbound proteins were eluted from the column with 30 mL of buffer, followed by a subsequent increase in the saline gradient to 0.4 M NaCl to elute bound α-amylases. A flow rate of 0.5 $mL.min^{-1}$ at 4°C was used and 1.5 mL fractions were collected. The fractions corresponding to the same activity peak were pooled and only the fractions corresponding to the main (i.e., more active) α-amylase isoform of each strain were used for subsequent characterization. Polyacrylamide gel electrophoresis (PAGE) was carried out following Laemmli (1970) using 12% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulphate (SDS) to check the enzyme purification and to estimate de molecular mass of the purified enzymes.

2.4. Protein determination and enzyme activity

Protein concentration was measured following the method of Bradford (1976). Bovine serum albumin (BSA) solutions of 0-0.02 mg mL^{-1} were used as the standard. Amidolytic activity of serine-proteinases was determined using *N*-α-benzoil-L-Arg-*p*-nitroanilide (L-BApNA) as substrate at a final concentration of 0.5 mM in 0.1 M Tris-HCl buffer (pH 8.2). Esterolytic activity of serine-proteinases was determined using *N*- α-*p*-tosyl-L-Arg methyl ester (L-TAME; 0.5 mM) as a substrate in 0.1M Tris-HCl buffer (pH 8.2). Amidolytic activity was also determined for the purified cysteine-proteinases as described by Erlanger et al. (1961), using L-BapNA as a substrate at 0.5 mM in 0.1 M Tris-HCl buffer (pH 8.0), with 20 mM $CaCl_2$ and 5 mM DTT, and 100 µL of the serine-proteinase inhibitor benzamidine at 1 mM in order to measure only the cysteine-proteinase activity. Enzyme activity was determined by formation of *p*-nitroanilide, through the measurement of absorbance at 405 nm, and using the molar absorption coefficient 8800 $M^{-1}.cm^{-1}$. α-Amylase activity was determined with the K003 BIOCLIN enzymatic kit containing a substrate solution (starch) and color reagent (iodine), following methods adapted from Caraway (1959). Activity values of amylase were expressed as amylase units ($AU.dL^{-1}$), which refers to the amount of amylase that hydrolyzes 10 mg starch in 30 min at 37°C.

2.5. Effect of enzyme inhibitors and enzyme kinetics

Selected proteinase and amylase inhibitors were tested for their effect on the purified enzyme activity using concentration ranges covering their estimated K_i from published papers. Partially purified enzyme samples were incubated for either 7.5 or 15 min (amylase and proteinase inhibitors respectively) with the different inhibitors and enzyme activity was subsequently determined as previously described. Determination of the kinetic parameters K_M and V_{max} was carried out with increasing substrate concentrations using the methods previously described subjected to non-linear regression using the curve-fitting procedure for enzyme kinetics of SigmaPlot (SPSS, 2000).

3. Results

3.1. Trypsin-like serine-proteinases

Serine-proteinases from the susceptible strain and the two resistant strains were partially purified using an aprotinin-agarose affinity column providing purification factors ranging from 36.5 to 51.2%, with yields between 10 and 15% and activity between 529 and 875 µM min^{-1} mg^{-1} protein with the substrate L-BApNA. SDS-PAGE of the purified fraction revealed a 56,000 Da molecular mass band in all strains and a 70,000 Da band more visible in the resistant no-cost strain. The purified proteinases from all strains were inhibited by *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), aprotinin, benzamidine and soybean trypsin inhibitor (SBTI) characterizing them as trypsin-like serine-proteinases (Table 1). Trypsin-like proteinases from the resistant strains exhibited higher affinity for L-BApNA. The resistant no-cost strain exhibited V_{max}-values 1.5- and 1.7-fold higher than the susceptible and resistance cost strains, respectively (Table 2). A similar trend was also observed when using L-TAME as substrate (Table 2).

3.2. Cysteine-proteinases

Purification of the cysteine-proteinases revealed a single 74,000 Da molecular mass band in the susceptible strain, two bands of 72,000 and 83,000 Da in the resistant cost strain, and two bands of

68,000 and 74,000 Da in the resistant no-cost strain. Purified cysteine-proteinases of the three strains were different regarding substrate degradation and inhibition; the proteinases least sensitive to inhibition by the specific cysteine-proteinase inhibitor E-64 were those from the resistant no-cost strain (Table 1). The pH and temperature profile of cysteine-proteinase activity differed among strains and although affinity (i.e. K_M) of the cysteine-proteinases were similar, the V_{max} value for cysteine-proteinases from the resistant cost strain was 3x and 5x higher than V_{max} values for the resistant no-cost and susceptible strains respectively (Table 2). Cysteine-proteinase activity was highest for the resistant cost strain rather than the resistant no-cost (Table 2).

Table 1 Concentration required to inhibit 50% of enzyme activity (I_{50}) (± SEM) for selected inhibitors towards the activity of proteinases and α-amylases purified from a susceptible and two pyrethroid-resistant strains (resistant cost and resistant no-cost) of *S. zeamais.* Results are reported as the mean ± standard error (n = 3). Means followed by the same letter in a row are not significantly different by Fisher's LSD test ($p < 0.05$).

		I_{50}		
Purified enzyme	**Inhibitor**	**Susceptible**	**Resistant cost**	**Resistant no-cost**
Trypsin-like serine-proteinases	Benzamidine (mM)	0.54 ± 0.02 a	0.41 ± 0.01 a	0.13 ± 0.01 b
	TLCK (mM)	0.56 ± 0.05 a	0.14 ± 0.03 b	0.11 ± 0.05 b
	Aprotinin (µM)	0.37 ± 0.10 a	0.60 ± 0.20 a	0.90 ± 0.31 a
	SBTI (µg.mL^{-1})	140.17 ± 0.50 a	75.20 ± 6.60 b	36.90 ± 3.10 c
Cysteine-proteinases	E-64 (mM)	0.0031 ± 0.0008 b	0.0057 ± 0.0021 b	0.0150 ± 0.0050 a
α-Amylases	Acarbose (mM)	41.01 ± 6.42 b	24.49 ± 2.37 c	63.80 ± 7.23 a
	Wheat amylase inhibitors (µg.mL^{-1})	4.89 ± 0.55 b	4.22 ± 0.58 b	6.38 ± 0.67 a

Table 2 Kinetic parameters (± SEM) of proteinases and α-amylases purified from a susceptible and two pyrethroid-resistant strains (resistant cost and resistant no-cost) of *S. zeamais.* Results are reported as the mean ± standard error (n = 3). Means for each enzyme followed by the same letter in a column are not significantly different by Fisher's LSD test ($p < 0.05$).

		K_M		V_{max}	
Enzyme	**Strain**	**L-BapNA (mM)**	**L-TAME (mM)**	**L-BapNA (µM/s/mg)**	**L-TAME (µM/s/mg)**
Trypsin-like serine-proteinases	Susceptible	0.34 ± 0.05 a	0.27 ± 0.03 a	0.044 ± 0.002 c	0.85 ± 0.03 b
	Resistant cost	0.26 ± 0.03 b	0.28 ± 0.04 a	0.050 ± 0.002 b	0.72 ± 0.03 b
	Resistant no-cost	0.22 ± 0.02 b	0.21 ± 0.03 a	0.076 ± 0.002 a	1.29 ± 0.05 a
Cysteine-proteinases	Susceptible	0.38 ± 0.04 a	-	10.40 ± 0.32 c	-
	Resistant cost	0.39 ± 0.04 a	-	53.10 ± 1.90 a	-
	Resistant no-cost	0.42 ± 0.05 a	-	32.04 ± 1.20 b	-
		K_M (starch; g.L^{-1})		**V_{max} (starch; AU.dL^{-1})**	
α-Amylases	Susceptible	0.24 ± 0.04 a		640.09 ± 26.67 a	
	Resistant cost	0.14 ± 0.02 b		500.12 ± 15.07 b	
	Resistant no-cost	0.23 ± 0.01 a		674.34 ± 7.18 a	

3.3. α-Amylases

The main α-amylase of each maize weevil strain was purified by glycogen precipitation and ion-exchange chromatography (≥ 70-fold purification, ≤ 19% yield). Single α-amylase bands with the same molecular mass (53,700 Da) were revealed for each insect strain. Higher activity was obtained at 35-40°C and at pH 5.0-7.0 for all of the strains. The α-amylase from the resistant no-cost strain exhibited higher activity towards starch and lower inhibition by acarbose and wheat amylase inhibitors (Table 1). Opposite results were observed for the α-amylase from the resistant cost strain (Table 1). Although the α-amylase from the resistant cost strain exhibited higher affinity to starch (i.e., lower K_m), its V_{max} value was the lowest among the strains, particularly the resistant no-cost strain (Table 2).

4. Discussion

The enzymes partially purified with aprotinin-agarose affinity column from the susceptible, resistant cost and resistant no-cost strains of *S. zeamais* were trypsin-like serine-proteinases because they were able to hydrolyze L-BApNA and L-TAME (Oliveira et al., 2005). Furthermore, they were inhibited by typical trypsin-like inhibitors, namely aprotinin, benzamidine, SBTI and TLCK (Oliveira et al., 2005). The resistant strains showed greater sensitivity to inhibition and the trypsin-like proteinases from the resistant no-cost strain were particularly sensitive to inhibition by benzamidine, TLCK and SBTI compared to trypsin-like proteinases from the susceptible strain. Trypsin-like proteinases purified from the resistant no-cost strain showed a nearly two-fold higher activity towards the two substrates investigated, which is in agreement with our previous finding in crude preparations (Araújo et al. 2008b).

The recognition of the purified proteinases as cysteine-proteinases was achieved through their subsequent characterization via kinetic studies and inhibitor analysis carried out with fractions eluted from thiol-sepharose affinity column. E-64 (a specific cysteine-proteinase inhibitor) efficiently inhibited such activity, unlike inhibitors of other proteinase classes (i.e., EDTA for metalloproteinases, pepstatin for aspartato-proteinases, and TLCK for serine-proteinases), as expected for cysteine-proteinases (D'Avila-Levy et al., 2003; Mohamed et al., 2005). The proteinases least sensitive to inhibition by the specific cysteine-proteinase inhibitor E-64 were those from the resistant cost strain. Although affinity (i.e. K_M) of the cysteine-proteinases was similar among them, the V_{max} value for cysteine-proteinases from the resistant cost strain was 3x and 5x higher than V_{max} values for the resistant no-cost and susceptible strains, respectively. These combined results indicate the existence of different isoforms of cysteine proteinases and consequently qualitative differences associated with the insect strains studied here.

The biochemical characterization of the purified α-amylases provided results consistent with insect α-amylases (Baker, 1983; Mendiola-Olaya et al., 2000; Cinco-Moroyoqui et al., 2008). They were similar among the maize weevil strains, although the activity levels of α-amylases obtained for the resistant no-cost strain were always higher, particularly when compared with the α-amylase from the resistant cost strain. The inhibition profile of the purified α-amylases from each strain were however significantly different not only for acarbose, but also for wheat amylase inhibitors. Both are recognized inhibitors of α-amylase, acarbose is an anti-diabetic drug (Brzozowski and Davies, 1997), while wheat amylase inhibitors are natural compounds obtained from wheat grains (Cinco-Moroyoqui et al., 2006).

We earlier hypothesized that higher proteinase and amylase activity in insecticide-resistant strains of maize weevil might contribute in mitigating the physiological costs (and consequently the fitness costs) usually associated with insecticide resistance, as initially suggested by studies with these insect strains (Guedes et al., 2006; Araújo et al., 2008ab). If so, higher activity levels and distinct inhibition profile of hydrolytic enzymes would occur in the resistant no-cost strain, particularly in contrast with the resistant cost strain. Indeed, higher levels of trypsin-like serine-proteinase and α-amylase activity were observed in the resistant no-cost strain, whose main isoforms also exhibited different inhibition profiles. The higher levels of α-amylase activity in the resistant no-cost strain were likely due to a higher expression of this enzyme rather than to a more efficient isoform of amylase, since it exhibits lower affinity to starch. In contrast, different isoforms of trypsin-like proteinases are probably the main determinants for the higher activity observed in the resistant no-cost strain. Such results provide support for the hypothesis that enhanced trypsin-like proteinase and α-amylase activity may be playing a major role in mitigating fitness costs associated with insecticide resistance (Araújo et al., 2008ab), unlike cysteine-proteinases, which may be playing only a secondary role, if any, in mitigating the costs usually associated with insecticide resistance.

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Physiological and behavioral resistance to esfenvalerate + fenitrothion in populations of the maize weevil, *Sitophilus zeamais*

Corrêa, A.S., Cordeiro, E.M.G., Braga, L.S., Pereira E.J.G., Guedes, R.N.C.*#
Setor de Entomologia, Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, MG 36571-000, Brazil. E-mail address: guedes@ufv.br

* Corresponding author
# Presenting author

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Abstract

The maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) is considered the main pest of stored maize in Brazil and its control is achieved mainly by insecticides. The massive and intensive use of these compounds may lead to selection of resistant populations and consequently compromise the control efficacy of this insect pest in Brazilian storage facilities. Therefore, we surveyed physiological and behavioral resistance to the insecticide mixture esfenvalerate + fenitrothion in 27 populations of *S. zeamais* collected in several Brazilian counties and Paraguay, and also investigated possible costs associated with this phenomenon. The insects were subjected to concentration-mortality bioassays to determine the lethal concentrations LC_{50} and LC_{95}. The populations were also subjected to two walking trials on surfaces fully-treated and partially-treated with dried insecticide residues for detection of behavioral resistance. We also determined the instantaneous rate of population increase (r_i), and body mass of individuals of each population. The concentration-mortality bioassays indicated resistance ratios (at LC_{50}) ranging from 1.00 to 5.02x for the insecticide mixture esfenvalerate + fenitrothion compared with the susceptible standard population (Sete Lagoas). Although the resistance ratios were modest at LC_{50}, they reached up to 232x at LC_{95} (Votuporanga county, São Paulo, Brazil). The behavioral trait of walking in treated arena varied among populations and sex, but there was no significant avoidance to the insecticide mixture. There was no correlation between physiological and behavioral resistance, indicating that physiological resistance is independent of behavioral resistance in the populations tested. There was no significant difference in the instantaneous rate of increase (r_i), and body mass among the insects. Therefore, we conclude that there was no fitness cost associated with the levels of resistance observed in the populations studied.

Keywords: Insecticide resistance, Locomotion, Behavioral avoidance, Fitness cost, Rate of population growth

1. Introduction

A major cause of losses in stored maize throughout the warm regions around the world is the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), which frequently requires control measures to reduce its infestation (Rees, 1996; White and Leesch, 1996). Pyrethroid and organophosphate insecticides are heavily used for maize weevil control (Guedes et al., 1995; Fragoso et al., 2003), due to the lack of other suitable control alternatives in warm climates (White and Leesch, 1996). The over-reliance on insecticides for controlling the maize weevil and other stored-grain pests in tropical areas turned insecticide resistance into a frequent problem (Champ and Dyte, 1976; Subramanyam and Hagstrum, 1996). The earlier use of individual organophosphate and pyrethroid insecticides was replaced by the use of tank mixtures of organophosphate + pyrethroid by the 1990's and more recently the commercial fenitrothion + esfenvalerate mixture became available for stored product use in Brazil (Braga et al., 1991; Ministério da Agricultura, Pecuária e Abastecimento, 2009). However, studies of resistance to such an insecticide mixture have yet to be carried out.

Insecticide resistance results from the enhancement of biochemical-physiological barriers to intoxication and decrease in target-site sensitivity (physiological mechanisms of resistance), or behavioral modifications leading to reduced insecticide exposure (behavioral mechanism of resistance) (Georghiou, 1972). However, most studies usually focus on biochemical-physiological mechanisms (here referred as physiological resistance), whereas relatively little attention is placed on behavioral responses leading to reduced insecticidal exposure. Behavioral resistance to insecticides is frequently neglected in studies of

storedproduct insect-pests, although potentially important for their control and design of suitable management programs (Georghiou, 1972; Lockwood et al., 1984; Guedes et al., 2009).

The major genes responsible for the individual adaptation to a new environment (e.g., insecticide-treated grains in the case of weevils) are usually associated with a fitness cost, since they may be at a disadvantage in the previous environment where independent selection pressures shaped the prevailing phenotypes (Coustau et al., 2000; Berticat et al., 2002). This rationale is based on the general view that a resource allocation takes place, affecting metabolic or developmental processes, and decreasing reproductive potential (Berticat et al., 2002; Guedes et al., 2006). However, there are cases without apparent disadvantages, or rather fitness advantages, observed in insecticide resistant individuals of some insect species, including *S. zeamais* (Guedes et al., 2006).

In this study, we carried out a survey of physiological and behavioral resistance to the insecticide mixture of an organophosphate (fenitrothion) with a pyrethroid (esfenvalerate) insecticide in representative populations of *S. zeamais*, which has not been a focus of attention so far. In addition, we determined behavioral responses of the maize weevil populations, their demographic performance, and correlated these responses with the observed levels of physiological resistance.

2. Materials and methods

2.1. Insects

Twenty seven populations of *S. zeamais* collected in eight Brazilian states and Paraguay were used in this study. These populations were randomly obtained from stored maize grains in representative stored product units between March and September of 2007. The standard susceptible population used was obtained from the EMBRAPA National Research Center of Maize and Sorghum (EMBRAPA Milho and Sorgo, Sete Lagoas, MG, Brazil), where it has been maintained for over 20 yrs in the absence of insecticides. They were maintained in glass containers (1.5 L) within growth chambers (25 ± 2°C, 70 ± 10% r.h., 12 h:12 h photoperiod), and reared on insecticide-free whole maize grains. The bioassays were carried out under these same environmental conditions, except for the behavioral trials, which were carried out at room temperature.

2.2. Concentration-mortality bioassays

Bioassays using technical grade fenitrothion (96.8% pure, Iharabrás/Sumitomo Corporation, São Paulo, SP, Brazil) and esfenvarelate (96.9% pure, Iharabrás/Sumitomo Corporation, São Paulo, SP, Brazil) were carried out using a completely randomized experimental design with five replicates. The insecticide mixture was used at the Brazilian registered and recommended proportion of 20 parts fenitrothion to 1 part esfenvalerate. Each replicate was constituted of a 20 mL glass vial treated with its inner walls covered with insecticide residue following Fragoso et al. (2003). Control vials were treated with the solvent only (acetone). Mortality was recorded after 48-h exposure considering dead those insects unable to walk when prodded with a fine hair brush.

2.3. Behavioral bioassays

The methods used were adapted from those of Guedes et al. (2009) and Pereira et al. (2009). Two behavioral bioassays were carried out – one with the whole filter paper disc treated with insecticide (and an untreated control where only acetone was used), and one with only half of the of filter paper disc treated with the insecticide mixture (the other half was treated with acetone only). These arenas were placed under a video tracking system (Viewpoint LifeSciences, Montreal, Canada). One adult of *S. zeamais* was released on the center of the arena and its movement behavior was tracked for 30 min, after an initial 1 min of waiting period. Measurements taken by the tracking system included the distance walked, ambulatory time (i.e. time walking), resting time, stationary time (time moving without walking), and the total time spent in each half of the arena. The walking speed was calculated by dividing the distance walked by the time spent walking. The experimental design was completely randomized with 16 replicates for each sex and insect population.

2.4. Instantaneous rate of population increase (r_i), and body mass

The experiment was carried out in glass jars (1.5 L) containing 250 g of whole maize with 14.4% m.c. (free of insecticide residues). Fifty unsexed adults of *S. zeamais* (2 wk old) were released in each jar and removed after 100 d. Three replicates were used for each population. The number of live insects, as well

as grain weight and its moisture content (13.3%), were recorded after the storage period (100 d). The instantaneous rate of population increase (r_i) was calculated using the formula $r_i = [\ln(Nf/Ni)/\Delta T$, where Nf and Ni are respectively the final and initial number of live insects, and ΔT is the duration of the experiment in days (Walthall and Stark, 1997). Sixty insects of each sex were randomly removed each population and individually weighed on an analytical balance for determining their (wet) body mass (Sartorius BP 210D, Göttingen, Germany).

2.5. Statistical analyses

Concentration-mortality data were subjected to probit analysis using the procedure PROBIT of SAS (SAS Institute, 2002). The resistance ratios with their 95% confidence intervals were calculated based on LC_{50} values for the insecticides (Robertson and Preisler, 1992). The resistance ratio is considered significant ($p < 0.05$) when its confidence limits do not include the value one (Robertson and Preisler, 1992).

Measurements on the locomotory behavior of different populations of *S. zeamais* on treated surfaces were subjected to multivariate analysis of variance (PROC GLM, MANOVA; SAS Institute, 2002), followed by univariate analysis of variance and Tukey's HSD test, when appropriate (PROC GLM; SAS Institute, 2002). Data on population growth and insect body mass were subjected to univariate analysis of variance, and the means were again compared using Tukey's HSD test (PROC GLM; SAS Institute, 2002). Pearson's correlation analyses between physiological and behavioral resistance to the insecticide mixture were also carried out to recognize any significant association between them (PROC CORR; SAS Institute, 2002). The assumptions of normality and homogeneity of variances were checked (PROC UNIVARIATE; PROC GPLOT; SAS Institute, 2002), and no data transformation was necessary.

3. Results

3.1. Physiological resistance

The probit model was suitable for the analyses and estimation of the intended toxicological parameters based on the low χ^2-values (< 11.00) and high *p*-values (> 0.05) obtained (Table 1). The insect populations exhibited low levels of resistance to the insecticide mixture, which ranged from 1.0 to 5.0-fold at LC_{50}. However, over a third of the tested population were significantly resistant to the mixture, and due to the low slope on the probit curves estimated for some populations, the resistance ratios at LC_{95} observed for some of them were fairly high (up to 232-fold, as observed for the Votuporanga population).

Population	No. individuals tested	Slope ± SEM	LC_{50} (FI 95%) µg/cm²	RR_{50} (IC 95%)	LC_{95} (FI 95%) µg/cm²	RR_{95} (IC 95%)	χ^2	*P*
Sete Lagoas	600	2.21 ± 0.39	0.0052 (0.0032 - 0.0066)	1.00 (0.75 - 1.34)	0.029 (0.018 - 0.105)	1.00 (0.51 - 1.98)	7.85	0.10
Piracicaba	780	3.61 ± 0.30	0.0052 (0.0047 - 0.0055)	1.00 (0.80 - 1.24)	0.014 (0.012 - 0.017)	0.51 (0.31 - 0.85)	9.03	0.17
Amambay	780	1.93 ± 0.32	0.0053 (0.0037 - 0.0067)	1.03 (0.77 - 1.36)	0.038 (0.022 - 0.136)	1.32 (0.63 - 2.79)	10.42	0.06
Rio Verde	700	2.17 ± 0.19	0.0057 (0.0049 - 0.0064)	1.09 (0.86 - 1.39)	0.032 (0.025 - 0.045)	2.20 (0.64 - 1.96)	3.20	0.67
Sacramento	700	2.16 ± 0.22	0.0057 (0.0050 - 0.0063)	1.10 (0.87 - 1.39)	0.033 (0.025 - 0.049)	1.15 (0.64 - 2.05)	8.96	0.11
Dourados - Bororó	540	1.40 ± 0.36	0.0060 (0.0019 - 0.0086)	1.15 (0.79 - 1.68)	0.089 (0.032 - 40.46)	3.08 (0.83 - 11.59)	8.49	0.08
Canarana	680	1.93 ± 0.29	0.0062 (0.0043 - 0.0079)	1.20 (0.89 - 1.61)	0.044 (0.026 - 0.131)	1.53 (0.75 - 3.11)	10.92	0.05
Maracaju	740	2.20 ± 0.19	0.0063 (0.0055 - 0.0070)	1.21 (0.95 - 1.53)	0.035 (0.027 - 0.048)	1.21 (0.70 - 2.11)	8.66	0.19
Guaxupé	600	2.02 ± 0.23	0.0064 (0.0057 - 0.0073)	1.24 (0.98 - 1.58)	0.042 (0.029 - 0.072)	1.45 (0.76 - 2.77)	5.15	0.27
Viçosa	640	1.81 ± 0.21	0.0069 (0.0049 - 0.0091)	1.33 (0.98 - 1.81)	0.056 (0.035 - 0.123)	1.93 (1.01 - 3.71)	9.82	0.08
São José do Rio Pardo	600	1.63 ± 0.26	0.0071 (0.0045 - 0.0100)	1.38 (1.00 - 1.90)	0.073 (0.036 - 0.390)	2.53 (1.10 - 5.84)	9.09	0.06
São João	760	2.55 ± 0.21	0.0075 (0.0068 - 0.0082)	1.44 (1.15 - 1.81)	0.033 (0.026 - 0.044)	1.15 (0.67 - 1.97)	4.50	0.61
Nova Era	600	1.62 ± 0.25	0.0076 (0.0047 - 0.0105)	1.46 (1.05 - 2.03)	0.078 (0.040 - 0.395)	2.71 (1.19 - 6.17)	8.91	0.06
Espírito Santo do Pinhal	580	1.15 ± 0.16	0.0076 (0.0057 - 0.0095)	1.46 (1.07 - 2.01)	0.204 (0.108 - 0.590)	7.03 (2.80 - 17.90)	1.22	0.88
Jacuí	600	0.75 ± 0.15	0.0076 (0.0045 - 0.0105)	1.47 (0.96 - 2.24)	1.165 (0.309 - 25.45)	40.20 (5.97 - 277.15)	6.74	0.15
Vicentina	700	1.75 ± 0.20	0.0078 (0.0068 - 0.0089)	1.50 (1.18 - 1.91)	0.067 (0.044 - 0.131)	2.33 (1.15 - 4.74)	9.19	0.10
Machado	700	1.07 ± 0.14	0.0084 (0.0066 - 0.0103)	1.62 (1.20 - 2.18)	0.291 (0.142 - 0.978)	10.06 (3.65 - 28.13)	9.09	0.11
Abre Campo	700	1.56 ± 0.16	0.0085 (0.0072 - 0.0098)	1.64 (1.27 - 2.11)	0.096 (0.064 - 0.173)	3.32 (1.69 - 6.57)	8.18	0.15
Juiz de Fora	600	2.58 ± 0.20	0.0096 (0.0086 - 0.0106)	1.84 (1.46 - 2.33)	0.041 (0.033 - 0.055)	1.44 (0.84 - 2.48)	5.42	0.25
Tunapolis	660	1.36 ± 0.15	0.0121 (0.0102 - 0.0146)	2.33 (1.78 - 3.05)	0.195 (0.112 - 0.463)	6.75 (2.96 - 15.57)	8.75	0.12
Dourados – Barrerinha	580	1.05 ± 0.16	0.0127 (0.0100 - 0.0161)	2.45 (1.80 - 3.33)	0.468 (0.201 - 2.183)	16.14 (4.93 - 53.64)	5.26	0.26
Guarapuava	700	0.93 ± 0.14	0.0175 (0.0138 - 0.0242)	3.37 (2.40 - 4.73)	1.020 (0.364 - 6.506)	35.20 (8.66 - 145.56)	2.70	0.75
Pedro Juan Caballero	600	1.21 ± 0.16	0.0177 (0.0145 - 0.0226)	3.41 (2.53 - 4.60)	0.407 (0.197 - 1.367)	14.06 (5.06 - 39.50)	5.14	0.27
Votuporanga	600	0.64 ± 0.15	0.0185 (0.0128 - 0.0320)	3.55 (2.27 - 5.56)	6.751 (0.923 -1402.00)	232.82 (12.47 - 4474.61)	5.99	0.20
Dourados – Proterito	780	1.36 ± 0.13	0.0204 (0.0170 - 0.0258)	3.93 (2.94 - 5.26)	0.331 (0.187 - 0.753)	11.41 (5.01 - 26.21)	2.58	0.86
Jacarezinho	560	1.18 ± 0.22	0.0211 (0.0128 - 0.0395)	4.06 (2.73 - 6.04)	0.519 (0.155 - 18.36)	17.91 (4.66 - 69.65)	9.44	0.05
Unaí	760	1.40 ± 0.13	0.0261 (0.0214 - 0.0340)	5.02 (3.70 - 6.83)	0.395 (0.221 - 0.913)	13.63 (5.92 - 31.64)	6.48	0.37

Table 1 Relative toxicity of insecticide mixture fenitrothion + esfenvalerate to 27 *Sitophilus zeamais* populations.

3.2. Behavioral resistance

The overall walking behavior of the insect population on fully-treated arenas was significantly different among populations ($df_{num/den}$ = 130/14781; Wilks' lambda = 0.83, F = 4.2; $p < 0.001$) and even sex ($df_{num/den}$ = 5/2999; Wilks' lambda = 0.99, F = 3.1; $p < 0.001$). The interaction population x sex was also significant ($df_{num/den}$ = 130/14781; Wilks' lambda = 0.95, F = 1.3; $p < 0.03$) based on the multivariate analysis of variance carried out. There was a highly significant correlation between distance walked and the other walking parameters observed (n = 27; r > |0.4|; $p < 0.001$). Therefore, distance walked was used to represent the observed trend (Fig. 1). Distance walked was significantly affected by sex and population ($p < 0.05$), although no significant difference was observed between sex in the arena without insecticide application ($p > 0.5$) (Fig. 1).

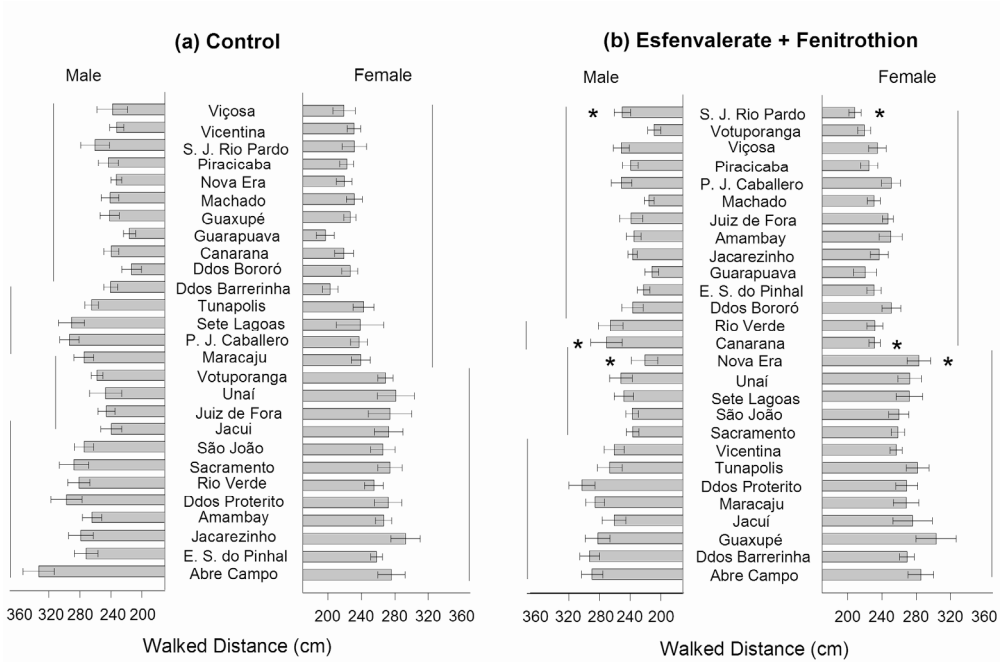

Figure 1 Distance walked (cm) during 10 min (± SEM) by individual insects from 27 populations of *Sitophilus zeamais* in arenas fully-treated with the insecticide mixture fenitrothion + esfenvalerate or not treated (control). Means grouped by the same vertical bar are not significantly different by Scott-Knott groupment analysis test ($p < 0.05$). The asterisk indicates sex differences for the given population also based on Scott-Knott groupment analysis test ($p < 0.05$).

Regarding the avoidance behavior of the maize weevil populations when exposed to partially-treated arenas, there were significant differences among sex and just for three insect populations ($p < 0.05$) (Fig. 2) There was no significant correlation ($p > 0.05$) between physiological and behavioral resistance to the fenitrothion-esfenvalerate mixture (between the resistance ratios and the differences in behavioral walking traits).

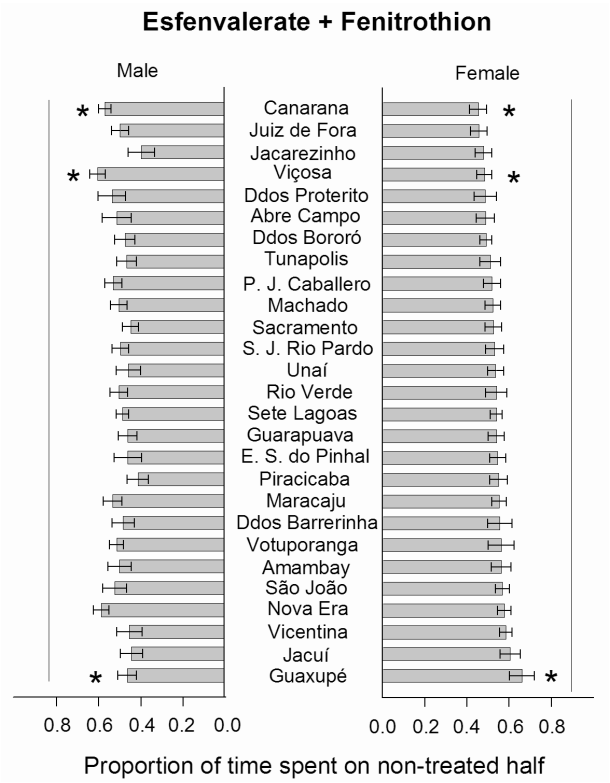

Figure 2 Proportion of time spent on the non-treated half of arenas partially treated with the insecticide mixture fenitrothion + esfenvalerate. Means grouped by the same vertical bar are not significantly different by Scott-Knott groupment analysis test ($p < 0.05$). The asterisk indicates sex differences for the given population also based on Scott-Knott groupment analysis test ($p < 0.05$).

3.3. Instantaneous rate of population increase (r_i), and body mass

The instantaneous rate of population growth (r_i) was not significantly affected by maize weevil strain (0.0213 ± 0.0004 insects/day; $F_{26,54} = 0.73$; $p = 0.80$). In addition, the observed differences in individual insect body mass were not significant either (2.96 ± 0.02 mg; $F_{53,108} = 0.94$; $p = 0.80$).

4. Discussion

Our results of concentration-response bioassays with the insecticide mixture fenitrothion + esfenvalerate indicate emerging problems of resistance to this insecticide mixture. The resistance levels were significant, although low at LC_{50}, among the populations of maize weevil. However, the high variation in response against the mixture in some populations led to high levels of resistance at the LC_{95} level suggesting likely control problems in the field in the near future if insecticide resistance management strategies were not employed. This should not come as a surprise given the history of control failures due to insecticide resistance in maize weevil populations in Brazil – earlier on with DDT and pyrethroids and more recently with organophosphates (Guedes et al., 1995; Fragoso et al., 2003; Pereira et al., 2009).

Locomotion plays a major role determining insecticide exposure and was therefore considered in investigating the behavioral responses of maize weevil populations to insecticide in fully- and partially-treated arenas. The overall mobility parameters of the maize weevils on the insecticide-treated surfaces varied among the populations, although they were not significantly repelled by the insecticides. These varied responses probably reflect the insecticide mode of action and the extent to which they influence the behavior, which varied with the insect population (Hoy et al., 1998). The inter-population variation observed in our tests might reflect differences in the insects' sensory perception of insecticides and could

lead to the development of behavioral resistance if such differences are inheritable. Behavioral avoidance to insecticides has yet to be detected in *S. zeamais* and, although potentially important, it is frequently neglected.

We did not detect significant correlation between the levels of physiological resistance of the populations and their mobility parameters. However, the low levels of physiological resistance in the *S. zeamais* populations probably contributed to their undetected correlation with the mobility parameters.

The body mass of individual insects and demographic performance was similar among the maize weevil populations. As a likely result, significant correlations between these variables and resistance ratios were not observed and so far there is no indication of fitness costs associated with resistance to the fenitrothion-esfenvalerate mixture in maize weevil. Such association may however take place with increased levels of insecticide resistance, as earlier reported for stored grain insects (e.g., Pimentel et al., 2007).

In summary, this research shows that the levels of resistance to the fenitrothion-esfenvalerate mixture in populations of *S. zeamais* are low and not yet associated with fitness costs. Such insecticide mixture remains a viable alternative in managing pyrethroid-resistant populations of *S. zeamais*. However, care must be taken to prevent insecticide over-reliance and consequently overuse against populations of the maize weevil, otherwise the current low frequency and low levels of resistance to this insecticide mixture is likely to increase, compromising its future use. This study is one of the few that investigated, in addition to insecticide lethality, the behavioral responses of different insect populations to insecticidal exposure. The variation in the behavioral responses observed were small, but may also evolve to behavioral resistance and reduce insecticide exposure compromising even further the future efficacy of such compounds. Behavioral responses should therefore be considered in future insecticide resistance surveys and management programs.

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Long term effectiveness of the mixture of diatomaceous earth and deltamethrin on wheat

Korunic, Z.*#[1], Kalinovic, I.[2], Liska, A.[2], Hamel, D.[3]
[1] Diatom Research and Consulting Inc., 14 Tidefall Dr. Toronto, ON, M1W 1J2, Canada.
Email: zkorunic@rogers.com
[2] Faculty of Agriculture in Osijek, Department for Plant Protection, University of Josip Juraj Strossmayer in Osijek, Trg Sv. Trojstva 3, 31000 Osijek, Croatia
[3] Croatian Centre for Agriculture, Food and Rural Affairs, Department of Plant Protection, Svetošimunska 25, 10 040 Zagreb, Croatia

* Corresponding author
# Presenting author

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Abstract

A mixture of diatomaceous earth (DE) and deltamethrin (DM) was developed to control stored-grain insect pests. The formulation contains a low quantity of DE and small amounts of insecticide deltamethrin technical powder dissolved in solvent and soap.

This study was initiated in order to determine how long the mixture DE/DM will provide acceptable protection against infestation by the rice weevil, *Sitophilus oryzae* (L.), the lesser grain borer *Rhyzopertha dominica* (F.) and the red flour beetle, *Tribolium castaneum* (Herbst), when applied to hard red spring wheat (HRSW) and stored under normal storage conditions. Immediately after treatment, all three species were controlled at 100 milligram per kg or parts per million (ppm) of DE/DM mixture. At 100 ppm, DE/DM mixture provided 100% population reduction of all three species for up to 12 months with little or no progeny produced.

Keywords: Diatomaceous earth, Deltamethrin, Mixture, Wheat, Protection

1. Introduction

Insects infesting grain after harvest cause economic loss to producers and the grain industry. Fewer options are available for providing long term protection of grain due to concern over pesticide residues in food, insecticide resistance and the loss or restricted use of conventional grain protectants and fumigants due to new regulations. Alternatives, such as diatomaceous earth (DE), as an integral component of an Integrated Pest Management strategy, can provide very effective extended protection (Stathers et al., 2004; Athanssiou et al., 2005). However, under certain circumstances DE requires high dosage rates that have adverse effects on bulk density (test weight) and handling properties of grain, potentially reducing the value of the commodity (Korunic, 1998; Korunic et al., 1998; Fields, 1999).

Grain protectants should be safe with low mammalian toxicity, be easy to apply with minimal residue issues in finished products, have a broad spectrum of activity towards stored-grain insects and reduce progeny production, have low adverse effects on grain handling and quality properties, and have a price that is acceptable in terms of efficacy and economic viability (FAO, 1981, 1983).

Following these principles and in order to control all stored-product insects and mites within at least 7 to 21 d after treatment, even in grain having relatively high moisture content (up to 15%), a new grain protectant was developed. This new formulation is a mixture of DE, a very low concentrations of deltamethrin technical powder (DM), the synergist PBO (piperonyl butoxide), and a safe and low toxicity solvent and emulsifier for deltamethrin. The mixture combined at least two different modes of action; desiccation and chemical toxicity. Because of this combined action, the required concentrations of DE and other substances used in these mixtures are much less than if any one component were used alone (Korunic, unpublished data). The selected substances are registered as grain protectants in many countries of the world. Deltamethrin and PBO are used at approximately 0.5 to 0.7 ppm and approximately 5 ppm, respectively, for long-term protection of stored grain (EXTOXNET, 1995). In most cases, diatomaceous earth is registered to be used at concentrations of 500 to 3,500 ppm (Subramanyam and Roesli, 2000). Due to the synergism (Korunic, unpublished data) the mixture DE and deltamethrin gave very high to complete mortality of tested stored grain insects and their progeny at application rates of 18 to 20% of the effective rates of any of the active ingredients when used alone.

This mixture also produced a synergistic effect at much lower concentrations in comparison with mixtures evaluated by Daglish (1994) and with the mixtures of insecticides, from different groups, that are currently in use. When the mixture of DE and deltamethrin is applied at the recommended concentration of 100 ppm, treated grain contains only approximately 0.1 ppm of deltamethrin and 90 ppm of DE.

The objective of this study was to determining if 100 ppm of the mixture of DE and deltamethrin can protect grain during one year of storage controlling the adults and the progeny of the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), and the red flour beetle,*Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae).

2. Materials and methods

Three species were used for these tests: *S. oryzae*. – rice weevil (RW), *R. dominica* – lesser grain borer (LGB) and *T. castaneum* – red flour beetle (RFB). A minimum of 25 adults of mixed age and gender were used for each replicate. All insect strains had been colonised in the laboratory for at least three years.

At the beginning of the test, grain was treated with 100 ppm DE/DM mixture (100 g of DE/DM mixture per 1000 kg of grain). Bioassays were initiated 0, 120, 180, 240, 300 and 360 d after the initial treatment. Comparisons were made with grain freshly treated with the same concentrations of DE/DM mixture. Treated and untreated grain was stored under ambient conditions (21.0 ± 1.0°C, 40-60% r.h.) in 11 L plastic containers with vented bottoms and lids. Grain moisture content and grain temperature were recorded throughout the experiment. Bioassays were conducted at 30° ± 1.0°C and 70% ± 5% air relative humidity (r.h.).

The variety of hard red spring wheat (HRSW) used was Quantum, grade No. 1, obtained from Palmerston Elevators, Palmerston, ON. Grain bulk density was determined using the Ohaus 0.5 L measure and Cox funnel. Grain moisture was measured using a dielectric moisture metre (AACC method 44-11). The percentage of dockage present was determined by sieving 100 g of wheat for 45 s in a 2.00-mm aperture sieve. At the beginning of the test, 3 to 6 kg of HRSW was weighed and grain moisture content, percent dockage and test weight were determined. One group was used for the initial 0-d treatment, one group was used for "fresh" treatments, and the remaining group was used as an untreated control.

The initial treatment was performed by adding 600 mg of the formulation to 6 kg grain (100 ppm) then mixing thoroughly in a plastic bag for 5 min. Test weight of the treated group was determined before transferring all groups into vented, plastic containers. Immediately after initial treatment, 500 g of grain was removed from the container for the initial treatment and from the container containing untreated control grain. Grain moisture content was determined for the untreated control and fresh treatment samples. Freshly treated grain was prepared by mixing 50 mg of DE/DM mixture with 500 g of untreated wheat (100 ppm) in a glass jar for 1 min and evenly divided into five 500 mL jars (replicates) . For each additional treatment at 120, 180, 240, 300 and 360 d after the beginning of the experiment, grain was also evenly divided into five replicates. After introducing 25-50 adults of each species to each jar, jars were maintained at 30° ± 1.0°C and 70% ± 5% r.h.

To determine mortality in each treatment, grain was sieved 7 and 14 d after insects were introduced, and the number of dead and living insects was recorded. All dead insects were removed 7 d post-introduction and all dead and live insects were removed after 14 d post-introduction; jars were maintained for an additional 35 d (total of 49 d post-introduction) before being sieved again to determine the number of adult offspring generated. One way ANOVA, means in tables within columns, followed by the same letter, were not significantly different; n = 5; $P>0.05$.

3. Results

The hard red spring wheat used in this study contained between 0.8 and 0.9% dockage (w/w), a mean bulk density between 78.75 and 78.79 kg hL^{-1}, and initial grain moisture content of 12.2%. Treatment with 100 ppm of DE/DM mixture reduced bulk density by 2.82 kg hL^{-1}. The mean grain temperature and grain moisture content, as measured in the untreated grain over 300 d of storage, were 21.2 ± 1.0°C and

12.3 ± 0.3%, respectively. The low moisture content of grain and very low percentage of dockage are the probable reasons for very low development of the red flour beetle progeny.

Mortality of *S. oryzae*, *R. dominica* and *T. castaneum* was 100% on grain stored for 360 d post-treatment. The same results were achieved on grain freshly treated with DE/DM mixture (Tables 1, 2 and 3). Also, no live progeny were observed on treated grain for 360 d post-treatment. The only exception was *S. oryzae* with 99.6% progeny reduction (Table 1, 2 and 3).

Table 1 *Sitophilus oryzae* mortality on DE/DM mixture treated grain, immediately after treatment (zero day), 180 and 360 days post-treatment.

Treatment	Conc. (ppm)	Mean percent *S. oryzae* mortality ± s.d. after days 7	14	Mean number of live progeny ± s.d.
Zero day-post-treatment				
Untreated	0	0.8 ± 1.8 b	-	410.4 ± 161.2 a
DE/DM mixture	100	100.0 ± 0.0 a	-	0.0 ± 0.0 b
180 days post-treatment				
Untreated	0	0.0 ± 0.0 a	0.8 ± 1.8 a	107.2 ± 20.0 a
DE/DM 180 days post- treatment	100	87.2 ± 3.3 b	100.0 ± 0.0 b	0.0 ± 0.0 b
DE/DM fresh treatment	100	84.8 ± 3.3 b	100.0 ± 0.0 b	0.0 ± 0.0 b
360 days post-treatment				
Untreated	0	0.8 ± 1.7 a	3.2± 1.7 **a**	54.4 ± 32.2 a
DE/DM 360 days post- treatment	100	97.2 ± 2.8 b	100.0 ± 0.0 b	0.0 ± 0.0 b
DE/DM fresh treatment	100	100.0 ± 0.0 b	-	0.0 ± 0.0 b

Table 2 *Rhyzopertha dominica* mortality on DE/DM mixture treated grain, immediately after treatment (zero day), 180 and 360 days post-treatment.

Treatment	Conc. (ppm)	Mean percent *R. dominica* mortality ± s.d. after days 7	14	Mean number of live progeny ± s.d.
Zero day-post-treatment				
Untreated	0	1.2 ± 1.1 a	2.0 ± 2.0 a	224.6 ± 44.1 a
DE/DM mixture	100	89.2 ± 5.0 b	100.0 ± 0.0 b	0.0 ± 0.0 b
180 days post-treatment				
Untreated	0	0.0 ± 0.0 a	0.8 ± 1.8 a	107.2 ± 20.0 a
DE/DM old treatment	100	87.2 ± 3.3 b	100.0 ± 0.0 b	0.0 ± 0.0 b
DE/DM fresh treatment	100	84.8 ± 3.3 b	100.0 ± 0.0 b	0.0 ± 0.0 b
360 days post-treatment				
Untreated	0	0.0± 0.0 a	0.0± 0.0 a	157.8± 80.2 a
DE/DM old treatment	100	62.4± 8.7 b	100.0± 0.0 b	0.0± 0.0 b
DE/DM fresh treatment	100	87.2± 7.6 b	100.0± 0.0 b	0.0± 0.0 b

Our results demonstrate that, under the conditions of grain storage (21°C and 12.2% m.c.), treatment of HRSW with 100 ppm of the mixture DE/DM provides effective protection against the adults and the progeny of three tested species for at least 12 months of storage.

4. Discussion

Deltamethrin is very stable on grain and shows little or no tendency to penetrate individual kernels, therefore, it is expected to be removed with the bran during processing. Results from a number of tests to determine the persistence of deltamethrin residue on stored wheat and maize were analyzed by Food and Agricultural Organization/World Health Organization (FAO/WHO, 1981, 1983). It is clear from this review that there was little or no degradation after 30 to 50 wks of storage when good storage practices were followed. For example, at 25°C, the half lives of deltamethrin on wheat at 12% m.c. and 15% m.c. were 114 and 90 wks, respectively. At 35°C, the half lives of deltamethrin on wheat at 12% m.c. and

15% m.c. were 70 and 35 wks, respectively. Degradation is faster at higher temperatures and grain moisture contents. The data for chemical residue levels and biological activity clearly indicate that deltamethrin should give prolonged residual action against grain insects. However, as some decay of the compound will occur during typical storage periods, the initial application rates will need to be at or below the maximum residue limits.

Table 3 *Tribolium castaneum* mortality on DE/DM mixture treated grain, immediately after treatment (zero day), 180 and 360 days post-treatment.

Treatment	Conc. (ppm)	Mean percent *T. castaneum* mortality ± s.d. after days 7	14	Mean number of live progeny ± s.d.
Zero day-post-treatment				
Untreated	0	14.4 ± 13.1 b	29.6 ± 17.3 b	0.8 ± 1.1 a
DE/DM mixture	100	95.2 ± 1.8 a	100.0 ± 0.0 a	0.0 ± 0.0 a
180 days post-treatment				
Untreated	0	0.0 ± 0.0 a	2.4 ± 5.4 a	17.0 ± 10.6 a
DE/DM old treatment	100	58.8 ± 10.0 b	100.0 ± 0.0 b	0.0 ± 0.0 b
DE/DM fresh treatment	100	68.0 ± 7.0 b	100.0 ± 0.0 b	0.0 ± 0.0 b
360 days post-treatment				
Untreated	0	0.8± 1.7 a	9.6 ± 4.5 a	0.6 ± 1.3 a
DE/DM old treatment	100	68.0 ± 7.0 b	100.0 ± 0.0 b	0.0 ± 0.0 a
DE/DM fresh treatment	100	72.6 ± 3.0 b	100.0± 0.0 b	0.0 ± 0.0 a

There is no degradation of deltamethrin when treated wheat is milled to produce either whole wheat flour or white flour. Generally, there is a reduction in the deltamethrin level to about 10 to 20% of the level applied to wheat when it is used in the production of white flour and bread. Residues of deltamethrin are not significantly reduced during baking (FAO/WHO, 1981, 1983).

The FAO/WHO (1983) recommended the following maximum residue limits in cereal grains and milled cereal products: cereal grains 2 mg kg^{-1} (2 ppm), raw wheat bran 5 mg kg^{-1}, whole wheat flour, wheat flour white 0.5 mg kg^{-1}. When DE/DM mixture formulation is applied at the recommended concentration of 100 ppm, it contains 0.01 ppm which is only 4.95% of the maximum residue limit in cereal grains recommended by FAO/WHO (1983).

Pyrethroids synergized with piperonyl butoxide, applied in a combination with organophosphates (OP), have been used in control programs in Australia, the United Kingdom, and other European countries for several years. Using a mixture of compounds from different insecticide groups seems to be a logical and economical approach. Unlike OP, pyrethroids do not rapidly break down at high temperatures and moisture contents. However, the concentrations of pyrethroids that control lesser grain borer do not completely control weevils and flour beetles. Similarly, the concentrations of OP that control many pest species do not completely control lesser grain borer.

One of the potentially valuable uses of DE is in an admixture where DE is impregnated with an insecticide. Effective insecticide concentrations can be significantly reduced in this form, and the insecticides are more readily removed from the grain (Desmarchelier, personal communication). Desmarchelier stated it was possible to halve the applied dose of insecticides such as chlorpyrifos methyl and deltamethrin by simultaneous application of small amount (100 g t^{-1}) of diatomaceous earth, without a reduction in efficacy.

According to the study of Arthur and Zehner (1994), the rates of degradation of the active ingredients in the mixtures of OP and pyrethroids are similar to their rates of degradation when they are applied alone. They evaluated the long term efficacy of deltamethrin applied to wheat at rates of 0.5, 0.75, and 1.0 ppm. Treated wheat was stored under ambient conditions for 10 months. Bioassays with *R. dominica* and *S. oryzae* were initiated every two months. *R. dominica* adult survival after the initial exposure was variable at all three concentrations tested throughout the 10 month period. However, no progeny were produced and the wheat was not damaged. Also, a significant number of *S. oryzae* adults survived on wheat treated

with 0.5 and 0.75 ppm. Survival decreased as the application rates increased. Survival rates were 1 to 3% at 1.0 ppm, and 92 to 97% on untreated wheat. Also, subsequent progeny production decreased with increasing rates of deltamethrin (1 to 7 adults at 1.0 ppm compared with 1198 to 1662 adults on untreated wheat).

When compared with mentioned results of the research, it is clear that the main advantage of using the DE/DM mixture is acceptable efficacy in grain treated with very low doses of deltamethrin (0.1 ppm) and DE (90 ppm). These doses are much lower in comparison with the doses required for effective control when these two insecticides are applied either alone or in previously evaluated mixtures. Deltamethrin and PBO are used at approximately 0.5 to 0.75 ppm and approximately 5 ppm, respectively, for the long-term protection of stored grain. In most cases, diatomaceous earth is registered to be used at concentrations of 500 to 3500 ppm (Korunic, 1998; Subramanyam and Roesli, 2000).

Confirmed synergism between the components in DE/DM mixture reduces the dosage rates of each required for effective control, significantly reducing the risk of harmful residues and adverse effects on grain bulk density.

The effectiveness of DE/DM mixture on wheat after 360 d after treatment and the effectiveness on freshly treated wheat for rice weevil, lesser grain borer and red flour beetle were not significantly different. The results demonstrate that, under the conditions of grain storage (30°C and 14% m.c.), treatment of wheat with 100 ppm of DE/DM mixture provides effective protection against the adults and progeny of *S. oryzae*, *R. dominica* and *T. castaneum* for at least 12 months of storage.

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Laboratory evaluation of insecticidal effectiveness of a natural zeolite formulation against *Sitophilus oryzae* (L.), *Rhyzopertha dominica* (F.) and *Tribolium castaneum* (Herbst) in treated wheat

Kljajić, P.*#[1], Andrić, G.[1], Adamović, M.[2], Pražić Golić, M.[1]
[1] Pesticide and Environment Research Institute, Banatska 31b, 11080 Belgrade, Serbia.
Email: petar.kljajic@pesting.org.rs (P. Kljajić)
[2] Institute of Technology of Nuclear and Other Mineral Raw Materials, Franše D'Eperea 86, 11000 Belgrade, Serbia

* Corresponding author
# Presenting author

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Abstract

Inert dusts are increasingly becoming an integral part of programs for protection of cereal grains from stored-product insects. The intention in this study was therefore to conduct preliminary tests of insecticidal potentials of the natural zeolite formulation Minazel SP (66% SiO_2, particle size ≤ 50 μm) originating from Serbia in controlling *S. oryzae*, *R. dominica* and *T. castaneum*. Dust effectiveness was tested in the laboratory (24±1ºC and 50-55% r.h. for parents and 24±1ºC and 60±5% r.h. for F_1 progeny) by exposing insects to wheat treated with 0.50, 0.75 and 1.00 g/kg of Minazel SP. Mortality was determined after 7, 14 and 21 days of insect contact with threated wheat, and total mortality after an additional 7 days of recovery on untreated broken wheat. Progeny production in F_1 generation was also determined for each insect species after 8-12 weeks. After seven days of exposure and 7 days of recovery of all tested species, the highest efficacy of 62% was observed after the highest application rate of 1.00 g/kg against *S. oryzae*. The highest efficacy after 14 and 21 days was achieved with the same application rate against *T. castaneum* (100%), *S. oryzae* (96-98%) and *R. dominica* (70-82%). Progeny reduction (IR – inhibition rate) of all tested species depended on the duration of parents exposure to treated wheat. After 7 days of exposure progeny reduction rates were 49-67% for *S. oryzae,* 42-68% for *R. dominica* and 47-78% for *T. castaneum.* After 14 days of exposure, inhibition rates were 55-78% for *S. oryzae,* 72-81% for *R. dominica* and 53-90% for *T. castaneum,* while progeny reductions of *S. oryzae* were 51-85%, *R. dominica* 80-96% and *T. castaneum* 87-99% after 21 days of exposure.

Keywords: Wheat grain, *Sitophilus oryzae, Rhyzopertha dominica, Tribolium castaneum*, Natural zeolite

1. Introduction

Modern protection of stored-products is seeking to apply some of nontoxic materials such as inert dusts which are increasingly becoming an integral part of programs for control of insect pests. Products based on diaromaceous earths (DEs) are highly effective even at low application rates, while other inert dusts require higher rates for successful control of stored-product insects (Subramanyam and Roesli, 2000; Zettler and Arthur, 2000). Environmental conditions (temperature, and relative humidity), as well as the content of silicon dioxide (SiO_2), and its particle shape and size, are known to have significant impact on the effectiveness of inert dusts against stored-product insects (Korunić, 1998; Fields and Korunić 2000; Arthur, 2001; 2002; Ferizli and Beris. 2005; Athanassiou et al., 2005; Arnaud et al., 2005; Vardeman et al., 2006).

Natural zeolite (alkaline aluminium silicates) is a sorption dust widely applicable in agriculture for remediation of polluted soils and as fodder additive due to its capacity to neutralize negative effects of mycotoxics, and together with DEs belongs to the same 4th group of inert dusts (Subramanyam and Roesli 2000; Đorđević and Dinić, 2007). However, findings in some studies have demonstrated that this material also has insecticidal potential for control of stored-product insects (Haryadi et al. 1994; Kljajić et al., 2010).

The purpose of this study was to conduct preliminary investigation of the insecticidal potential of the natural zeolite formulation Minazel SP (originating from Serbia) with 66% of SiO_2 and particle size ≤50 μm against rice weevil *Sitophilus oryzae* (L.), lesser grain borer *Rhyzopertha dominica* (F.) and red flour

beetle *Tribolium castaneum* (Herbst) in treated wheat grains. Progeny production/reduction in F_1 generation was also determined for each insect species.

2. Materials and methods

2.1. Test insects and applied natural zeolite

Laboratory populations of *S. oryzae*, *R. dominica* and *T. castaneum*, reared in the insectary of the Pesticides and Environment Research Institute, Belgrade, were used in a trial which employed methods described by Harein and Soderstrom (1966), and Davis and Bry (1985). Two-to-four-week-old unsexed insects were used.

The natural zeolite dust Minazel SP used in bioassay contained: SiO_2 (65.7%), Al_2O_3 (14.1%), CaO (3.6%), Fe_2O_3 (2.4%) and up to 1.5% of MgO, Na_2O and K_2O, respectively. Particle size ≤ 50 μm was predominant.

2.2. Bioassay

A bioassay was conducted in the laboratory at 24±1ºC, and 50-55% r.h. for parents and 60±5% r.h. for F_1 progeny using a modified method of OEPP/EPPO (2004 a,b) and a method described by Collins (1990). Soft wheat (var. Takovčanka) with 11.5±0.2% grain moisture, determined by a Dickey–John moisture meter (Dickey-John Mini GAC, Dickey-John Co., USA) was used in the assay.

We chose to apply Minazel SP at the rates of 0.50, 0.75 and 1.00 g/kg wheat. The dust was applied per 500 g of whole-grain wheat (+ 1% of broken grains only in tests involving *T. castaneum*), hand mixed for 2 minutes and then left to mix on a rotary mixer for another 10 minutes. The next day, portions of 50 g of treated wheat were placed in 200 mL plastic vessels with four replicates and 25 adults of *S. oryzae*, and *T. castaneum* and 20 adults of *R. dominica* were placed separately in each vessel. The same procedure was followed on untreated wheat that was used as a control. Insect mortality was determined after 7, 14 and 21 days of contact with treated wheat, and total mortality after additional 7 days of recovery on untreated broken wheat grains.

The effect of natural zeolite on insect progeny production in F_1 generation was determined in the following manner. After recording parental mortality, wheat was seived to remove all insects, and wheat containers were then covered with cotton cloth and fixed with rubber bands. Progeny production was determined by counting live insects in treated and control wheat grains seived after a total of 8 weeks for *S. oryzae*, 10 weeks for *R. dominica* and 12 weeks for *T. castaneum*.

2.3. Data analysis

The acquired mortality data for treated insects were corrected for the mortality of control insects using Abbott's formula (1925) and they are presented as percentages with standard error. The data were statistically compared, separately for each species, using one-way ANOVA and the significance of mean differences between treatments and control was determined by Fisher's LSD test at $P > 0.05$ (Sokal and Rohlf, 1995).

3. Results

The results presented in Table 1 show that after 7 days of contact of all test insect species with all application rates of natural zeolite mortality did not exceed 40%. After 14 days of contact with the highest application rate (1.0 g/kg) mortality reached 87% for *S. oryzae* and 83% for *T. castaneum*, while mortality of *R. dominica* was notably lower, merely 61%. After 21 days of contact with the highest application rate, only the mortality of *T. castaneum* reached 100%, while mortality of *S. oryzae* and *R. dominica* was 94% and 79%, respectively.

After an additional seven-day period of recovery on untreated broken wheat grains, total mortality of all exposed insect species increased with the duration of their contact with treated grains (Table 2). After seven days of exposure and 7 days of recovery, the highest application rate (1 g/kg) of natural zeolite achieved high mortality only against *S. oryzae*, 62%, while the efficacy after 14 days of exposure and 7 days of recovery was 100% against *T. castaneum*, and 96% and 70% against *S. oryzae* and *R. dominica*, respectively. Total mortality after 21 days of exposure to the highest application rate and 7 days of recovery was again the highest against *T. castaneum* (100%), while efficacy against *S. oryzae* and *R. dominica* was 98% and 82%, respectively.

Table 1 Mortality of *S. oryzae, R. dominica* and *T. castaneum* adults after 7, 14 and 21 days of exposure to wheat treated with natural zeolite Minazel SP.

Insect	Rate (g/kg)	Mortality (% ± SE) after exposure 7 days	14 days	21 days
Sitophilus oryzae	1.00	40.0±1.4 d **	87.0±1.7 d	94.0±1.0 c
	0.75	28.0±0.8 c	72.0±0.8 c	67.0±2.4 b
	0.50	16.0±0.8 b	52.0±2.4 b	65.0±2.8 b
	0 *	0.0±0.0 a	1.0± 0.5 a	2.0±0.6 a
Rhyzopertha dominica	1.00	18.8±1.0 b	61.2±0.5 c	78.8±4.4 c
	0.75	11.2±2.2 a	47.5±2.1 b	61.2±1.3 bc
	0.50	3.8±1.0 a	42.5±2.1 b	56.2±4.7 b
	0	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
Tribolium castaneum	1.00	28.0±0.8 c	83.0±1.7 d	100.0 d
	0.75	4.0±0.8 b	66.0±1.7 c	80.0±2.3 c
	0.50	1.0±0.5 ab	25.0±1.7 b	47.0±2.4 b
	0	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

* Control; ** Means within columns (separately for each species) followed by the same letter are not significantly different (LSD test $p > 0.05$)

Table 2 Mortality of *S. oryzae, R. dominica* and *T. castaneum* adults after 7, 14 and 21 days of exposure to wheat treated with natural zeolite Minazel SP and 7 days of recovery on untreated broken wheat grains.

Insect	Rate (g/kg)	Mortality (% ± SE) after exposure 7 days	14 days	21 days
Sitophilus oryzae	1.00	62.0±1.9 c **	96.0±1.2 d	97.9±0.6 c
	0.75	53.0±1.0 b	86.0±1.5 c	74.7±2.2 b
	0.50	37.0±3.8 b	63.0±2.2 b	72.6±2.9 b
	0 *	0.0±0.0 a	1.0±0.5 a	5.0±1.3 a
Rhyzopertha dominica	1.00	21.2±2.1 c	70.0±1.4 c	82.5±3.7 c
	0.75	18.8±1.7 bc	56.2±3.0 bc	63.8±0.9 b
	0.50	8.8±0.5 ab	50.0±1.6 b	58.8±2.8 b
	0	0.0±0.0 a	5.0±0.6 a	0.0±0.0 a
Tribolium castaneum	1.00	40.0±1.2 c	100.0 d	100.0 d
	0.75	13.0±1.5 b	75.0±1.0 c	82.0±1.7 c
	0.50	6.0±3.0 ab	36.0±3.9 b	60.0±2.4 b
	0	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

* Control; ** Means within columns (separately for each species) followed by the same letter are not significantly different (LSD test $p > 0.05$)

The data on progeny production of stored-product beetles in F_1 generation, presented in Table 3, show that reproduction of all three species occurred in all cases examined, meaning that none of the applied natural zeolite rates caused IR 100%. According to mortality data, the highest application rate of Minazel SP achieved high inhibition of reproduction. After 7 days of exposure, progeny reduction rates were 49-67% for *S. oryzae.* 42-68% for *R. dominica* and 47-78% for *T. castaneum.* After 14 days of exposure, inhibition rates were 55-78% for *S. oryzae,* 72-81% for *R. dominica* and 53-90% for *T. castaneum,* while progeny reduction after 21 days of parental exposure was 51-85% for *S. oryzae,* 80-96% for *R. dominica* and 87-99% for *T. castaneum.*

Table 3 Progeny individuals of *S. oryzae*, *R. dominica* and *T. castaneum* per vessel in F_1 generation after 7, 14 and 21 days of parents exposure to wheat grains treated with natural zeolite Minazel SP.

		Average number of progeny individuals (± SE) and inhibition rate (%) after exposure periods					
	Rate	7 days		14 days		21 days	
Insect	(g/kg)	Av. No.	IR	Av. No.	IR	Av. No.	IR
Sitophilus oryzae	1.00	31.8±2.8 d **	66.8	22.5±8.4 c	78.2	16.8±3.9 c	85.1
	0.75	44.2±4.8 c	53.8	33.5±9.6 bc	67.7	29.0±9.0 c	74.4
	0.50	55.2±6.1 b	48.3	46.5±10.0 b	55.2	55.2±7.8 b	51.3
	0 *	95.8±6.4 a	-	104.0±18.1 a	-	113.5±24.6a	-
Rhyzopertha dominica	1.00	12.2±2.5 b	68.4	10.8±2.6 b	80.8	3.2±1.8 b	96.1
	0.75	17.0±4.9 b	56.1	18.0±2.6 b	67.8	13.8±3.9 b	83.7
	0.50	22.5±6.6 b	41.9	15.5±2.5 b	72.3	16.8±5.3 b	80.2
	0	38.8±13.1 a	-	56.0±12.4 a	-	84.5±22.9 a	-
Tribolium castaneum	1.00	14.2±9.9 c	78.2	7.8±3.3 c	89.9	1.2±1.0 c	98.8
	0.75	19.8±6.7 bc	69.7	19.0±5.5 c	77.7	5.2±2.6 c	95.0
	0.50	34.2±8.9 b	47.4	36.2±5.0 b	52.5	13.5±1.9 b	87.2
	0	65.0±15.6 a	-	76.2±16.8 a	-	105.5±8.4 a	-

* - Control; ** Means within columns (separately for each species) followed by the same letter are not significantly different (LSD test $p > 0.05$)

4. Discussion

Many earlier studies have demonstrated that species of the genus *Sitophilus* are considerably more susceptible to DEs than *Tribolium* species (Korunić, 1997; Fields et al., 2003; Athanassiou et al., 2005; 2007) but such findings were not confirmed in our studies of the natural zeolite formulation Minazel SP, especially over the longest period of exposure and additional 7 days of recovering.

The data in our study show that 7 days is an insufficient exposure period for any significant effects to be achieved with natural zeolite, and confirm earlier findings that the effectiveness of inert dusts increases with the duration of exposure (Arthur, 2001; Fields et al., 2003; Athanassiou et al., 2005; Kljajić et al., 2010). Also, our data confirm reports by Collins and Cook (2006a, b) on the mortality of several stored-product insect and mite species growing significantly after contact with DEs applied to different surfaces and a subsequent period of recovery.

After 21 days exposure to wheat grains treated with 0.75 mg/kg of the natural zeolite product Minazel, and additional 7 days of recovering, Kljajić et al. (2010) observed 100% mortality of *S. oryzae* and *T. castaneum,* and 49-79% mortality of *R. dominica*, which is significantly higher effectiveness than the effectiveness of the natural zeolite product Minazel SP used in the present study. Comparing the results of progeny reduction after 21 days of parent exposure to wheat treated with 0.75 mg/kg of Minazel SP, we also found a significantly lower level of Inhibition rates for *S. oryzae* and *R. dominica* and a slightly higher inhibition rate of *T. castaneum*. Higher relative air humidity in the bioassay involving Minazel SP may explain the difference in effectiveness between the two natural zeolites, a factor that neither the large fraction of small-size particles (≤ 50 μm) in that dust nor its higher application rate (1 g/kg) were able to compensate for.

In conclusion, our experiment has confirmed the practical importance of adjusting the application rates of inert dusts chosen to provide in different environmental conditions successful control of several stored-product insect pests with different characteristics and natural levels of susceptibility. Under our laboratory conditions, the natural zeolite formulation Minazel SP showed moderate to high insecticidal effectiveness against *S. oryzae* and *T. castaneum* in treated wheat grain.

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Susceptibility of red flour beetle *Tribolium castaneum* (Herbst) populations from Serbia to contact insecticides

Andrić, G.*#, Kljajić, P., Perić, I., Pražić Golić, M.
Pesticides and Environment Research Institute, Banatska 31b, 11080 Belgrade, Serbia.
Email: goran.andric@pesting.org.rs

* Corresponding author
# Presenting author

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Abstract

Contact insecticides remain the principal option for controlling stored-product insects. Unsatisfactory results of insecticide applications are caused by several factors, one of the most important being resistance of stored-product insects. The objective of this study was to examine the susceptibility in several populations of red flour beetle *Tribolium castaneum* (Herbst) from Serbia to different contact insecticides. Toxicity of the insecticides dichlorvos, malathion, chlorpyrifos-methyl, pirimiphos-methyl, deltamethrin and bifenthrin to adults of a laboratory population of *T. castaneum* was investigated in the laboratory by topical application. At the LD_{50}, deltamethrin was the most toxic and malathion the least toxic of the insecticides. Discriminating dose data for the laboratory population were used to test the susceptibility of 10 other populations originating from different storage facilities (silos, warehouses and flour mills) in Serbia. The discriminating dose of malathion caused mortality of up to 85% in seven populations, indicating malathion resistance in those populations. For two populations of *T. castaneum* from Nikinci and Jakovo LD values, *ld-p* lines and levels of susceptibility/resistance (RRs) were determined. The most toxic insecticide for adults from Nikinci and Jakovo was deltamethrin, while malathion was least toxic. The resistance ratios (RRs) for malathion at the LD_{50} were 17.6 for beetles from Nikinci, and 26.0 for beetles from Jakovo.

Keywords: *Tribolium castaneum* adults; Different populations; Insecticide toxicity; Susceptibility resistance

1. Introduction

Red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a cosmopolitan species and one of the most widespread insects in storage facilities worldwide, especially in mills, and it develops most rapidly under favourable conditions (Rees, 2004). In Serbia, *T. castaneum* is common in grain storages, mills and flour depots, as well as in foodstuff production plants (Almaši, 2008). Application of contact insecticides and fumigants continues to be the most significant tools for protection of stored products from insect pests. Contact insecticides ensure long-term protection and prevent infestation of treated products. Several formulations based on malathion, pirimiphos-methyl and synergized or nonsynergized deltamethrin have been registered in Serbia for treatment of grains, and dichlorvos for treatment of vacant storage areas (Kljajić, 2008).

The efficacy of contact insecticides in protecting stored products is affected by several factors, primarily by altered susceptibility or resistance of stored-product insects (Subramanyam and Hagstrum, 1996; Kljajić and Perić, 2005). According to data collected globally by the FAO organization (Champ and Dyte, 1976), susceptibility to malathion had changed in 87% of the examined populations of *T. castaneum*. In the USA, many populations collected from peanut, barley and wheat storages were resistant to malathion (Halliday et al., 1988; Subramanyam et al., 1989; Zettler and Cuperus, 1990). Populations of *T. castaneum* from mills were resistant to malathion, and also dichlorvos and chlorpyrifos-methyl (Zettler, 1991; Zettler and Arthur, 1997). For the purpose of monitoring *T. castaneum* resistance to insecticides in Serbian storage facilities, we determined the toxicity parameters (LD levels and *ld-p* lines) for a laboratory population of *T. castaneum* by topical application and determined levels of susceptibility/resistance (resistance ratios, RRs) in populations collected from different silos, warehouses and flour mills.

2. Materials and methods

2.1. Populations tested and contact insecticides used

A laboratory population of *T. castaneum* was used in the experiment, as well as populations collected in 2008 and 2009 from various storage facilities in Serbia: (1) Bačka Topola (silo and flour mill) and Čurug (silo) in the north of Serbian province of Vojvodina; Novo Miloševo (silo) and Kikinda (flour mill) in the northeast; and Adaševci (silo), Nikinci (warehouse) and Irig (silo) in the southwest part, and (2) Belgrade Port (silo) and Jakovo (silo) of Belgrade area.

All populations were reared using methods described by Harein and Soderstrom (1966) and Davis and Bry (1985). Two-to-four-week-old unsexed insects were used in all tests and F_3 generation of beetles for the susceptibility tests of collected *T. castaneum* populations. Technical concentrates of the following insecticide active ingredients were used in bioassay: dichlorvos 98%, malathion 96%, chlorpyrifos-methyl 97%, pirimiphos-methyl (product Actellic 50 EC containing 50% a.i.), deltamethrin 98% and bifenthrin 94.7%.

2.2. Bioassay

Insecticide toxicity to *T. castaneum* adults and susceptibility/resistance of the collected populations were tested by topical application in the laboratory at 24±1ºC and 60±5% r.h. (Halliday et al., 1988). To immobilize the insects, they were anesthetized with CO_2 for about 30 seconds before 0.5 µL of each insecticide, disolved in acetone (6-8 concentrations), was applied to the thorax of each insect by Burkard microapplicator (needle No. 18 in 1.0 mL syringe). Control insects were treated with aceton alone. After treatment of 25 insects in four replications, the beetles were transferred to clean Petri dishes after 4-6 h, each was filled with approximately 1 g of wheat flour. Lethal effects were determined after 7 d of microapplication. Based on discriminating doses causing 100% mortality in the laboratory population and reactions of each collected population to them, the populations from Nikinci and Jakovo were chosen for a full-range testing of insecticide toxicity or susceptibility.

2.3. Data analysis

Mortality data for treated insects were corrected for mortality in the control using Abbott's (1925) formula. Data were processed by probit analysis according to a method described by Finney (1971) and using a computer software developed by Raymond (1985). Statistical significance of differences between toxicity indicators for the insecticides investigated was assessed based on the overlapping/non-overlapping of intervals of confidence.

3. Results

Data on the toxicity of contact insecticides to adults of the laboratory population of *T. castaneum* (Table 1) show that deltamethrin was the most toxic insecticide at the LD_{50} (0.0069 µg insect $^{-1}$), i.e., 40.6 times more toxic than the lowest values recorded for malathion (0.28 µg insect $^{-1}$). At the LD_{95}, however, pirimiphos-methyl (0.0018 µg insect $^{-1}$) demonstrated the highest toxicity, being as much as 3,300 times more toxic than malathion at the lowest level (5.97 µg insect $^{-1}$) of the tested insecticides.

Table 1 Insecticide toxicity to *T. castaneum* adults from a laboratory population assayed by topical application after 7 d of exposure.

Insecticide	LD_{50} (µg insect $^{-1}$) Fiducial Limits (0.05)	LD_{95} (µg insect $^{-1}$) Fiducial Limits (0.05)	Slope *ld-p* line (±SE)	DD* (µg insect $^{-1}$)
Dichlorvos	0.040 (0.038-0.043)	0.087 (0.079-0.097)	4.98±0.34	0.12
Malathion	0.28 (0.21-0.36)	5.97 (4.08-9.66)	1.24±0.08	10.0
Chlorpyrifos-methil	0.011 (0.0104-0.0115)	0.019 (0.018-0.021)	6.52±0.42	0.030
Pirimiphos-methil	0.0091 (0.0087-0.0095)	0.0018 (0.017-0.019)	5.68±0.27	0.025
Deltamethrin	0.0069 (0.0062-0.0075)	0.024 (0.021-0.027)	3.05±0.15	0.050
Bifenthrin	0.049 (0.041-0.056)	0.27 (0.22-0.35)	2.19±0.16	0.50

*Discriminating dose

Discriminating doses of the insecticides dichlorvos, pirimiphos-methyl, chlorpyrifos-methyl, deltamethrin and bifenthrin (Table 2) caused high mortality in all collected populations of *T. castaneum*, ranging from 92-100%. The discriminating dose of malathion caused 100% mortality only in a population from Bačka Topola (flour mill), and high mortality in another Bačka Topola population (silo) and one from Belgrade Port (silo), 95 and 99%, respectively. Mortality in all other populations was below 85%, and the lowest in the Jakovo population (silo), up to 64%.

Table 2 Effects of discriminating doses of insecticides to *T. castaneum* adults from different populations assayed by topical application after 7 d of exposure.

	Mortality (%±SE)					
Population	**DDVP**	**PM**	**CPM**	**MAL**	**DEL**	**BIF**
Bačka Topola-FM	99±0.5	99±0.5	100	100	94±1.3	96±0.8
Bačka Topola-S	97±1.0	93±1.0	92±0.8	95±0.5	96±0.8	100
Belgrade Port-S	99±0.5	93±1.5	100	99±0.5	96±1.2	100
Čurug-S	99±0.5	92±2.2	99±0.5	80±0.8	98±0.6	100
Nikinci-W	98±0.6	94±1.3	100	70±2.5	99±0.5	100
Jakovo-S	100	99±0.5	100	64±2.9	100	100
Adaševci-S	99±0.5	97±0.5	100	79±2.2	100	100
Novo Miloševo-S	99±0.5	99±0.5	98±0.6	80±0.8	98±0.6	100
Irig-S	93±1.0	94±0.6	96±1.2	74±2.4	98±0.6	97±1.0
Kikinda-FM	100	99±0.5	98±1.0	84±2.2	96±1.4	100

S-silo; W-warehouse; FM-flour mill

Tables 3 and 4 show the results of insecticide toxicity (LD levels and *ld-p* lines) and resistance ratios (RRs) for beetles from Nikinci and Jakovo. For the *T. castaneum* from Nikinci, deltamethrin was the most toxic insecticide (0.0066 µg insect $^{-1}$) at the LD_{50}, and chlorpyrifos-methyl (0.016 µg insect $^{-1}$) was most toxic at the LD_{95}, which were 748.5 and 4097.5 times more toxic, respectively, than the lowest value for malathion (LD_{50} 4.94 µg insect $^{-1}$and LD_{95} 65.56 µg insect $^{-1}$).

For *T. castaneum* adults from Jakovo deltametrin (0.0054 µg insect $^{-1}$) was the most toxic insecticide at the LD_{50}, while pirimiphos-methyl (0.0163 µg insect $^{-1}$) was most toxic at the LD_{95}, and they were 1348.1 and 2238.0 times more toxic, respectively, than the lowest toxicity for malathion (LD_{50} 7.28 µg insect^{-1} and LD_{95} 36.48 µg insect $^{-1}$). The RRs show that both populations were resistant to malathion as its RRs were 17.6 and 26.0 at the LD_{50}, and 11.0 and 6.1 at the LD_{95} for beetles from Nikinci and Jakovo, respectively.

Table 3 Insecticide toxicity to *T. castaneum* adults from Nikinci population assayed by topical application after 7 d of exposure and resistance ratios found (RR = LD tested/LD laboratory population).

Insecticide	**LD_{50} (µg insect $^{-1}$) Fiducial Limits (0.05)**	**RR for LD_{50} level**	**LD_{95} (µg insect $^{-1}$) Fiducial Limits (0.05)**	**RR for LD_{95} level**	**Slope *ld-p* line (±SE)**
Dichlorvos	0.042 (0.039-0.044)	1.0	0.087 (0.080-0.096)	1.0	5.15±0.33
Malathion	4.94 (4.23-5.72)	17.6	65.56 (49.25-93.58)	11.0	1.46±0.01
Chlorpyrifos-methil	0.0098 (0.0093-0.010)	0.9	0.016 (0.015-0.018)	0.8	7.21±0.60
Pirimiphos-methil	0.011 (0.010-0.011)	1.2	0.025 (0.022-0.029)	1.4	4.48±0.34
Deltamethrin	0.0066 (0.0058-0.0073)	0.9	0.029 (0.025-0.035)	1.2	2.53±0.17
Bifenthrin	0.054 (0.049-0.060)	1.1	0.14 (0.11-0.17)	0.5	4.07±0.37

Table 4 Insecticide toxicity to red flour beetle adults from Jakovo population assayed by topical application after 7 d of exposure and resistance ratios found (RR = LD tested/LD laboratory population).

Insecticide	LD_{50} (µg insect $^{-1}$) Fiducial Limits (0.05)	RR for LD_{50} level	LD_{95} (µg insect $^{-1}$) Fiducial Limits (0.05)	RR for LD_{95} level	Slope *ld-p* line (±SE)
Dichlorvos	0.035 (0.033-0.037)	0.8	0.079 (0.071-0.090)	0.9	4.65±0.34
Malathion	7.28 (6.54-8.03)	26.0	36.48 (30.85-44.85)	6.1	2.35±0.14
Chlorpyrifos-methil	0.012 (0.0113-0.0127)	1.1	0.024 (0.022-0.027)	1.3	5.55±0.40
Pirimiphos-methil	0.0090 (0.0085-0.0094)	0.9	0.0163 (0.0154-0.0176)	0.9	6.31±0.38
Deltamethrin	0.0054 (0.0047-0.0060)	0.7	0.024 (0.020-0.029)	1.0	2.55±0.19
Bifenthrin	0.040 (0.034-0.045)	0.8	0.17 (0.14-0.21)	0.6	2.65±0.23

4. Discussion

As in many other studies worldwide, our experiments confirmed resistance to malathion. This is based on the effects of discriminating doses of contact insecticides on the ten tested populations of *T. castaneum,* and toxicity parameters and RRs for populations from Nikinci and Jakovo. However, compared to data on species resistance to insecticides summarized by Subramanyam and Hagstrum (1996) and Kljajić and Perić (2005), the level of resistance to malathion was low in the two examined populations in our experiment (RRs 17.6 and 26.0 at the LD_{50} for beetles from Nikinci and Jakovo, respectively).

Subramanyam et al. (1989) had examined the susceptibility of *T. castaneum* and *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) populations collected from barley warehouses using discriminating doses of malathion, pirimiphos-methyl and chlorpyrifos-methyl applied to filter paper. All tested populations of *T. castaneum* displayed resistance to malathion, but no cross resistance to either pirimiphos-methyl or chlorpyrifos-methyl.

In a study by Halliday et al. (1988) using topical application of discriminating doses of malathion, dichlorvos, pirimiphos-methyl, chlorpyrifos-methyl and synergized pyrethrins, several populations of *T. castaneum* from stored peanuts were resistant to malathion alone, mortality in 12 of the 15 tested population below 10%. Zettler (1991) used the same method to test susceptibility of *T. castaneum* and *Tribolium confusum* Jacquelin du Val populations collected from mills in the USA to malathion, dichlorvos, chlorpyrifos-methyl, synergized pyrethrins and resmethrin. Of the 28 tested populations of *T. castaneum*, 93% were resistant to malathion, 64% to dichlorvos, 36% to chlorpyrifos-methyl, while none were resistant to synergized pyrethrins or resmethrin.

More recently, Zetller and Arthur (1997) tested the susceptibility of 14 populations of *T. castaneum* and 10 populations of *T. confusum* from various mills to malathion and dichlorvos and found all *T. castaneum* populations to be resistant to dichlorvos and even more to malathion (top RR=29,081). They found a positive correlation between insect survival after exposure to discriminating doses of insecticides and lethal dose values.

In conclusion, our results confirmed the importance monitoring the susceptibility/resistance of *T. castaneum* and other stored-product insects to insecticides as an important element of pest management programs.

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Bioefficacy of plant derivatives on the repellency, damage assessment and progeny production of the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)

Murugan K.*#
Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore -641046, India.
E-mail: kmvvk@yahoo.com

* Corresponding author
# Presenting author

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Abstract

A laboratory experiment was conducted to investigate the efficacy of different plant derivatives that affect the development of the cowpea weevil, *Callosobruchus maculatus* fed on cowpea, *Vigna unigulculata* seeds. The leaf extracts of the aromatic plant, *Anisomeles malabarica* and *Azadirachta indica* (neem) were evaluated for their repellency, damage assessment and progeny production of *C. maculatus*. The results revealed that the extracts of the two plant species caused a considerable reduction in the number of weevils. The combination of neem seed kernel extract and leaf extracts of *A. malabarica* was the most effective in checking the insect infestation and allowing the least number of F_1 adults emerging from the seeds over the other treatments. Acetone extracts of leaves of *A. malabarica* were more toxic to adult beetles compared to ethanol plant extracts. It was concluded that the botanical products acted as insect antifeedant and the order of repellency of the two plant leaf and kernel extracts on cowpea weevil were: combination of neem seed kernel extract + *A. malabarica* leaf extract > neem > *A. malabarica.*

Keywords: *Callosobruchus maculatus, Anisomeles malabarica, Azadirachta indica*, Repellency, Damage assessment, Progeny production

1. Introduction

Grain crops are commonly stored on-farm in a small scale due to their valuable nutrient content and relative ease in storage when they are dried after harvest (Duke, 1981). However, storage is one of the most crucial post-harvest operations because insects can infest grain all year round under favorable conditions. All storage insect pests undergo complete metamorphosis, have short developmental periods from egg to imago, and can complete several generations a year (Zakladnoi, 1987). The fast development, high fecundity and fertility of stored grain insects under optimal conditions and their ability to adapt to a range of habitat conditions, i.e. temperature/humidity variations, can lead to very high damage during storage (Zakladnoi, 1987).

The cowpea bruchid, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) is a cosmopolitan post-harvest pest. It causes quantitative and qualitative losses manifested by seed perforation, reduction in weight, market value and reduced germination of seeds (Anonymous, 1989). About 4% of the total annual production or about 30,000 t values at over 30 million US dollars are lost annually in Nigeria alone to *C. maculatus* (Casewell, 1980).

With the limitations on the use of current pest control methods, there is scope for the discovery of safe, non-polluting, bio-rational pest management technologies for stored products. Extrapolation of the number of plant species studied and the number of compounds known suggests that millions of different compounds possess activity against pests which could be isolated from different plant species (Harborne, 1998).

The use of plant-based pesticides in grain protection has a long history (Chimbe and Galley, 1996) and there are a number of bibliographic databases on the use of different botanicals or parts of plants (leaves, twigs, roots, seeds) or their extracts (hot/cold water) or residues (ash, husk) by farmers in developing countries against stored product insect pests (Dales, 1996; Murugan et al., 1999). These databases provide information on the plant materials, target organism, toxic level of the compound or extract or whole plant material, economic value and active principles when they are known.

Naturally occurring compounds can affect the physiology of insects or they can modify the behavior of insects (Bell et al., 1984). Either the compounds in the vapor phase (volatile) or non-volatile compounds can affect insects to change their behavior. Those compounds can impede development, kill insects or cause losses in fecundity or viability of egg production and, therefore, reduce the number of offspring. They may act by ingestion, cuticle contact or fumigant action (Stadler, 1983). The objectives of the present study was to test the efficacy of Neem seed kernel extract (NSKE) and *Anisomeles malabarica* leaf extracts (AMLE) on repellency, damage assessment and progeny production of *C. maculatus*.

2. Materials and methods

2.1. Rearing of Callosobruchus maculatus

A small population of *C. maculatus* was reared and bred under laboratory conditions on the seeds of cowpea (*Vigna unguiculata*) inside a growth chamber at 30 ± 2^0C, 12:12 L: D and with 70% r.h.. Initially, 50 pairs of 1-2 day old adults were placed in a jar containing cowpea seeds. The jars were sealed and a maximum of 7 d were allowed for mating and oviposition. Then parent stocks were removed and cowpea seeds containing eggs was transferred to fresh cowpea seeds in the breeding jars that were covered with pieces of cloth fastened with rubber band to prevent the contamination and escape of beetles. The subsequent progenies of the beetles were used for all experiments.

2.2. Test plant materials

The leaves of *A. malabarica* and *Azadirachta indica* (A. Juss) seeds were collected from plants growing in and around the Bharathiar University Campus, Coimbatore, Tamil Nadu, India. The leaves were thoroughly washed and air-dried in the shade; the dried leaves were manually ground into powder with the help of a mortar and pestle.

2.3. Soxhlet extraction

Fifty g of leaves were extracted in 500 ml acetone, ethanol by ***soxhlation*** for 48 h. The extracts were filtered and filtrate was evaporated under reduced pressure to obtain crude. After complete solvent evaporation, one gram of each concentrated solvent extract was dissolved in 9 ml of acetone and used in repellent bioassays and respective concentrations were prepared by dilution.

2.4. Bioassays

2.4.1. Repellency bioassay

An olfactometer was constructed from a large (19 cm diameter) plastic Petri dish. Ten small (5 cm diameter) Petri dishes were attached to the central dish around its circumference and a small hole was made to allow free passage between each small Petri-dish and the large central dish. In the lid of the large dish, a small hole was made to allow the release of insects into the chamber. This hole was closed during the experimental tests preventing insect escape. Twenty seeds were thoroughly mixed with 2 mL of each plant extract. Residual extract was allowed to evaporate from the seeds. This experimental procedure was repeated for plant extract in each of the solvent. Each experimental test was replicated 4 times. For each replicate, fifty *C. maculatus* adults were released into the large dish and twenty grains, soaked in individual plant extract were placed in the small peripheral dishes. The direction of movement of beetles was recorded at 15 min, 30 min, 1 hr, 2 hr and 24 hr intervals.

2.4.2. Damage assessment

Damage assessment was carried out on treated and untreated grains. Samples of 100 g of grains were taken from each jar and the number of damaged grains was counted and weighed.

Percentage seed weight loss = $\frac{UNd - Dnu}{U (Nd + Nu)} X\ 100$

Where U = weight of undamaged grain
D = weight of damaged grain
Nd = number of damaged grains
Nu = number of undamaged grains

2.4.3. Progeny production

Twenty pairs of beetles were introduced into treated and control grains and after 30, 15 and 7 d after the oviposition period for *C. maculatus* respectively, the parent adults were removed. Insect subsequently emerging were counted to estimate the F1 progeny production. Counting was stopped after 63, 42 and 42 days *C. maculatus*, respectively to avoid overlapping of generation (Mian and Mulla, 1982).

2.5. Statistical Analyses

All data were subjected to analysis of variance (ANOVA) and the means were separated using Duncan's multiple range test (Duncan, 1955).

3. Results

3.1. Repellency bioassay

The repellent activity of extracts of neem seed kernel (NSKE) and AMLE at three concentrations against *C. maculatus* at one-hour intervals showed that maximum repellent activity (81%) was observed for NSKE at 2% concentration after 1 h. (Table 1) with increasing time, the repellent activity was decreased for all the other extracts. When the plant powders of neem seed kernel and powdered leaves of *A. malabarica* at three different concentrations against *C. maculatus* at one-hour intervals were treated, maximum repellent activity (76%) was observed for NSKP (2%) after 1 h, followed by 62% repellency with AMLP (Table 2).

Table 1 Repellent activity of different plant extracts on *Callosobruchus maculatus*

Treatment	Conc. (%)	Repellency (%) 1 HAT	2 HAT	3 HAT	4 HAT
NSKE	0.5	61 c	55 d	50 d	47 c
	1	72 ab	64 ab	60 ab	55 b
	2	81 a	72 a	67 a	64 a
AMLE	0.5	52 bc	50 cd	46 cd	43 cd
	1	61 c	56 c	51 c	46 d
	2	73 b	65 b	62 b	54 ab
Control	0	0	0	0	0

Within a column means followed by a same letter is not significantly different 5% level of DMRT. HAT: Hours After Treatment. *NSKE* = Neem Seed Kernel Extract, *AMLE = Anisomeles malabarica* Leaf Extract.

Table 2 Repellent activity of different plant powders on *Callosobruchus maculatus*.

Treatment	Conc. (%)	Repellency (%) 1 HAT	2 HAT	3 HAT	4 HAT
NSKE	0.5	52 d	41 d	34 cd	27 cd
	1	68 b	53 c	40 c	35 c
	2	76 a	64 a	59 a	42 ab
AMLE	0.5	46 cd	40 cd	37 d	32 d
	1	54 c	53 c	50 ab	45 b
	2	62 ab	58 b	54 b	49 a
Control	0	0	0	0	0

Within a column means followed by a same letter is not significantly different 5% level of DMRT. HAT: Hours After Treatment. *NSKE* = Neem Seed Kernel Extract, *AMLE = Anisomeles malabarica* Leaf Extract

3.2. Damage assessment

The results of damage assessment of *C.maculatus* on cowpea after the treatment of acetone plant extracts were shown in Table 3. Damage decreased with increasing concentration of extract. Among the acetonic extracts studied, NSKE showed better activity (14%) than AMLE (19%). The damage assessment of *C. maculatus* on cowpea after the treatment of ethanol plant extracts showed that increase in concentration

causes a decrease in damage of the seeds (Table 4). Among the ethanol extracts tested, NSKE (18%) showed higher activity than AMLE (23%). However, the acetone extracts of NSKE showed maximum protection than the ethanol extracts of NSKE. The damage assessment of *C. maculatus* on cowpea after the treatment of plant powders of neem seed kernel and powdered leaves of *A. malabarica* showed that a decrease in percentage of damage was observed while increasing the concentration of the powder. Among the treatments, NSKP exhibited better activity (12%) than AMLP (33%), but the NSKE treated grains caused less seed damage than NSKP treated grains (Table 5).

Table 3 Damage assessment of *Callosobruchus maculatus* on cowpea after the treatment of acetone plant extracts.

Treatment	Conc. (%)	Damage (%)
NSKE	2	35 b
	4	27 c
	6	20 d
	8	14 e
AMLE	2	64 b
	4	48 c
	6	33 d
	8	19 e
Control	0	97 a

Within a column means followed by a same letter is not significantly different 5% level of DMRT. *NSKE* = Neem Seed Kernel Extract, *AMLE = Anisomeles malabarica* Leaf Extract.

Table 4 Damage assessment of *Callosobruchus maculatus* on cowpea after the treatment of ethanol plant extracts.

Treatment	Conc. (%)	Damage (%)
NSKE	2	42 c
	4	36 d
	6	27 cd
	8	18 f
AMLE	2	72 b
	4	51ab
	6	42 c
	8	23 e
Control	0	97 a

Within a column means followed by a same letter is not significantly different 5% level of DMRT. *NSKE* = Neem Seed Kernel Extract, *AMLE = Anisomeles malabarica* Leaf Extract.

3.3. Progeny production

The various plant powders caused a significant reduction of progeny of *C. maculatus*. NSKP significantly reduced the progeny production of. *C. maculatus* compared to AMLP, but the combined treatment of NSKP and AMLP showed maximum progeny reduction (Table 6). The acetone plant extracts of NSKE was more effective in reducing the F_1 progeny than the ethanol extracts (Table 7). Among the extracts tested, the combinations of acetone extracts of NSKE and AMLE proved to be the best plant materials in controlling the emergence of F_1 individuals.

4. Discussion

In the present investigation, the efficacy of neem seed kernel and *A. malabarica* afford better protection to the infestation of the cowpea weevil, *Callosobruchus maculatus*. Pradhan et al. (1963) reported that neem seed kernel possess an extra ordinary gustatory repellent properties, much higher than neem leaf powder against the desert and migratory locusts. Rouf et al. (1996) showed that mixing of neem leaf powder with lentil seeds resulted in reduced oviposition and adult emergence of the pulse beetle, *Callosobruchus chinensis* (Linnaeus) Pandey et al. (1986) reported that a petroleum ether extract of neem leaves and twigs mixed with green gram seeds inhibited the oviposition of *C. chinensis*. Butterworth and Morgan (1971) revealed that the most active antifeedant is reported to occur in neem seed kernel powder,

further, the results were confirmed by who also reported that azadirachtin is a major compound in the seed kernel responsible for the reduced oviposition and adult emergence in beetles. Neem has many other activities against insects disrupting or inhibiting development of eggs, larvae or pupae, preventing the molting of larvae or nymphs, disrupting mating and sexual communication, repelling larvae and adults, deterring females from laying eggs, sterilizing adults, poisoning larvae and adults, feeding deterrent, blocking the ability to swallow by reducing the motility of the gut preventing metamorphosis, thus preventing adult maturation, inhibiting the formation of chitin, the substance essential for the insect to form an exoskeleton. This huge array of insecticidal properties of neem is thought to be due to it's adversely affecting the insect's hormone system (Joshi and Sitaramaiah, 1979; Murugan et al., 2009).

Table 5 Damage assessment of *Callosobruchus maculatus* on cowpea after the treatment of certain plant powders.

Treatment	Conc. (%)	Damage (%)
NSKP	2	45 b
	4	31c
	6	20 d
	8	12 e
AMLP	2	75 b
	4	56 c
	6	42 d
	8	33 e
NSKP+AMLP	2+2	36 b
	4+4	24 c
	6+6	15 d
	8+8	9 e
Control	0	95 a

Within a column means followed by a same letter is not significantly different 5% level of DMRT. *NSKP*= Neem Seed Kernel Powder, *AMLP = Anisomeles malabarica* Leaf Powder

Table 6 Emergence of F_1 progeny of *Callosobruchus maculatus* on cowpea after the treatment of certain plant powders.

Treatment	Number of F_1 adults
NSKP	7 c
AMLP	9 b
NSKP+AMLP	6 ab
Control	18 a

Within a column means followed by a same letter is not significantly different 5% level of DMRT. *NSKP*= Neem Seed Kernel Powder, *AMLP = Anisomeles malabarica* Leaf Powder.

This study also demonstrated the potential of using *A. malabarica* to control *C. maculatus* in stored cowpea. *A. malabarica*, commonly called as Malabar catmint is a highly aromatic plant belonging to the family Lamiaceae (Joshi, 2000). The plant powder obtained from this plant in this study can be used in the same manner as most conventional insecticides, and could be less toxic to humans (Duke, 1985). This may be due to the presence of volatile compounds such as anisomelic acid, betulinic acid, citral, gerainic acid and ovatodiolide in the plant extract (Guha Bakshi et al., 1999). Of the above phytochemicals, it is supposed that citral plays a key role as an insecticide in controlling the bruchid population and disrupting the physiology of *C. maculatus* (Prajapati and Kumar, 2003). Our treatments of various plant extracts and powders were repellent to *C. maculatus*. The phytochemicals like azadirachtin present in neem extract and citral present in *A. malabarica* plant extract may suppress the phagostimulation and arrest the physiological events of the beetle. The antifeedant effects of azadirachtin are well known (Jacobson, 1989; Schmutterer, 1990; Ascher, 1993; Mordue and Blackwell, 1993; Murugan et al., 1988). Both primary and secondary antifeedant effects have been observed with azadirachtin (Ascher, 1993).

Table 7 Emergence of F_1 progeny of *Callosobruchus maculatus* on cowpea after the treatment of certain plant extracts.

Treatment	Number of F_1 adults	
	Acetone extract	Ethanol extract
NSKE	5 ab	6 ab
AMLE	7 b	8 b
NSKE+AMLE	4 c	5 c
Control	15 a	15 a

Within a column means followed by a same letter is not significantly different 5% level of DMRT. *NSKE* = Neem Seed Kernel Extract, *AMLE = Anisomeles malabarica* Leaf Extract.

Among the treatments, NSKE and AMLE showed higher activity than other combinations. This clearly suggests that the plant extracts contain powerful phytochemicals, which suppress the chemoreceptors in the mouthparts of the beetle and reduced the feeding in *C. maculatus*. Neem's efficacy to non-target and beneficial organisms has been documented (Schmutterer, 1995; Ascher, 1993; Murugan *et al*., 1999). Many biologically active compounds can be extracted from neem, including triterpenoids, phenolic compounds, carotenoids, steroids and ketones. The tetranortriterpenoid azadirachtin has received the most attention as a pesticide, because it is relatively abundant in neem kernels, and has shown biological activity on a wide range of insects. Azadirachtin is actually a mixture of seven isomeric compounds labeled as azadirachtin-A to azadirachtin-G with azadirachtin-A being present in the highest quantity and azadirachtin-E regarded as the most effective insect growth regulator (Verkerk et al., 1993). Many other compounds have been isolated that shows antifeedant activity as well as growth regulating activity on insects. This cocktail of compounds significantly reduces the chances of tolerance or resistance developing in any of the affected organisms. However, only four of the compounds in neem have been shown to be highly effective in their activity as pesticides: azadirachtin, salannin, meliantriol, and nimbin (Jacobson, 1990; National Research Council, 1992; Murugan et al., 1998.).

The emergence of F_1 progeny of *C. maculatus* on cowpea after the treatment of NSKE, AMLE, NSKP and AMLP suggests that the acetone plant extracts of NSKE was more effective in reducing the F_1 progeny than the ethanol extracts. The insect growth regulatory effect of azadiracthin and citral causes various developmental, post-embryonic, reproductive and growth inhibitory affects in insects so that the emergence of F_1 generation is prevented.

Botanical materials tested here could be useful and further studies are recommended to determine if these plant species control other storage pests through direct effects and also indirect effects. The plants tested in our study are a possible source of natural products that could be used as an alternative to synthetic insecticides (Zebitz, 1987; Murugan et al., 2004).

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Efficacy of diatomaceous earth and botanical powders against the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) on maize

Nukenine, E.K.*#[1], Goudoungou, J.W.[1], Adler, C.[2], Reichmuth, C.[2]
[1] Department of Biological Sciences, University of Ngaoundere, PO Box 454, Ngaoundere, Cameroon.
Email: ennukenine@fulbrightmail.org
[2] Federal Research Centre for Cultivated Plants – Julius Kühn - Institut, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Königin-Luise-Str. 19, 14195 Berlin, Germany

* Corresponding author
# Presenting author

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Abstract

The effectiveness of the diatomaceous earth SilicoSec, neem seed powder and *Plectranthus glandulosus* leaf powder, applied at four different rates with four exposure intervals (1, 3, 7 and 14 d) for the control of maize weevil, *Sitophilus zeamais* Motschulsky, on maize in the laboratory was determined. Treatment with SilicoSec was the most effective followed by neem seed powder and *P. glandulosus* powder. The highest tested content (2 g/kg) of SilicoSec caused 81.1% and 100% mortality of *S. zeamais* within 3 and 14 days of exposure, respectively. The application of the highest content (40 g/kg) for neem seed powder and *P. glandulosus* powder resulted in 86.8% and 59.5% mortality, respectively 14 days after exposure. Seven-day LC_{50}-values were 0.56 g/kg for SilicoSec, 19.7 g/kg for neem seed powder and 45.24 g/kg for *P. glandulosus* powder. The treatments reduced progeny emergence, percentage of grain damage, percentage of weight loss and percentage of germination loss, although *P. glandulosus* powder was less active for these parameters. Results suggest that SilicoSec can be considered as a potential component of an integrated pest management strategy against the maize weevil. However, in the poor tropical countries were the plant powders are widely available and food production dominated by subsistence agriculture, neem seed powder and *P. glandulosus* powder could be adopted also for the protection of stored maize against the infestation of *S. zeamais*.

Keywords: Diatomaceous earth, Botanical powders, Maize, Integrated weevil management, *Sitophilus zeamais*

1. Introduction

The maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), is one of the most serious cosmopolitan pests of stored cereal grain, especially of maize (*Zea mays* L.), in tropical and sub-tropical regions (Throne, 1994). The weevil has been reported to cause up to 80% grain damage in Cameroon during storage, where maize constitutes the most important food crop (Nukenine et al., 2002). Damaged grain has reduced nutritional value, low percentage germination, reduced weight and lowered market value (Demissie et al., 2008). Cheap and effective methods for reducing *S. zeamais* damage are needed in Africa to reduce food insecurity.

Control of *S. zeamais* populations around the world is primarily dependent upon continued applications of synthetic insecticides, which are often the most effective treatments for the disinfestations of stored food, feedstuffs and other agricultural commodities from insect infestation. Although effective, their repeated use for decades has disrupted biological control by natural enemies and led to outbreaks of other insect species and sometimes resulted in the development of resistance (Park et al., 2003). There are also serious concerns about environmental degradation and human health. Furthermore, the majority of farmers in Africa are resource-poor and have neither the means nor the skills to obtain and handle pesticides appropriately. Therefore, an environmentally safe and economically feasible weevil control practice needs to be available. The use of chemically inert materials, such as diatomaceous earths or plant products in large quantities to fill up the interstitial space in grain bulks and provide a barrier to insect movement, is quite widespread (FAO, 1999). Diatomaceous earths have proven to be very effective in smaller quantities. There is limited published work on the efficacy of diatomaceous earths in Africa (Stathers et al., 2002; Dimessie et al., 2008) In Africa, much research has focused on botanical pesticides (plant powders and extracts) and the mixing of grains with plant materials is an age-old practice among rural framers in the continent (Poswal et al., 1991; Shaaya et al. 1997; Tapondjou et al., 2000).

Plectranthus glandulosus Hook f. (syn. *Coleus laxiflorus* (Benth.) Roberty) (Lamiaceae) is an annual, glandular and strongly aromatic herb, used in folk medicine for the treatment of colds and sore throat in the Adamawa region of Cameroon, (Ngassoum et al., 2001). The few studies on the insecticidal effect of the plant reported high efficacies of the leaf powder (Nukenine et al., 2007; 2010a) and essential oils against *S. zeamais* (Nukenine et al., 2010b). Insecticidal activity from products of the neem tree, *Azadirachta indica* A. Juss (Meliaceae) is widely reported in the literature (Schmutterer, 1990; Isman, 2006). However, in northern Cameroon the medicinal use of the plant seems to shield its employment in storage protection.

The objective of this study was, therefore, to evaluate the insecticidal and reproduction inhibitory effects of SilicoSec (a diatomaceous earth of fresh water origin), neem seed powder and *P. glandulosus* leaf powder against the maize weevil in the laboratory.

2. Materials and methods

2.1. Test insects

Maize weevil was reared on maize grains under fluctuating laboratory conditions. Adult weevils were obtained from a colony kept since 2005 in the Applied Chemistry laboratory at the University of Ngaoundere.

2.2. Insecticide materials

The insecticide materials used were SilicoSec (Biofa AG, Münsingen, Germany), neem seed powder and *P. glandulosus* leaf powder. Neem fruits were collected on the ground below the trees in Maroua (10°33, 16' N, longitude 14°15, 04' E and altitude 356 masl), Far-North region, Cameroon in November 2008 (dry season). The seeds were removed from the fruits, sundried, and cracked to remove the husks. The kernels were crushed in a mortar until the powder passed through a 1-mm sieve mesh. The powder was stored in opaque containers inside a refrigerator at 4°C until needed for bioassay.

The leaves of *P. glandulosus* were collected in October (end of wet season) of 2008 around Ngaoundere (latitude 7° 22' North and longitude 13° 34' East, altitude of 1100 masl), located in the Adamawa region (plateau) of Cameroon. The plants were less than one-year old and only the green leaves were harvested. The leaves were dried at room temperature for seven days, and then crushed in a mortar until the powder passed through a 0.4-mm mesh sieve. The powder was stored in opaque containers inside a refrigerator at 4°C.

2.3. Toxicity tests and F1 progeny production

The application rates of the powders were 0.5, 1, 1.5 and 2 g/kg for SilicoSec (Demissie et al., 2008), 5, 10, 20 and 40 g/kg for neem seed powder (Chouka, 2007) and *P. glandulosus* powder (Nukenine et al., 2007). These rates were obtained by adding 0.025, 0.05, 0.075 and 0.1 g powder for SilicoSec and 0.25, 0.5, 1 and 2 g powder for neem seed powder and *P. glandulosus* powder to 50 g maize in a glass jar and shaken well to get uniform coating. Twenty 7 to 14-d old adult weevils of mixed sex were introduced into each jar. Untreated controls were included. The experiments were laid out in a completely randomized design on shelves. The treatments had four replications. All treatments were maintained in the laboratory under ambient conditions, and the daily temperature and r.h. in the laboratory ranged from 17.3 – 28.8°C and 56.3 – 97.8%, respectively. Mortality was recorded 1, 3, 7 and 14 days after infestation. On the 14th day post-infestation, all insects were removed and the different jars containing grains were kept under the same experimental conditions. The counting of F1 adults was done once a week for five weeks commencing 6 weeks post-infestation. The insect rearing had shown that emergence started only after the 5th week post-infestation

2.4. Population increase and damage

Three rates of the insecticidal materials (1, 1.5 and 2 g/kg for SilicoSec and 10, 20 and 40 g/kg for the plant powders), for 200 g seed were admixed as described above. A lot of 30 seven to 14-days-old insects of mixed sexes were introduced into each jar containing treated or untreated seed. Each treatment with the same dosage was repeated three times. After four months, the number of live and dead insects was determined for each jar. Damage assessment was performed by measuring the weight of the sieved powder and that of the grains without powder (final weight). The amount of grain powder (frass plus

faeces) was expressed as the total powder minus the weight of plant powder used. Percent weight loss was determined as follows: (initial weight-final weight/100) x 100.

2.5. Seed germination

In order to assess the viability of seeds, seed germination was tested using 30 randomly picked seeds from undamaged grains after separation of damaged and undamaged grains in each jar. The seeds were placed on moistened sand in perforated plastic trays and the number of germinated seeds was recorded after 10 days (Rao et al., 2005).

2.6. Statistical analysis

Data on % cumulative mortality and % reduction in F_1 progeny were arcsine-transformed [(square root(x/100)] and the number of F_1 progeny produced was log-transformed (x + 1). The transformed data were subjected to the ANOVA procedure using the Statistical Analysis System (Zar, 1999; SAS Institute, 2003). HSD test (P = 0.05) was applied for mean separation. Probit analysis (Finney, 1971; SAS institute, 2003) was applied to determine lethal dosages causing 50% (LC_{50}) and 95% (LC_{95}) mortality of *S. zeamais* at 3, 7 and 14 d after treatment application. Abbott's formula (Abbott, 1925) was used to correct for control mortality before probit analysis and ANOVA.

3. Results

The results of the toxicity tests showed that the insecticidal materials caused significant mortality to *S. zeamais* (Table 1). Mortality increased with powder content level and time post-exposure for all the insecticidal materials. In general, SilicoSec was more toxic to the weevil than the plant powders and neem seed powder caused higher mortality to *S. zeamais* than *P. glandulosus* powder. At the highest tested powder content, SilicoSec (2 g/kg), neem seed powder (40 g/kg) and *P. glandulosus* powder (40 g/kg) caused 100, 86, and 59% mortality to *S. zeamais*, respectively, within 14 days of exposure.

Table 1 Corrected cumulative mortality of *Sitophilus zeamais* exposed to SilicoSec and two plant powders with LC_{50} values under fluctuating laboratory conditions (t = 17.3 – 28.8°C, r.h. 56.3 – 97.8%).

Insecticide	Content (g /kg)	%Mortality (mean ± SE) - Exposure period (days)			
		1	3	7	14
SilicoSec	0	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	0.5	5.00 ± 2.04^{ab}	20.86 ± 12.74^{bc}	44.47 ± 8.61^{b}	44.36 ± 12.44^{b}
	1	5.00 ± 2.04^{ab}	48.75 ± 9.12^{ab}	71.19 ± 8.20^{ab}	75.70 ± 14.31^{ab}
	1.5	10.00 ± 4.08^{ab}	$59,21 \pm 15,25^{ab}$	85.00 ± 10.21^{a}	93.75 ± 4.73^{a}
	2	13.75 ± 4.27^{a}	81.12 ± 10.84^{a}	86.12 ± 3.71^{a}	100.00 ± 00.00^{a}
	F	4.02*	8.57***	25.25***	22.24***
	LC_{50}	–	1.06	0.56	0.58
A. indica	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	5	2.5 ± 1.44^{a}	7.83 ± 6.27^{a}	15.60 ± 7.44^{bc}	$20.33 \pm 13.12b^{c}$
	10	2.5 ± 1.44^{a}	10.23 ± 10.53^{a}	36.72 ± 6.53^{ab}	50.46 ± 13.06^{ab}
	20	2.5 ± 1.44^{a}	14.28 ± 7.61^{a}	50.42 ± 18.03^{ab}	54.34 ± 14.67^{ab}
	40	3.75 ± 2.39^{a}	23.16 ± 8.79^{a}	66.61 ± 15.97a	86.12 ± 7.44^{a}
	F	0.76ns	1.27*	5.23**	8.98***
	LC_{50}	–	–	19.74	12.72
P. glandulosus	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}
	5	0.00 ± 0.00^{a}	1.25 ± 1.25^{b}	8.82 ± 4.25^{bc}	11.38 ± 8.25^{ab}
	10	1.25 ± 2.50^{a}	6.38 ± 1.21^{ab}	17.83 ± 5.87^{abc}	22.84 ± 15.78^{ab}
	20	2.5 ± 1.44^{a}	9.01 ± 5.35^{ab}	$30.46 \pm 8,84^{ab}$	40.27 ± 18.34^{a}
	40	2.5 ± 1.25^{a}	11.52 ± 1.17^{a}	47.17 ± 17.65^{a}	59.48 ± 10.42^{a}
	F	0.48^{ns}	4.36*	3.88*	3.64*
	LC_{50}	–	–	$45.24^{\#}$	28.48

Means ± S.E. in the same column for the same category of insecticide, followed by the same letter do not differ significantly at *P* = 0.05 (Tukey's test)). Each datum represents the mean of four replicates of 20 insects each.
ns P > 0.05 * P < 0.05, *** P < 0.001. # LC value obtained by extrapolation.
- Estimated LC values are too large or estimation impossible due to inadequate mortality

For the same time-point, and in the same order, the lowest tested powder contents caused 44, 20 and 11% weevil mortality. Within 1 d of exposure 10, 4 and 3% mortality was recorded for SilicoSec, neem seed powder and *P. glandulosus* powder, respectively, for the highest tested powder contents. The 7-d LC_{50} values (Table 1) clearly demonstrate that SilicoSec (0.56 g/kg) was more toxic to *S. zeamais* than neem seed powder (17.74 g/kg) and *P. glandulosus* powder (45.24 g/kg)

All the three treatments generally caused significant reduction in progeny production relative to the control, which was dose dependent (Table 2). SilicoSec at 1 g/kg and neem seed powder at 5 g/kg caused >90% suppression of F1 progeny emergence. Higher concentration levels of these two powders roughly achieved complete suppression of progeny emergence. The highest and lowest tested concentration level of *P. glandulosus* powder reduced F1 progeny production by 69.6 and 18.5%, respectively.

Table 2 Progeny production of *Sitophilus zeamais* in grains treated with SilicoSec, Neem seed powder and *Plectranthus glandulosus* leaf powder under fluctuating laboratory conditions (temperature = 17.3 – 28.8°C and r.h. =56.3 – 97.8%).

Insectcide	Content (g/kg)	Mean number of F_1 adult progeny	% reduction in adult emergence relative to control
SilicoSec	0	91.50 ± 16.68^{a}	0.00 ± 0.00^{c}
	0.5	29.75 ± 8.01^{b}	63.95 ± 13.00^{b}
	1	8.50 ± 3.52^{b}	92.05 ± 2.53^{a}
	1.5	4.50 ± 2.33^{b}	95.85 ± 1.83^{a}
	2	2.00 ± 2.00^{b}	98.38 ± 1.63^{a}
	F	19.35 ***	47.55 ***
A. indica	0	91.50 ± 16.68^{a}	0.00 ± 0.00^{c}
	5	5.25 ± 1.25^{b}	94.125 ± 0.89^{b}
	10	0.50 ± 0.29^{b}	99.20 ± 0.47^{a}
	20	0.25 ± 0.25^{b}	99.55 ± 0.45^{a}
	40	0.00 ± 0.00^{b}	100.00 ± 0.00^{a}
	F	29.02 ***	8008.49 ***
P. glandulosus	0	91.50 ± 16.68^{a}	0.00 ± 0.00^{c}
	5	76.25 ± 19.52^{a}	18.85 ± 11.66^{bc}
	10	69.50 ± 16.45^{a}	20.85 ± 13.37^{bc}
	20	37.00 ± 15.13^{a}	57.00 ± 13.37^{ab}
	40	28.00 ± 13.95^{a}	69.60 ± 10.67^{a}
	F	2.68 ns	6.89 *

Means ± S.E. in the same column for the same category of insecticide, followed by the same letter do not differ significantly at $P = 0.05$ (Tukey's test)). Each datum represents the mean of four replicates of 20 insects each.
ns $P > 0.05$ * $P < 0.05$, *** $P < 0.001$

Apart from percentage seed damage which did not differ among content levels for neem seed powder, there were significant differences in the number of live insects, percentage seed damage, percentage weight loss and percentage germination among powder content levels for all the three insecticide materials (Table 3). SilicoSec and neem seed powder were effective in reducing the rate of the weevil population increase, seed damage, seed weight loss and germination losses, while *P. glandulosus* powder was less effective for the four parameters.

4. Discussion

Our experiments showed that the three insecticide powders possess some toxic components which could cause a noticeable weevil mortality. However, the action of the botanical powders was slower than that of SilicoSec. Between the botanical powders, *P. glandulosus* was less toxic than neem seed powder. This contention is supported by the lower 7-d LC_{50} values for SilicoSec (0.56 g/kg) relative to those of neem seed powder (19.74 g/kg) and *P. glandulosus* powder (45.24 g/kg). Although the results with SilicoSec are encouraging, other authors recorded higher mortalities of *S. zeamais* caused by the diatomaceous earth. Demissie et al. (2008) reported that SilicoSec caused 99 and 100% mortality of *S. zeamais* within 3 and 7 d exposure periods, respectively, at the rate of 2%, whereas within the same time-point and

content rate, the present study recorded 81 and 86% mortality, respectively. The difference in mortality between the two studies could be linked at least in part to variation in environmental conditions and insect strains. Our laboratory temperature and r.h. varied respectively between 17.3 – 28.8°C and 56.3 – 97.8% and those of Demissie et al. (2008) between 20 – 30° C and 66.5 – 76.5 %. The efficacy of diatomaceous earth reduces with increasing relative humidity (Korunic, 1988; Vayias et Athanassiou, 2004). Within 14 d of exposure, and at a concentration rate of 40 g/kg, neem seed powder caused respectively 30% (Pereira and Wohlgemuth, 1982), 38% (Chouka, 2007) and 86% (present study) mortality to *S. zeamais*. With similar concentration level and time post-exposure, Nukenine et al. (2007), Chouka (2007) and the present study recorded respectively 100, 83 and 29% mortality of *S. zeamais* caused by *P. glandulosus* powder. These discrepancies in the toxicity of the plant powders are not surprising, since geographical location, time of harvest, r.h. among others, greatly influence the activity of botanicals against insects (Schmutterer, 1990; Korunic, 1988; Vayias and Athanassiou, 2004). However, these differences show that efficacy data with botanicals require the determination of contents of pure compounds

Table 3 Population increase (mean number of progeny for three jars ± SE) and damage parameters of *Sitophilus zeamais*, and percentage germination in maize admixed with different contents of SilicoSec, Neem seed powder and *Plectranhus glandulosus* leaf powder and stored for four months under fluctuating laboratory conditions (temperature = 17.3 – 28.8°C and r.h. = 56.3 – 97.8%).

Insecticide	Content (g/kg)	Number of live insects	Seed damage(%)	Weight loss (%)	Germination (%)
SilicoSec	0	463.7 ± 35.4^{a}	94.5 ± 0.2^{a}	26.0 ± 3.0^{a}	0.0 ± 0.0^{b}
	1	16.7 ± 4.4^{b}	4.4 ± 1.4^{b}	0.6 ± 0.2^{b}	70.0 ± 6.7^{a}
	1.5	6.7 ± 4.1^{b}	2.7 ± 1.5^{b}	0.4 ± 0.2^{b}	68.4 ± 2.9^{a}
	2	1.0 ± 1.0^{b}	0.6 ± 0.2^{b}	0.1 ± 0.0^{b}	66.7 ± 10.2^{a}
	F	160.9 ***	1906.9 ***	87.9 ***	29.9***
A. indica	0	463.67 ± 35.4^{a}	94.5 ± 0.2^{a}	94.5 ± 0.2^{a}	0.0 ± 0.0^{b}
	10	0.7 ± 0.7^{b}	4.1 ± 1.6^{b}	4.1 ± 1.6^{b}	17.8 ± 6.9^{a}
	20	0.0 ± 0.0^{b}	0.4 ± 0.4^{c}	0.4 ± 0.4^{c}	12.2 ± 5.5^{a}
	40	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	6.7 ± 1.9^{ab}
	F	171.3 ***	3127.3 ***	3127.3 ***	3.2 ns
P. glandulosus	0	463.7 ± 35.4^{a}	94.50 ± 0.2^{a}	94.5 ± 0.2^{a}	0.0 ± 0.0^{b}
	10	621.0 ± 25.2^{a}	93.92 ± 0.3^{a}	93.9 ± 0.3^{a}	0.0 ± 0.0^{b}
	20	287.0 ± 28.6^{ab}	64.04 ± 6.8^{b}	64.0 ± 6.8^{b}	0.0 ± 0.0^{b}
	40	218.3 ± 116.1^{b}	48.12 ± 7.6^{b}	48.1 ± 7.6^{b}	27.8 ± 6.7^{a}
	F	8.1 *	20.5 **	20.5 **	10.2*

Means ± S.E. in the same column for the same category of insecticide, followed by the same letter do not differ significantly at $P < 0.05$ (Tukey's test)). Each datum represents the mean of four replicates of 30 initial insects each. ns $P > 0.05$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Concerning the mode of action, diatomaceous earth absorbs the lipids of the insect's epicuticle and dead ensues from loss of water and desiccation (Vayias et al., 2008). Neem powder is rich in alkaloids (azadiracthtin) and other molecules like salanine and melandriol, which after ingestion, cause digestive disorders and loss of appetite (antifeedant activity) (Schmutterer, 1990). *Plectranthus glandulosus* powder contains several monoterpenes (Ngassoum et al., 2001; Nukenine et al., 2007) which could be toxic to the weevil by reversible competitive inhibition of acetyl cholinesterase by occupation of the hydrophobic site of the enzyme's active centre (Ryan and Byrne, 1988). These mechanisms are all possible in the present study apart from the antifeedant mechanism of neem seed powder which was less apparent, since up to 23 and 67% mortality was recorded within the relatively short exposure periods of 3 and 7 days, respectively.

In addition to causing adult mortality, the insecticide powders either completely hindered or significantly reduced progeny emergence, indicating their potential for use in the management of the maize weevil. Kavallieratos et al. (2005) reported that SilicoSec was effective against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) in maize, wheat, barley, oats and rice. In previous studies, *P. glandulosus* powder and neem seed powder also greatly inhibited progeny production of *S. zeamais* (Nukenine et al., 2007; 2010b; Chouka, 2007). Reductions in insect population growth rate, percentage damaged grains, percentage weight loss and percentage germination losses were observed in all the treatments although *P.*

glandulosus powder did not affect insect population growth and the rate of seed germination. Stathers et al. (2000; 2002) reported that diatomaceous earth products did not have negative effects on seed germination. Diatomaceous earths are very promising alternatives to traditional pesticides, as they have low mammalian toxicity, low or zero residual effects in food and are effective against the target pests (Korunic, 1988). However, at present their cost and lack of availability is preventing their widespread use in developing countries (Stathers et al., 2000; 2002). In some communities in Africa, grains with holes were unacceptable for either planting or food and were discarded by consumers (Dunkel et al., 1986). For the neem and *P. glandulosus* powders, the dosage of 20 g/kg reduced grain damage by almost 100 and 30%, respectively. At that powder application rate, F1 progeny emergence was reduced by roughly 100% and over 50% respectively for neem seed powder and *P. glandulosus* powder. This indicates that at the practicable powder content of 20 g/kg, there will be few or no holes on grains, from feeding or emergence, and that the net losses in stored maize caused by *S. zeamais* to subsistence farmers may be greatly reduced by using neem seed powder. The neem plant is widely available in the northern parts of Cameroon, but the use of neem products in stored product protection is insignificant in the country. Therefore, the presented data encourage the production and promotion of insecticidal products from this plant in the country.

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Australian national residue survey – closing the loop on pesticide residue risk management for Australian grain

Reichstein, I.*#[1], Healy, K.[1], James, A.[1], Murray, B.[2]
[1] National Residue Survey, Australian Government Department of Agriculture, Fisheries and Forestry, PO Box 858, Canberra, ACT, 2601, Australia. Email: ian.reichstein@daff.gov.au
[2] WJ Murray Consulting Services, 22 Thornley Close, Ferntree Gully, Victoria, 3156, Australia

* Corresponding author
# Presenting author

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Abstract

Australia exports a major proportion of its agricultural production and is highly dependent on maintaining and developing access to, and competitiveness in, export markets. To preserve Australia's status as a provider of high quality grain, the majority of Australian primary producers rely on pesticides to protect their crops from pests and diseases, particularly in post-harvest situations. The Australian Pesticides and Veterinary Medicines Authority (APVMA) supports Australian agriculture by registering and allowing the supply of safe and effective animal health and crop protection products.

A residue risk management continuum is established when the effectiveness of chemical registration and control of chemical use regulations is assessed through residue monitoring programs. Programs assess good agricultural practice and provide traceback capacity to investigate areas of concern. Risk communication provides opportunities for continuous improvement. In the early 1960s, the Australian Government established a non-regulatory body, the national residue survey (NRS). In 2008-2009, random monitoring programs were conducted for over 50 commodities (21 grains, five horticultural commodities, 11 fish species, 12 animal species, honey and egg with over 20,000 samples collected for analytical testing. The NRS grain residue monitoring program is presented as a case study of the residue risk management continuum demonstrating to overseas markets the high level of residue integrity of Australian grain. Over 4,000 grain samples are collected and analysed per annum. Most of the samples are collected in the bulk export program where samples are collected from every hatch of every ship loaded at the seventeen Australian grain export terminals. The chemical screens have expanded beyond the multi-residue screen (MRS) insecticides, fungicides and herbicides, to include phosphine, additional herbicides (not included in the MRS), heavy metals and mycotoxins. In its current form, the NRS grains program provides 15 years of residue testing data which demonstrates a very high degree of conformance with Australian MRLs and the import tolerances of overseas trading partners. In addition, trends in residue testing data demonstrate a decline in the frequency of residue detections and the levels of residue detected. To be confident that residue testing results meet the requisite standards, the reliability of the Australian analyses must be assured. The NRS laboratory performance evaluation system has been developed to provide that assurance, using a range of proficiency tests and other techniques in the selection of laboratories for NRS work. Residue testing results are reported against both Australian MRLs and the international MRLs which apply in the relevant export market. NRS maintains databases of overseas MRLs and compares its residue testing results of exported commodities against those standards. Grain marketers receive certificates of analysis, in the form of NRS residue testing results, for each shipment prior to arrival at the overseas market.

Keywords: Grain, Pesticide, residue, monitoring program, residue risk management

1. Introduction

Pesticides (insecticides, fungicides, herbicides and fumigants) are widely used in Australia as an essential tool in broadacre farming. Pesticides are used at all stages of crop production from seeding through to post-harvest protection from stored grain pests, to maximise production and improve productivity from weed control and control of micro- and macro-organism pests. Pesticides are registered for use by the Australian Pesticides and Veterinary Medicines Authority (APVMA) which takes into account the potential risks from the incorrect use of pesticides. The APVMA has responsibility to ensure that pesticide products registered for use in Australia are suitably formulated and properly labelled and, when used according to instructions, are safe to the plant host, the user, consumers and the environment;

efficacious (that is, the product does the job it claims it shall do); and not unduly prejudicial to trade. Registration helps to ensure that unacceptable residues from the chemicals used in agriculture do not appear in food for human or animal consumption in Australia or in Australia's export markets. As part of the registration process, APVMA recommends maximum residue limits (MRLs). MRLs reflect the maximum residues of pesticides which may occur in foods when registered chemicals are used in accordance with Good Agricultural Practice (GAP). Good Agricultural Practice is defined as the nationally recommended, authorised or registered use-pattern of chemicals, that is necessary for effective and reliable pest control under actual conditions at any stage of the production, storage, transport, distribution and processing of food commodities and animal feed. MRLs are not health standards *per se*, however they are assessed against health standards, to ensure that foods containing residues at the MRL are fit for human consumption. These considerations apply to the current methodology used to set Australian MRLs and the MRLs of other countries, as well as MRLs set by the Codex Alimentarius Commission.

Residue monitoring programs are an essential component of the Australian national residue risk management framework. The closed loop approach ensures that pesticide registration and farm chemical use is audited through residue monitoring programs which in turn provide feedback with information supporting the original registration decision. Traceback investigations on non-compliant residues provides additional information which allows regulators to address problems and minimise reoccurrences.

The key objective of Australia's national grain residue testing program is to audit the effectiveness of regulatory controls that are in place to ensure that pesticides applied in broadacre farming are used according to GAP. This provides assurances to the grain industry that farming practices are appropriate and that pesticide residues levels in grain are compliant with relevant MRLs, do not pose a threat to human health and have no adverse impact on trade.

The specific pesticides registered for use in each country and GAP for the use of those chemicals varies from country to country on the basis of differing agricultural pest pressures and diseases and their significance in food production. GAP is also affected by the nature of agricultural business, geography and environmental considerations. Therefore, variances in MRL values for particular chemical-commodity combinations from country to country can be expected and commodity exporters need to be aware of those differences.

The grain industry is becoming increasingly aware of overseas MRLs and varying overseas marketing requirements when considering pesticide use in crops and in post-harvest situations. Given the high importance placed on food safety and market access, the National Residue Survey (NRS) in cooperation with the grain industry have established a comprehensive national grain residue monitoring program to verify the pesticide residue integrity of Australian grain from farm receival to point of export. The NRS Export Grains Residue Testing Program involves grain sampling at container and bulk out-turn as a final check of residue integrity prior to export and is considered the primary residue testing program for Australian grain. The NRS is a government agency, located in Canberra within the Australian Government Department of Agriculture, Fisheries and Forestry. Since 1993, the NRS has operated on the basis of full cost recovery.

In 1993, the Grains Council of Australia, in full consultation with the grain industry, elected to establish a 0.015% ad valorem grain grower levy to fund the operation of the NRS Grains Residue Testing Program. The grain industry requested that the program be operated primarily as a market access project focussed on collecting grain samples at the point of export and at the point of domestic receival for processing.

2. Materials and methods

2.1. Pesticide residue management in Australia grain

The responsibility, in regard to pesticide residue matters in grain, is shared between the Commonwealth and State governments and the grain industry. The Commonwealth government's responsibilities cover registration of pesticides, establishment of MRLs and the inspection and phytosanitary certification requirements for export. State government agencies are responsible for the control-of-use of pesticides and the inspection and certification requirements for the domestic market. Grain marketers and bulk

handling companies (BHCs) within Australia conduct rigorous testing from the first point of farm receival through to grain aggregation into bulk storage facilities. BHCs monitor all grain quality parameters including pesticide residues.

2.2. The grains pesticide residue testing program

The NRS Grains Program is designed to provide an unbiased estimate of the frequency of residues in Australian grain as a whole. The program requires randomised sampling of grain from throughout Australia from as many grain streams as is possible. The NRS Grains Program is structured to give due consideration to the number of different grain commodities and the many export and domestic grain streams. The NRS Grains Program currently consists of the following series of sub-programs:

- Bulk Export - All 17 grain exports terminals with a representative grain sample collected from every hatch loaded on every ship leaving Australia
- Export Container - Representative grain samples collected whilst grain is loaded into bags and shipping containers destined for export
- Domestic (Milled Products) - Samples of wheat, maize, soybean, triticale and corresponding milled derivatives are collected.
- Domestic (Maltsters) - Samples at grain receival to malting barley plants.
- Domestic (Oat Processors) - Samples collected at grain receival to rolled oat processing plants
- Domestic (Oilseed Crushers) - Samples collected at grain receival to crushing plants.
- Domestic (Feed mills) - Samples collected at grain receival to stockfeed-mill site.
- Domestic (Feedlots) - Samples collected at grain receival to cattle feedlots

All tradable grains are included in the NRS Grains Program. These are wheat (including durum), barley, oat, sorghum, maize, triticale, canola, soybean, sunflower, safflower, linseed, chickpea, field pea, cow pea, lentil, lupin, mung bean, faba bean, navy bean, vetch and pigeon pea. This paper focuses on the NRS Export Grain Program covering bulk shipments.

2.3. Sampling methodology – bulk export program

Approximately 3,000 samples of export grain are collected annually on the basis of a sample from every hatch from every bulk shipments of grain loaded at the 17 export terminals around Australia. The sample numbers are therefore determined by shipping throughput. Grain is collected at outturn at a rate of 2.25 L per 33 t using automated sampling equipment and a representative composite sample is collected from each hatch. Sample collectors are provided with a sample collection and despatch manual, sample forms, plastic sample bags, security satchel, freight satchels addressed to NRS contract laboratory and reply paid envelopes for return of the 'original' form.

2.4. Analytical methodology

A 20-g portion of each grain sample is subjected to a 30 minute sonication extraction in acetone with 12 h soaking. All extracted samples are applied to the contract laboratory's LCMSMS technique. The analytical parameters are as follows: Flow rate: 0.22 mL min^{-1}, Oven temp: 40°C, Column: Luna 3*u* PFP (150 x 3.00 mm), Mobile phase A: 0.1% FA in water, Mobile phase B: 0.1% FA in MeOH: ACN (2:3), LOQ: 0.01 mg kg^{-1}. If required, the laboratory utilises its confirmatory LCMSMS technique for purposes. The analytical parameters are as follows: Flow rate: 0.40 mL min^{-1}, Oven temp: 40°C, Column: Luna 3*u* PFP (150 x 3.00 mm), Mobile phase A: 0.2% FA in 10 mM NH_4OAc, Mobile phase B: 0.1% FA in MeOH: CAN (2:3), LOQ: 0.01 mg kg^{-1}.

2.5. Analytical screen

All grain samples are subjected to a multi-residue screen which is based on a risk profile that considers the following criteria:

- all pesticides registered for use in Australia on grain;
- known use patterns including timing of application in the growing season and repeat applications
- potential for residues in grain commodities;
- availability of suitable analytical methods, testing capacity and laboratory testing proficiency arrangements; and
- perceived risks to international trade and overseas market concerns.

- The analytical screen is as follows:
- Post-harvest grain protectants: chlorpyrifos-methyl, fenitrothion, pirimiphos-methyl, dichlorvos, methoprene, deltamethrin, spinosad
- Organophosphate insecticides: azamethiphos, chlorfenvinphos, chlorpyrifos, diazinon, dimethoate, ethoprofos, malathion, methacrifos, omethoate, phosmet, profenofos, terbufos, trichlorfon
- Synthetic pyrethroids insecticides: bifenthrin, bioresmethrin, cyfluthrin, cyhalothrin, cypermethrin, fenvalerate, permethrin, phenothrin, piperonyl butoxide
- Other insecticides: acetamiprid, amitraz, carbaryl, diflubenzuron, endosulfan, fipronil, imidacloprid, indoxacarb, methomyl, thiodicarb, pirimicarb, pyriproxyfen, triflumuron.
- Fungicides: azoxystrobin, captafol, carbendazim, cyproconazole, difenoconazole, epoxiconazole, etridiazole, fluquinconazole, flutriafol, hexaconazole, iprodione, penconazole, propiconazole, prothioconazole, tebuconazole, thiabendazole, triadimefon, triadimenol, triticonazole
- Herbicides: 2,4-D, atrazine, bromoxynil, carfentrazone-ethyl, chlorsulfuron, clethodim, clodinafop-propargyl, clopyralid, dicamba, diflufenican, diuron, iodosulfuron-methyl, MCPA, metolachlor, metosulam, metsulfuron-methyl, pendimethalin, picloram, propyzamide, simazine, tralkoxydim, triasulfuron, triclopyr, trifluralin
- Environmental contaminants: aldrin, chlordane, DDT, dieldrin, endrin, HCB, HCH, heptachlor, lindane, methoxychlor, mirex, oxychlordane
- Randomly selected grain samples are also subjected to additional analytical screens which include analytes not able to be included in the MRS. These analytical screens are as follows:
- Fumigants: phosphine
- Additional herbicides: amitrole, diclofop-methyl, diquat, fenoxaprop-P-ethyl, flamprop-M-methyl, fluazifop-P-butyl, glufosinate, glyphosate, haloxyfop, paraquat
- Heavy metals: cadmium, lead, mercury

2.6. Analytical laboratory proficiency

NRS awards analytical laboratories contracts on the basis of: performance in a pre-requisite NRS proficiency testing round; assessment of laboratory management; National Association of Testing Authority accreditation; accreditation for the relevant analytical test; quality assurance and control systems; previous performance; and value for money. The NRS laboratory performance evaluation activities are designed to maintain an up-to-date and continuing assessment of the proficiency of laboratories analysing samples for NRS. In simple terms, proficiency testing involves sending verified residue-free grain samples, which have been spiked with known concentrations of pesticides, to analytical laboratories which have indicated interest in tendering for the NRS Grains Program laboratory contract. Analytical laboratories must demonstrate the capacity to identify all spiked pesticides and quantify to 85 to 115 percent of the spike level. Ongoing proficiency of contract laboratories is closely monitored by NRS through a structured quality assurance program that includes biannual proficiency tests, bimonthly check samples, random audits and the use of 'blind' samples. Blind samples contain incurred residues and are indistinguishable from normal grain samples. These blind samples are sent to contract laboratories through the routine courier system in place for normal grain samples. Failure to meet the required standard during ongoing proficiency testing can result in the termination of a laboratory contract.

2.7. Reports

The NRS is bound by Australian privacy legislation which stipulates that detailed specific residue testing data must only be disseminated to the grain marketer and the grain handler. By agreement with the grain industry, NRS is required to provide a certificate of analysis within 14 d of an export sample being collected. This timeframe takes into account sample courier from grain establishment to the contract laboratory and a laboratory turn-around time of three working days. In addition, NRS publishes a Export Grains Program results reports each financial year. Each year, the NRS published its Annual Report which covers all residues testing program managed by NRS. These Annual Reports are available on the NRS website (www.daff.gov.au/nrs).

3. Results

As shown in Table 1, 3012 grain samples were collected for analyses during the 2008-2009 financial year from throughout Australia as part of bulk and container export residue testing programs. In 2008-2009, all bulk export grain samples were 100% compliant with relevant Australian Standards. Overall, the export container grain samples were 98.2% compliant with the relevant Australian standards. A Phosphine Residue Testing Program has been conducted since 2002 with no residues detected in export grain samples above the Australian MRL. There has been a progressive decline in the frequency of violative residues detected in Australian grain exported in bulk shipments as shown in Table 2. The table illustrates the very high compliance rates against Australian standards. These figures provide sound evidence that pesticide use in-crop and post-harvest applications are in accordance with good agricultural practice as specified on the pesticide chemical product label and instructions for use. Tighter marketing controls and improved grain storage facilities have contributed to this nation-wide trend towards residue-free exported grain. The introduction of smaller production volume grains into the export container program in 2006 has had a minor impact on the compliance rate trend shown in Table 3. The grain industry and NRS expect a return to 100 percent compliance when traceback investigations, education programs and quality assurance feedback mechanisms with grain growers take effect.

Table 1 NRS Export Grains Program for 2008-2009.

Commodity	Bulk export samples	Container export samples
wheat	1684	258
barley	505	44
sorghum	246	1
Other cereals	15	16
oilseeds	134	31
pulses	37	41
TOTAL	2621	391

Table 2 Residue violations in bulk export grain from 1996-2009.

Year	Samples	>MRL	Compliance %
1996-97	5746	22	99.6
1997-98	4420	20	99.7
1998-99	4972	6	99.9
1999-00	4758	13	99.8
2000-01	4559	2	99.9
2001-02	4436	0	100.0
2002-03	3233	0	100.0
2003-04	3822	0	100.0
2004-05	3659	2	99.9
2005-06	2953	0	100.0
2006-07	2085	0	100.0
2007-08	2055	0	100.0
2008-09	2621	0	100.0

Table 3 Residue violations in container export grain from 2004-2009.

Year	Samples	Compliance %
2004-05	77	100.0
2005-06	89	100.0
2006-07	168	100.0
2007-08	565	99.6
2008-09	391	98.2

When a pesticide residue is detected in a grain sample that is above the relevant Australian MRL, the contract laboratory immediately notifies NRS. Under NRS-laboratory contract arrangements, the contract laboratory is required to retest the grain sample to confirm the first result. If confirmation is received, the relevant Australian state or territory government and the grain owner/handler and marketer are notified of the residue violation. Should the need arise, the NRS Export Grains Program includes a traceback investigation function which allows State/Territory government officers, in cooperation with the particular grain company, to conduct an examination of any residue detection in grain over the MRL. The investigators trace the grain sample back through transporter to the property of origin to determine the cause of the residue violation. Subsequent actions depend on both the chemical detected, the levels detected and the commodity in which it is found, and are specified by the relevant state and territory government authority legislation. Action can very from simple advice to the grain grower in the case of a minor problem to legal action where the residue violation has resulted from gross misuse of an agricultural chemical. NRS is provided with report of the traceback investigation and general details are provided in annual reports to industry. NRS forwards residue monitoring results along with traceback investigation report to the APVMA to assist existing chemical reviews processes and close the risk management framework loop.

4. Conclusions

The approach taken by the Australian grain industry to manage pesticide residues has evolved over the past 17 years. The consultative and cooperative framework established between the APVMA, NRS, state government departments of primary industries, Grains Council of Australia and grain marketing organisations has facilitated enhancements to the comprehensiveness of the NRS Grains Program. The expansion of the NRS Grains Program to include all tradable grains and to cover all trading streams has helped raise the program's profile both within Australia and with trading partners. Moreover, the move to assessing and reporting residue testing results against relevant international MRLs, in addition to the standard checks against Australian standards, has provided Australian grain marketers with a complete set of information with which to make appropriate grain marketing decisions. The grain industry has progressively become more involved in managing pesticide residue issues and residue testing data provided by the NRS Grains Program. This has assisted the development of on-farm quality assurance programs and enabled industry to demonstrate long term improvements in pesticide residue management. The residue testing results derived from the NRS Grains Program over the past 12 years and the integration of this information into market access and quality assurance initiatives gives strong guarantees that Australia's grain contains very low and ever declining levels of pesticide residues.

A synergistic mixture of diatomaceous earth and deltamethrin to control stored grain insects

Korunic, Z.*[1], Rozman, V.#[2]
[1] Diatom Research and Consulting Inc., 14 Tidefall Dr. Toronto, ON, M1W 1J2, Canada.
Email: zkorunic@rogers.com
[2] Faculty of Agriculture in Osijek, University of J. J. Strossmayer in Osijek, Trg Sv. Trojstva 3, 31100 Osijek, Croatia

* Corresponding author
# Presenting author

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Abstract

In order to mitigate the negative effect of diatomaceous earth (DE) on bulk density and grain flowability, DE was mixed with other insecticides. This paper investigates the efficacy of a mixture of DE and deltamethrin against Sitophilus zeamais, the maize weevil, *Rhyzopertha dominica*, the lesser grain borer, and *Tribolium castaneum*, the red flour beetle. Five mixtures of DE and deltamethrin were prepared in the laboratory containing the same quantity of DE and different concentrations of the active ingredient of deltamethrin. The ratio of DE and deltamethrin in formulations were: DE 1 part: deltamethrin 0.00025, 0.00050, 0.00075, 0.0010 and 0.00125 parts. Co-toxicity and Co-efficient values higher than 100 (S. zeamais 170–386, R. dominica 188–601, and *T. castaneum* 157–285) indicated synergism between DE and deltamethrin.

Keywords: Diatomaceous earth, Deltamethrin, Ready to use mixture, Co-toxicity, Co-efficient, Synergism, Grain insect pests

1. Introduction

Insects infesting grain after harvest cause economic loss to producers and the grain and food industry. During the past few decades application of synthetic pesticides to control agricultural stored-products insect pests has been a standard practice. However, with the growing evidence regarding detrimental effects of many of synthetic pesticides on health and environment, the grain industry wants to reduce the use of synthetic pesticides because of insecticide deregulation, resistant populations and consumer concerns over insecticide residues. Therefore, there is a need to evaluate alternatives to conventional synthetic pesticides.

Diatomaceous earth (DE) is a natural product registered in some countries for use directly on stored grain and in empty grain stores to control insects (Korunic, 1998; Subramanyam and Roesli, 2000). The main advantages of DEs are its low-toxicity to mammals and its stability. Amorphous SiO_2, the main active ingredient in natural DE, is considered Generally Recognized as Safe (GRAS), and is a registered food additive (21 CFR 182.90, 182.1711) in the United States. Several tests with DE have shown there were no effects on end use quality in baking, malting or pasta production (Aldryhim, 1990; Korunic et al., 1996).

However, DE does have some disadvantages that hinder its widespread use (Fields, 1999). DE significantly reduces the bulk density (test weight) and flowability of grain. Because of high concentrations needed to control insect pests it is dusty to apply and it has a low efficacy against some insect species. High grain moisture contents and high air relative humidity significantly reduce its efficacy (Fields and Korunic, 2000). Currently used DEs are applied between 100 (only against *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), the most sensitive insect against DE) and 1000 ppm (Subramanyam and Roesli, 2000). Rates above 300 ppm cause a considerable bulk density reduction (test weight) and significant reduction in grain flowability (Korunic et al., 1998; Korunic, 1998). Also, the presence of high DE concentration with increased quantity of crystalline silica may cause respiration problems (silicosis) to workers after long exposure. Hence, it is essential for researches to evaluate the use of novel DE formulations that are effective against insects at lower dose rates.

Deltamethrin is used in stored-grain protection, plant protection, livestock protection and public health. Deltamethrin has a very broad spectrum of control, and is effective in controlling *Rhyzopertha dominica*

(F.) (Coleoptera: Bostrichidae) resistant to organophosphate (OP) insecticides, but not *Sitophilus* spp. (Coleoptera: Curculionidae) and *Tribolium* spp. (Coleoptera: Tenebrionidae) (Daglish et al., 1995). Deltamethrin resistance has also been reported for *R. dominica* (Lorini and Galley, 1996).

Several studies using insects of stored products have shown synergism between OPs and pyrethroids (Daglish eta al., 1995; Dalglish, 1998). To date there are no published data regarding synergism between DE and deltamethrin. The mixture of DE and deltamethrin was developed to mitigate the disadvantages of DE and reduce deltamethrin residues in grains. It addition to efficacy benefits, the combination of different modes of action could also reduce the development of resistance. The objective of this research was to determine if mixtures of DE and deltamethrin acted synergistically against three storage insects, *Sitophilus zeamais* Motschulski, *R. dominica* and *Tribolium castanum* (Herbst).

2. Materials and methods

Mixed-sex adult *S. zeamais*, *R. dominica* and *T. castaneum*, 7 to 21 d old, were used for all experiments. *Sitophilus zeamais* and *R. dominica* were cultured on wheat with approximately 14% m.c. *Tribolium castaneum* was cultured on white flour with 5% brewer's un-activated yeast. Rearing was conducted at 30 $\pm 1^0$C and 70±5% r.h.

Uninfested clean Canadian Western Hard wheat with 14.4% m.c. was used in the experiment. In each replication there was 100 g of clean whole wheat kernels in 500 ml glass jars sealed with filter paper. The DE (Celite Corporation, USA) contained 89% SiO_2 with median particle size of 10 µm and specific gravity of 2.2. Moisture (as shipped) was 3% and crystalline silica was less than 0.1%. It contained Al_2O_3 4%, Fe_2O_3 1.7%, CaO 1.4%, Na_2O 1.2%, MgO 0.6% and K_2O 0.5% (Technical Data, Celite Corporation, USA). Delta tech (AgrEvo USA Company, Wilmington, DE, USA) is a powdered product contained 99% deltamethrin as the active ingredient. A determined quantity of powder was dissolved in a solvent and emulsifier (EPA List of Other Pesticide Ingredients Inert – List 4) and in piperonyl butoxide (PBO). The ratio of deltamethrin and PBO was 1:4. These concentrated emulsions contained different quantities of deltamethrin were mixed with DE for grain dusting and used alone for grain spraying.

Five formulations of mixtures of DE and deltamethrin emulsion were prepared in the laboratory containing one part of DE and different quantities of deltamethrin (0.00025, 0.00050, 0.00076, 0.0010 and 0.00125 parts). Deltamethrin alone was used in the following concentrations (ppm): 0.05, 0.1, 0.2, 0.3 and 0.4; DE alone (ppm): 100, 200, 300, 400 and 500; and mixtures of DE and deltamethrin (ppm): 50, 75, 100, 125 and 150. The various formulations at different concentrations were added to grain in each of the large jars containing 300 g of wheat. Jars were tightly closed with lids and thoroughly shaken by hand for one min. The grain from each jar was divided into three jars (sub-replications) with 100 g each. Untreated grain serves as control (0 ppm). Then 50 unsexed 7- to 21-d-old adults of either *S. zeamais*, *R. dominica* or *T. castaneum* were added into each jar. There was only one species per jar.

To test deltamethrin alone, deltamethrin at various doses was sprayed with a 0.6 ml emulsion on 300 g of wheat and thoroughly mixed in a tightly closed jar. After mixing, grain from each jar (300 g) was divided into three jars (sub-replications) with 100 g each. Grain sprayed with water served as control (0 ppm). After 6 d the entire contents of each jar was sieved to separate insects from the grain. The number of dead and live adult insects was counted. The corrected mortality was calculated according to Abbott's formula (Abbott 1925), and LD_{50} and fiducial limits (with 95% CIs) and X^2 analysis according to probit software.

The joint action of DE and deltamethrin individually in the insecticide mixture was determined on the basis of LC_{50} values of each insecticide and was determined on the basis of Co-toxicity Co-efficient (CTC) values of the mixture (Sun and Johnson, 1960). DE served as a standard insecticide. If the values of the CTC are higher than 100, this is an indication that the substances in the mixture have a synergistic action (Sun and Johnson, 1960; Ramasubramanian and Regupathy 2003).

3. Results

All mixtures with all insects show synergism (Tables 1-3). The dosages of both substances in the mixture are significantly reduced in a comparison with dosages when the substances are used alone. For example, based on the LD_{50} results, deltamethrin dosages in formulation D are reduced for seven, nine and four times and dosages of DE are reduced for 10, 20 and 11 times in controlling *S. zeamais*, *R. dominica* and

T. castaneum, respectively, in the comparison with dosages of DE and deltamethrin applied alone (Tables 1-3).The LD_{50} for *S. zeamais* for DE combined with deltamethrin is from 40 to 167 ppm compared to 376 ppm when DE is used alone. Similarly for the same species, the LD_{50} for deltamethrin combined with DE is 0.042 to 0.062 ppm compared to 0.28 ppm of deltamethrin used alone (Table 1).

Table 1 Co-toxicity Co-efficient (CTC) of ready to use insecticide mixture of DE and deltamethrin against *Sitophilus zeamais.*

Formulation	Ratio DE: deltamethrin	LC_{50} (ppm)	Fiducial limit 95% (ppm)	Deltamethrin alone and in mixture LC_{50} (ppm)	DE alone and in mixture LC_{50} (ppm)	Regression equation*				
						m	b	r	X^2	CTC
DE	1:0	376	353-404	-	376	39005	-5.05	0.98	17	-
deltamethrin	0:1	0.29	0.25-0.33	0.29	-	2.04	+6.11	0.95	18	-
Formulation A.	1:0.00025	167	160-176	0.042	150	5.08	-6.31	0.97	19	170
Formulation B.	1:0.00050	125	118-133	0.062	113	3.79	-2.96	0.94	20	182
Formulation C.	1:0.00075	66	61-71	0.049	59	3.15	-0.74	0.97	20	288
Formulation D.	1:00100	42	39-46	0.042	38	3.24	-0.27	0.96	18	386
Formulation E.	1:00125	40	37-43	0.050	36	3.68	-0.92	0.96	6	353

*Y = mx + b

Table 2 Co-toxicity Co-efficient (CTC) of ready to use insecticide mixtures of DE and deltamethrin against *Rhyzopertha dominica.*

Formulation	Ratio DE: deltamethrin	LC_{50} (ppm)	Fiducial limit 95% (ppm)	Deltamethrin alone and in mixture LC_{50} (ppm)	DE alone and in mixture LC_{50} (ppm)	Regression equation*				
						m	b	r	X^2	CTC
DE	1:0	468	442-495	-	468	3.88	-5.37	0.93	19	-
deltamethrin	0:1	0.23	0.20-0.28	0.23	-	1.40	+5.88	0.28	18	-
Formulation A.	1:0.00025	166	151-190	0.041	149	1.40	-0.01	0.94	6	188
Formulation B.	1:0.00050	166	151-190	0.083	149	2.25	-0.01	0.94	6	270
Formulation C.	1:0.00075	45	38-54	0.033	40	1.25	+2.94	0.96	9	419
Formulation D.	1:00100	26	19-32	0.026	23	1.02	+3.55	0.99	6	601
Formulation E.	1:00125	31	26-36	0.038	28	1.34	+3.00	0.99	5	433

*Y = mx + b

Table 3 Co-toxicity Co-efficient (CTC) of ready to use insecticide mixtures of DE and deltmethrin against *Tribolium castaneum.*

Formulation	Ratio DE: deltamethrin	LC_{50} (ppm)	Fiducial limit 95% (ppm)	Deltamethrin alone and in mixture LC_{50} (ppm)	DE alone and in mixture LC_{50} (ppm)	Regression equation*				
						m	b	r	X^2	CTC
DE	1:0	1142	1076-1222	-	1142	3.85	-6.78	0.99	7	-
deltamethrin	0:1	0.47	0.45-0.49	0.47	-	5.33	+6.72	0.99	8	-
Formulation A.	1:0.00025	453	426-487	0.11	408	3.58	-4.51	0.97	5	157
Formulation B.	1:0.00050	183	176-196	0.09	165	6.14	-8.89	0.99	3	283
Formulation C.	1:0.00075	180	173-186	0.13	162	6.55	-9.77	0.97	4	227
Formulation D.	1:00100	118	112-124	0.12	106	4.13	-3.56	0.95	21	285
Formulation E.	1:00125	132	123-142	0.13	119	3.23	-1.86	0.96	12	217

*Y = mx + b

The LD_{50} of *R. dominica* for DE combined with deltamethrin is from 26 to 166 ppm compared to 468 ppm when DE is used alone. Similarly for the same species, the LD_{50} for deltamethrin combined with DE is 0.026 to 0.082 ppm compared to 0.23 ppm of deltamethrin used alone (Table 2).The LD_{50} of *T. castaneum* for DE combined with deltamethrin is from 118 to 453 ppm compared to 1142 ppm when DE is used alone. Similarly for the same species, the LD_{50} for deltamethrin combined with DE is 0.09 to 0.13 ppm compared to 0.47 ppm of deltamethrin used alone (Table 3).

4. Discussion

Several DE formulations are now commercially available, and many studies document that their effective action against a wide range of stored-product insect species (Subramanyam and Roesli, 2000). In order to reduce the dosages of DE it can be mixed with other compounds such as silica gel, dry honey, un-activated yeast and sugar to increase the efficacy (Korunic and Fields, 1995; Subramanyam and Roesli, 2000). However, high doses of these mixtures still have a significant negative effect on grain bulk density and flowability (Korunic et al., 1998). One of the possible solutions to the implications that are caused by the use of DE in high doses is the combined use of DE with other reduced-risk methods. Such methods include extreme temperatures (Fields et al., 1997), grain cooling with a DE surface treatment (Nickson et al., 1994), in a mixture with entomopathogenic fungi (Michalaki et al., 2007) or with synthetic insecticides (Arthur, 2004), or in a mixture with plant extracts and a bacterial metabolite (Korunic, 2007; Athanassiou and Korunic, 2007). Experimentation with these components often revealed a synergistic or enhanced effectiveness effect (Lord, 2001; Korunic, 2007; Athanassiou and Korunic, 2007).

The combination of insecticides from different groups may overcome resistance and the weak effectiveness of pyrethroids against some stored-grain insect species. Several OPs have been mixed with pyrethroids (Arthur, 1994; Daglish, 1998). However, concentrations used in these mixtures were still high and often half the recommended concentration of each compound was used in the mixture. Despite the increased efficacy and lower concentrations in some cases, high concentrations of both OP and pyrethroids in mixtures could cause residue issues with consumers.

The main advantage of a DE + deltamethrin mixture is similar effectiveness against tested insect species which is not the case when DE and deltamethrin are applied alone. The new mixture generated 95 to 100% mortality of all species exposed to approximately 100 ppm containing 0.1 ppm of deltamethrin and 90 ppm of DE (Korunic, unpublished data). This was well below the concentrations of DE + pyrethroids or OPs + pyrethroids. One probable cause of this synergism is the combination of different modes of action: physical (desiccation) and chemical (toxicity). Insects desiccate from the DE and could become weaker and less resistant to the toxic action of deltamethrin. Therefore, lower concentrations of deltamethrin in combination with DE are needed for control. Deltamethrin and PBO are used at approximately 0.5 to 1 ppm and 2 ppm, respectively, for long-term protection of stored grain. In most cases, DE formulations are registered at concentrations of 500 to 1000 ppm. Another advantage of using the new developed mixture of DE and deltamethrin is acceptable efficacy in grain treated with very low doses of both active ingredients. These concentrations are much lower in comparison with concentrations required for effective control when these two insecticides are applied either alone or as mixtures with other insecticides (Daglish, 1998; Athanassiou, 2006), in a mixture with entomopathogenic fungi (Michalaki et al., 2007). These concentrations are similar in efficacy to concentrations of the mixture of DE + chlorpyrifos-methyl + deltamethrin + PBO (Arthur, 2004), DE + abamectin (Athanassiou and Korunic, 2007) or DE + plant extract (Korunic, 2007). However, the substances in the mentioned mixtures are not yet registered as grain protectants in any country or perhaps only a few. Deltamethrin and DE are registered as grain protectants in many countries, which could simplify the process of the adoption of this mixture as a grain protectant.

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Field trials with the diatomaceous earth SilicoSec® for treatment of empty rooms and bulk grain

Schöller, M.*#, Reichmuth, C.
Julius Kühn-Institut - Federal Research Centre for Cultivated Plants, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Section Stored Product Protection; Königin-Luise-Str. 19, D-14195 Berlin, Email: Matthias.Schoeller@jki.bund.de

* Corresponding author
# Presenting author

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Abstract

Diatomaceous earths (DE) are fossil diatoms (phytoplankton) that contain silicon dioxide (SiO_2), the particles absorb the waterproof lipids from the arthropod cuticle resulting in death through desiccation. The DE SilicoSec® is registered in Germany. The field trials presented here were performed in order to determine (1) the efficacy of SilicoSec® under temperate Central European climatic conditions, (2) the distribution in empty rooms and (3) the possible effect of different surface materials. For empty room treatment, a 41 m² storage room was treated with 20 g/m², the total surface treated including walls was 145 m². At 19 sampling points the amount of DE on the floor was determined. Distinctly less DE attached to the walls compared to the floor, and an uneven distribution on the floor ranging from 2.6 to 49.5 g/m² with a mean±SD of 15.4±14 g/m² per sampling point was measured. Additionally, test pieces with 5 different surface types were placed in the treatment room prior to the treatment. Adult *Tribolium confusum* and *Cryptolestes ferrugineus* were placed on all surfaces at 15 to 19°C and 65-81% r.h.. Mean corrected mortality after 14 d in *T. confusum* and *C. ferrugineus* was 94% and 65%, respectively. No significantly different mortality was recorded for *T. confusum* depending on surface type, but in *C. ferrugineus* significantly less beetles (20%) died on concrete flagstone compared to natural flagstone, glazed ceramic flagging, plywood and porcelain stoneware. For bulk grain, 10 t of wheat were treated with either 0.7 kg/t or 2 kg/t DE. *Sitophilus granarius, Oryzaephilus surinamensis* and *C. ferrugineus* adults were placed in vials with treated wheat together with data loggers and placed deep within the bulk for 19 d. Corrected mortality was around 90% for all treatments except for *S. granarius* at the lower dose where 60% mortality was achieved only.

Keywords: Diatomaceous earth, Efficiency, *Cryptolestes ferrugineus, Sitophilus granarius, Oryzaephilus surinamensis*

1. Introduction

Diatomaceous earth (DE) originates from fossil diatoms (Eucaryota: Bacillariophyta. The living diatoms are single-celled algae that secrete silica, and only the silica remains after the diatoms die and decay. The insecticidal effect of DE is due to physical damage to the protective wax layer of the insect's cuticle (Zacher and Kunike, 1931; Korunic, 1998; Subramanyam and Roesli, 2000; Mewis and Ulrichs, 2001). DEs applied for pest control are amorphous dusts and are not considered hazardous to human health in contrast to crystalline dusts. DE have been reported to be effective pesticides for control of stored product pests, e.g. when applied to grain or to the floor and walls of storage facilities (Korunic, 1998; Arthur, 2003).

Several DE products are commercially available and registered for stored product protection, the product SilicoSec (Biofa GmbH) was first approved for use in Germany in 1997 (Mewis and Ulrichs, 2001). However, still few data from field trials are available. The field trials presented here were performed in order to determine the efficacy of SilicoSec® under temperate Central European climatic conditions, to estimate the distribution in empty rooms and to elucidate possible effects of different surface materials.

2. Materials and methods

2.1. Surface treatment.

The field trials were conducted in a store in Temmen, Uckermark, Brandenburg that had not been treated with synthetic chemical insecticides for several years. The storage rooms, untreated control and room for

trial, were cleaned. The treated room had a total surface including walls of 145 m² (floor: 41.12 m²). SilicoSec® was applied with the help of a sucking and forcing pump gun, at a concentration of 20 g/m² calculated for the total surface (3 kg SilicoSec® total).The amount of DE was determined at 19 sampling points on the floor. For each sampling point, a filter paper (15 cm diameter, surface 177 cm^2) and a sealable plastic bag was weighed and numbered in the laboratory. After deposition of the DE in the field trial, the filter papers were carefully transferred into the plastic bags and weighed again in the laboratory to determine the amount of DE on the filter paper.

Additionally, test pieces (15 cm x 15 cm) with 5 different surface types were placed in the treatment room prior to the treatment, i.e. concrete flagstone, natural flagstone, glazed ceramic flagging, plywood and porcelain stoneware (Fig. 1). The test pieces were placed horizontally on the floor prior to the DE treatment (Fig. 2). After treatment, plastic cages were placed on top of the pieces and glued onto the pieces. The cages had an opening for inserting the test insects, and lateral perforations (diameter 1 mm) for aeration. Adults originating from the laboratory colonies of *Tribolium castaneum* (Herbst) maintained at the Institute (50 adults per trial) and *Cryptolestes ferrugineus* (Stephens) (100 adults per trail), also from laboratory colonies, were introduced into the cages and the opening sealed with tape. After 14 d, the insects were removed from the cages, transferred to glass vials and brought to the laboratory.

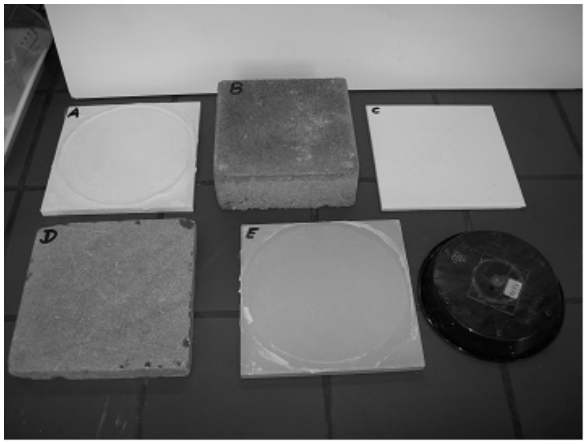

Figure. 1 Surface materials studied, (A) plywood, (B) concrete flagstone, (C) glazed ceramic flagging, (D) natural flagstone, and (E) porcelain stoneware, right to (E) plastic top for caging the insects.

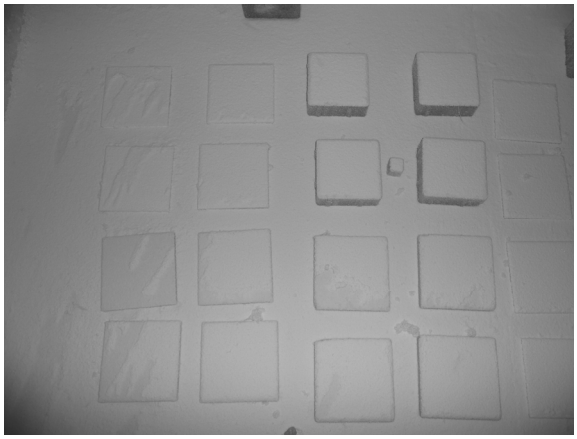

Figure. **2** Different surface materials after empty room treatment with diatomaceous earth SilicoSec in field trial, before exposing and caging of insects.

A total of 10 data loggers were distributed close to the test pieces at random on the floor. The untreated controls also received a data logger. Another logger transported together with the test insects to the laboratory. The temperature ranged between 15 and 19°C and the relative humidity between 65 and 81% r.h. during the field trial.

2.2. Grain treatment

Field trials were conducted on two farms in Germany, in Temmen-Ringenwalde, Brandenburg, and Finkenthal Güstrow, Mecklenburg-Western Pomerania, respectively. Ten tones of wheat were treated with SilicoSec on each of the farms by mixing the DE into the whole bulk. The wheat was treated with 0.7 kg/t and 2.0 kg/t in Temmen and Finkenthal, respectively. Wheat was taken from the treated bulk out of various depths and filled into 250 ml glass jars. Laboratory-reared beetles were placed into the jars onto the wheat and the jars closed insect-tight, but had a metal gauze for ventilation. A piece of string was attached to the jars, the jars were placed ca. 40 cm deep into the bulk, and the string tied to a rod in order to recover the samples. Consequently, the samples were exposed to the temperature and humidity conditions in the bulk for a period of 19 days. A data logger was placed close to each sample.

Three beetle species were tested, *C. ferrugineus*, *Oryzaephilus surinamensis* (L.) and *Sitophilus granarius* (L.). In every experiment, 100 adults of *S. granarius*, and 50 adults of *C. ferrugineus* and *O. surinamensis* were tested, respectively. For every species, ten replications and one untreated control (wheat not treated with SilicoSec) were exposed to the environmental conditions in the bulk and left there for four weeks. One untreated control was brought back to the laboratory in Berlin immediately after treatment. The mortality of the exposed adults was recorded. During the field trial, the temperature ranged between 15°C and 20°C and the relative humidity between 51 and 70% r.h. in Temmen, in Finkenthal environmental conditions were 18.5 to 24.8°C and 55.1 to 60.2% r.h.

2.3. Data analysis

For the mortality on different surface materials, the mortality counts were corrected by using Abbot's (1925) formula. The data were analysed with the help of SigmaStat-software (version 3.11.0) by using the non-parametric Kruskal-Wallis one way-analysis of variance, with insect mortality as the response variable and type of surface as main effect. Means were separated by using the Tukey-test at $p = 0.05$. Data from the results of the field trial with bulk grain were also corrected by using Abbot's (1925) formula. The GLM procedure was applied with insect mortality as the response variable and dose rate and insect species as main effects. Means were separated by using the Holm-Sidak test at $p = 0.05$.

3. Results

3.1. Surface treatment

A mean and SD of 0.027±0.25 g SilicoSec® was traced per filter paper (15 cm diameter), equalling 15.4 g/m². The distribution of SilicoSec® in the empty room fitted a normal distribution (Shapiro-Wilk-Test, $p = 0.002$) but was uneven, illustrated by the range of DE found (minimum: 0.05 g, maximum: 0.88 g) and the standard deviation. The distribution of the amount found at the 19 sampling points converted to g/m² is given in Figure 3. Calculated for all surfaces, the amount of SilicoSec® was 20 g/m² (total surface: 41.12 m²), however, the floor received much more DE compared to the walls. The maximum dosage on the floor was 73 g/m².

The different surface materials did not affect the mortality of *T. confusum* (Kruskal-Wallis One Way ANOVA on Ranks, $P = 0.427$) (Fig. 4), but had significant impact on the mortality of *C. ferrugineus* (Kruskal-Wallis One Way ANOVA on Ranks, $P = 0.034$) (Fig. 5). However, the latter was only due to the low mortality on concrete flagstone.

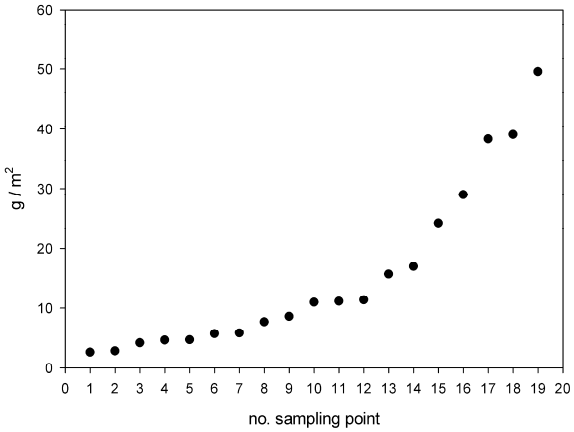

Figure. 3 Amount of DE at 19 sampling points (15 cm diameter, surface 177 cm^2) on the floor of a grain store converted to g/m².

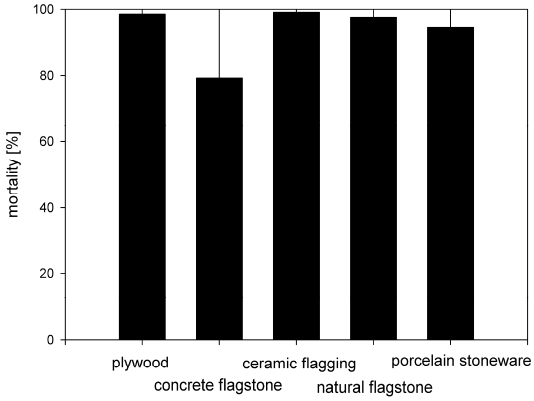

Figure. 4 Mortality of *Tribolium confusum* on five different surface types treated with 15 g/m² diatomaceous earth SilicoSec in a field trial.

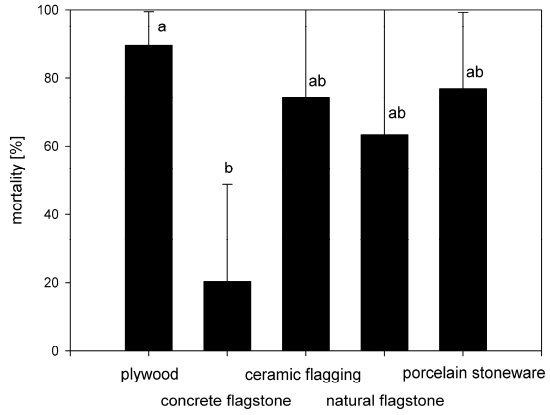

Figure. 5 Mortality of *Cryptolestes ferrugineus* on five different surface types treated with 15 g/m² diatomaceous earth SilicoSec in a field trial.

3.2. Grain treatment

The mortality of beetles in bulk grain was significantly different depending on species (P<0.001) and dosage of DE (P=0.001). There was an interaction between species and dosage (P<0.001). Combining the data of both sites, significantly higher mortality was recorded for *O. surinamensis* and *C. ferrugineus* compared to *S. granarius*, but no different mortality was found between *O. surinamensis* and *C. ferrugineus*. Comparing the two experimental sites, significantly more beetles of all species were controlled in Finkenthal (2.0 kg/t DE) compared to Temmen (0.7 kg/t DE), and specifically more *S. granarius* were controlled at 2.0 kg/t DE. However, no different mortality was found between *O. surinamensis* and *C. ferrugineus* depending on dosage.

The analysis of the mortality within the respective experimental sites revealed no difference in mortality for the three species in Finkenthal (2.0 kg/t DE) (Fig. 6) were mortality exceeded 90%. In Temmen (0.7 kg/t DE) mortality of *S. granarius* was significantly lower compared to *O. surinamensis* and *C. ferrugineus* (Figure. 7).

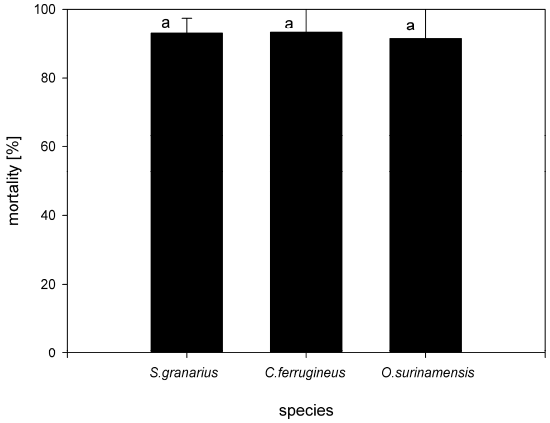

Figure. 6 Mortality of *Sitophilus granarius*, *Cryptolestes ferrugineus* and *Oryzaephilus surinamensis* in grain treated with 2.0 kg/t diatomaceous earth SilicoSec in a field trial in Finkenthal, Germany.

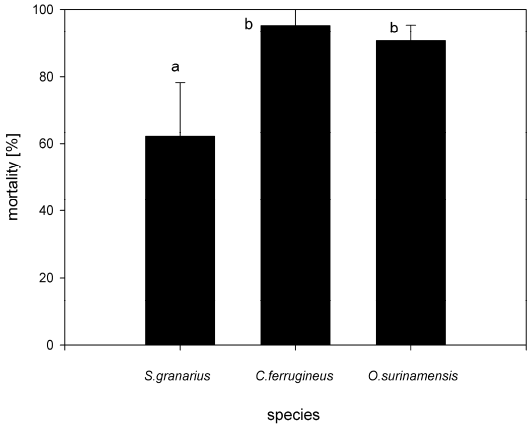

Figure. 7 Mortality of *Sitophilus granarius*, *Cryptolestes ferrugineus* and *Oryzaephilus surinamensis* in grain treated with 0.7 kg/t diatomaceous earth SilicoSec in a field trial in Temmen, Germany.

4. Discussion

The empty room treatment was effective against *T. confusum*, but only partly effective against *C. ferrugineus*. This result was not expected, because *T. confusum* was found to be less susceptible to diatomaceous earths than other stored-product beetles (Korunic, 1998; Arthur, 2000; Athanassiou et al., 2005) and *Cryptolestes* spp. are thought to be generally the most sensitive stored-product insects (Korunic, 1998). Complete control of *C. ferrugineus* at 300 ppm (300 g/t) within 24 h was achieved, while 100% mortality did not occur even after 21 days in *T. castaneum* under the same conditions (Korunic, 1998). Trials with DE produced different and often completely opposite results for stored-product insects (Korunic, 1998). On the one hand, this shows the need for further standardisation of experimental design, but conditions of temperature, humidity and substrate also have to be carefully examined. For the case of *T. confusum* and *T. castaneum*, a greater tolerance towards DE was found with a temperature of 30°C than at 22 to 24°C (Maceljski & Korunic, 1972; Aldryhim, 1990). However, this result was not confirmed in more recent studies by Arthur (2000) and Vayias and Athanassiou (2004) and discussed in detail in Vayias and Athanassiou (2004). An exceptional susceptibility at low temperatures could be the reason for the good control effect in the field trial presented here.

A low amount of DE adhered to the vertical surfaces only, this could be a problem for the control of stored-product pests seeking refuge on walls, especially for last-instar larvae of moths. The distribution on the floor was very variable, 1/3 of the sample points had less than 10 g/m². The application technique has to be improved to get a more even distribution for empty room treatment. However, it was not shown yet if an uneven distribution really affects efficiency of DE. Insects moving around from spots with low dosages to those with high dosages could still pick up sufficient amounts of DE to achieve control.

DE effectiveness is known to be influenced by the type of commodity treated, i.e. effectiveness is lower in maize than in wheat or barley (Athanassiou et al., 2008), but such an effect was not known for the type of surface material in empty rooms yet. In this study, a significant lower efficiency of DE against *C. ferrugineus* was found on concrete flagstone compared to plywood. The lowest mortality of *T. confusum* was found on concrete flagstone, too, even though this treatment did not significantly differ from the other surface materials. These results suggest the study of the influence of surface materials under more controlled laboratory conditions. An influence of surface materials on effectiveness is well known for synthetic chemical contact insecticides (Arthur and Peckman, 2006). However, the possible mechanism of influence is not clear for DE, as this light material piles up to several centimetres at 20 g/m² or more, preventing the contact of the insects moving on this layer to the ground.

Many studies examined the efficiency of DE at relatively high temperatures of 25°C or more, and relatively low relative humidity of 55 to 65 % (e.g. Athanassiou et al., 2005; 2008), because DE is known to be generally less effective at cool and humid conditions (Mewis and Ulrichs, 2001). However, in Central and Northern Europe cool and humid periods may occur even in summer at time of harvest. These field trials showed that 2.0 kg/t DE results in sufficient control even if temperatures are in the range of 15 to 20°C. If less than 1.0 kg/t DE are applied, *S. granarius* cannot be sufficiently controlled.

Acknowledgements

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Efficacy of propionic acid against the granary weevil *Sitophilus granarius* (L.)

Lorenz, S., Schöller, M.*#, Reichmuth, C.
Julius Kühn-Institut - Federal Research Centre for Cultivated Plants, Institute for Ecological Chemistry, Plant Analysis and Stored Product Protection, Section Stored Product Protection; Königin-Luise-Str. 19, 14195 Berlin, Germany. Email: Matthias.Schoeller@jki.bund.de

* Corresponding author
# Presenting author

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Abstract

Propionic acid is used to preserve feed grain, especially against fungal attack, and is known to affect stored product insects as well. In the study presented here, the effect of wheat treated with different amounts of pure propionic acid on both adult *Sitophilus granarius* and its progeny was investigated. Propionic acid (99.5% purity) was added to samples of 150 g of wheat at the doses of 0.5, 0.7 and 1% by weight. Subsequently, 100 adult *S. granarius* were released into each vial with treated wheat. Each trial was repeated three times. The untreated controls received water instead of propionic acid. Dead weevils were counted after 7 and 14 days. Insects surviving 7 days were placed back into the vials, all adults were removed after 14 days. During the period of 8 and 11 weeks after start of the experiment, the number of progeny was counted weekly. In the trials with 0.5%, 0.7% and 1% by weight, after 14 days 73.7, 37.3 and 3.7% of the adults were alive, respectively. While the mean number of progeny was 1549 in the untreated control, 1.3 and 0.3 progeny on average emerged from the grain treated with 0.5% and 0.7% propionic acid, respectively. No progeny survived in the treatment with 1% by weight. Even though complete control of adult *S. granarius* could not be achieved with the tested conditions, under practical situations of storage of feed grain, the described application of propionic acid will effectively suppress the mass-development of *S. granarius*.

Keywords: Granary weevil, *Sitophilus granarius*, Propionic acid, Control, Feed storage

1. Introduction

Propionic acid serves for prevention of fungal growth and control of insects especially in storage of moist feed grain (Reichmuth and Richter, 1991). The acid occurs in grain also naturally at low content (Mara, 1978) and has a repellent effect (Germinara et al., 2007; 2008) as well as a toxic effect on *Sitophilus granarius* (L.) (Reichmuth and Richter, 1991; Germinara et al., 2007). Aim of the presented study was to demonstrate the possible lethal effect of wheat treated with various amounts of propionic acid on adult granary weevils and their progeny. The lethal content of the acid was to be identified.

2. Materials and methods

Pure propionic acid with a purity of 99.5% served for the treatment of the wheat. The test comprised three dosages: 0.5, 0.7 and 1.0% by weight. Dosages of 5, 7 and 10 mL/kg, respectively, were added as portions of 0.75, 1.05 and 1.5 mL to wheat samples of 150 g insect-free whole wheat kernels *Triticum aestivum*. Exposure of the grain to -18°C for 10 days prior to the experiments ensured that the grain was free of living pests. The grain was adjusted to 14% mc before starting the experiments.

The insects originated from cultures maintained at the Julius Kühn-Institute in Berlin. One hundred 2-3 week old granary weevils per 400 mL glass jar served as test insects for the experiments. Throughout the experiments, temperature was kept at 22 ± 2°C and relative humidity at 67 ± 4% in a climatic chamber.

The temperature in untreated wheat samples (without granary weevils) was 22.5 ± 0.6°C and the relative humidity in the grain 70.4 ± 2.1%. Data loggers (MINIDAN CLIMA, ESYS GmbH, Berlin, Germany) served to determine the climatic conditions in the grain samples with four measurements per day. Figure 1 contains the data for temperature and relative humidity in reference glass jars.

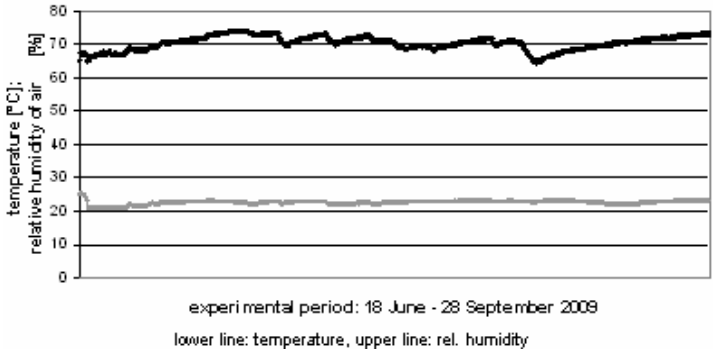

Figure 1 Temperature and relative humidity in grain samples throughout the experimental period; recorded with data loggers of the MINIDAN CLIMA company; 4 data points per day, linear scale.

The required amount of acid was pipetted to the grain (Table 1). To ensure equal distribution of the acid in the grain sample, the glass jars with grain were closed with plastic cap and rotated for one hour on a special device (Multifix, Alfred Schwinherr, Feinmechanische Spezialgeräte, Schwäbisch Gmünd, Germany). Subsequently, 100 adult *S. granarius* were added to the grain, and the glass jar was closed with a piece of cotton cloth and rubber bands. Three jars with 150 g wheat and 100 insects served as references at the same climatic conditions. Instead of the acid, water was added to the grain and the jar rotated as described above.

Seven and 14 days later, wheat samples were checked for surviving weevils. After the first period of 7 days, the surviving adults were counted and transferred back into the jars. Dead insects were removed. After 14 days, surviving weevils were counted again and all insects were removed from the jars.

After 8 weeks, emerging progeny were counted and removed together with dead insects for the first time, followed by further weekly counting for another 6 weeks. Each dosage was tested in three replicates.

Table 1 Survival rate of 100 adult *Sitophilus granarius* after treatment of wheat (mc = 14%) with propionic acid at dosages of 0.5, 0.7 and 1.0% by weight and in untreated reference samples at 22 ± 2°Cand 67 ± 4% r.h.; number of surviving *Sitophilus granarius* after 7 and 14 days and percentage of survivors in relation to the number of progeny in untreated reference samples, three replicates per dosage.

		Number of surviving *Sitophilus granarius*			
		Propionic acid in % by weight on wheat			
Days after start	**Glass jar No.**	**0**	**0.5**	**0.7**	**1.0**
	1	100	91	79	12
	2	100	90	68	10
7	3	98	90	74	11
	Total	298	271	221	33
	Survival rate (%)	99.3	90.3	73.7	11.0
	1	96	78	44	2
	2	100	62	33	5
14	3	98	81	35	4
	Total	294	221	112	11
	Survival rate (%)	98.0	73.7	37.3	3.7

3. Results and discussion

3.1. Survival of adults

Table 1 contains the results on the efficacy of propionic acid against adult *S. granarius* after 1 and 2 weeks of exposure at 22 ± 2°C. After the treatment with 1% by weight of propionic acid per kg wheat only few insects (11.0 and 3.7%) had survived 7 days and 14 days, respectively.

Figure 2 reveals that the influence of the exposure time on the survival rate of adult *S. granarius* with 1% content of propionic acid in wheat is not very pronounced. This is different with lower contents of acid. An expansion of the exposure of a few days beyond 2 weeks should have controlled all adult weevils with 1% propionic acid. In the situation of a feed store, full control might be achieved with 1% by weight of propionic acid since the grain is often stored for a period of months. Conversely longer exposure at lower dose did not give complete control. A slight increase in dose might as well improve the situation significantly and give full control.

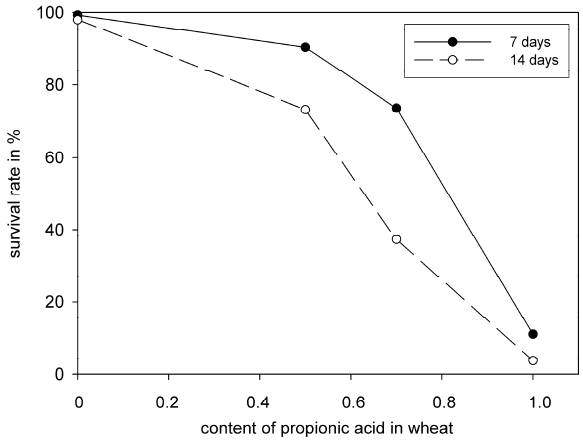

Figure 2 Survival rate [% of numbers in untreated samples] of adult *Sitophilus granarius* after treatment of wheat with propionic acid at dosages of 0.5, 0.7 and 1% by weight and in untreated reference samples after 7 days and 14 days of exposure at 22 ± 2°C and 67 ± 4% r.h., three replicates.

3.2. Production of offspring

Table 2 contains the results for the numbers of progeny of *S. granarius* (F_1 generation), that where collected 8 to 11 weeks after the beginning of the experiment with parent weevils and treated wheat. Only a few single eggs managed to survive and develop into adults in kernels of wheat that had been treated with 0.5 and 0.7% by weight, respectively. An acid content of 1% by weight totally interrupted the development of any F_1 imago. The lethal effect of this does was very pronounced.

Table 2 Number of progeny of *Sitophilus granarius* (F_1-generation) after 11 weeks of exposure at 22° ± 2 C and 67 ± 4% r.h. on wheat treated with either 0.5, 0.7 or 1% by weight.

Weeks after start	Glass jar No.	Number of *Sitophilus granarius* progeny Propionic acid in % by weight on wheat 0	0.5	0.7	1.0
11	1	1435	1	1	0
	2	1681	2	0	0
	3	1532	1	0	0
	Total	4648	4	1	0
	Average	1549.3	1.3	0.3	0
	Standard deviation	123.9	0.6	0.6	0

4. Conclusions

Control of adult granary weevils in stored wheat at 22 ± 2°C with propionic acid requires contents of about 1% by weight. A little bit more than 2 weeks are needed for this effect. The progeny is affected, too, and will not survive these conditions, even though the exposed adults may lay some eggs before dying. In so far, stored wheat is protected also against invading granary weevils by this treatment. Beside the fungicidal effect this acid brings along a lethal effect against this important insect pest. The progeny may also be suppressed at lower contents like 0.5% by weight because only few weevils survived this treatment, but this aspect requires further studies. Reichmuth and Richter (1991) mentioned that moist grain can be protected against mould growth and infestation by *S. granarius* at costs of about 0.3 €/t without loss of the quality of the grain for feeding purpose. An interesting question might be how long the treated grain is protected against newly invading weevils and development of progeny some weeks after treatment.

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A novel natural insecticide molecule for grain protection

Rajashekar, Y., Shivanandappa, T.*#[1]
Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Council of Scientific and Industrial Research, Mysore-570 020, India. Email: tshivanandappa@yahoo.com

* Corresponding author
# Presenting author

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Abstract

We have isolated a novel bioactive molecule from the roots of *Decalepis hamiltonii* that shows insecticidal properties against the stored-product insects *Sitophilus oryzae*, *Rhyzopertha dominica* and *Callosobruchus chinensis* by contact bioassay (LC_{50}: 0.033-0.044 mg/cm^2). This compound proved effective as a grain protectant against stored product insects of wheat and green gram. The compound is a novel insecticidal molecule and a promising grain protectant of natural origin.

Keywords: *Decalepis hamiltonii*, Decaleside- I, Insecticidal activity, Stored-product, insect pests, Grain protection

1. Introduction

Damage to stored grains and their products by insects may amount to 5-10% in the temperate countries to 20-30% in the tropical zone (Nakakita, 1998). Infestation control of stored grain is primarily achieved by the use of synthetic gaseous insecticides such as methyl bromide and phosphine. In several countries including India, mixing of any synthetic insecticide with stored grain is not permitted. Due to environmental concerns and human health hazards, several chemical insecticides have either been banned or restricted for use (Subramanyam and Hagstrum, 1995; Tapandjou et al., 2002). Further, due to the problem of resistance to insecticides, there is an urgent need for safer alternatives to conventional chemical insecticides particularly from natural sources, for the protection of grain against insect infestation. In view of all the aspects in grain protection and these problems have highlighted the urgent need to develop newer ecofriendly safer and effective stored-product insecticide

The highly successful and currently used synthetic pyrethroids were originally derived from the flowers of pyrethrum (Casida et al., 1975). Azadiraechtin, the active principle from the plant *Azadiraechta indica* (Indian neem), is an antifeedant and insect growth regulator but lacks contact toxicity (Isman et al., 1990; Islam and Talukder, 2005). At present, there is no botanical insecticide to replace pyrethrum for protection of stored grain from insect infestation. Bioinsecticides of plant origin often show selectivity to insect species, are biodegradable, have high chance of acceptability and, therefore, it is considered that plants could be the best source of newer chemical structures for development of new, ecofriendly, safer insect control agents (Saxena et al., 1992).

Decalepis hamiltonii Wight & Arn (family: Asclepiadaceae), a wild climber, grows in the hilly forests of peninsular India. The tuberous aromatic roots of *D. hamiltonii* are used in folk medicine and consumed as pickles and as a health drink for the alleged health benefits. Earlier work in our laboratory has shown that roots of *D. hamiltonii* are the source of novel bioactive compounds and more than a dozen compounds have been isolated and identified (Harish et al., 2005, Srivastava et al., 2006a, b, 2007). Previous studies from this laboratory have indicated that roots of *Decalepis hamiltonii* possess biopesticidal properties (George et al., 1999). In this study, we have investigated the insecticidal activity and grain protection potential of a novel bioactive compound isolated from the roots of *D. hamiltonii*.

2. Materials and methods

2.1. Preparation of the bioactive compound

Fleshy outer part of the roots was separated from the inner woody core, dried and powdered. The powder was extracted with organic solvents and the extracts were screened for insecticidal activity. The active extracts showing the insecticidal activity (aqueous extracts) were selected for the isolation of the bioactive compounds. The extracts were subjected to column chromatography using silica gel and eluted with various combinations of solvents (chloroform, ethyl acetate, acetone and methanol). The active

fraction was collected and subjected to second round of fractionation on silica gel. Purity of the compound was tested by TLC and by HPLC on a C_{18} column with methanol: water (1:1) and 0.1% trifluoro acetic acid as the mobile phase. Based on NMR and MS data, the structure of the purified compounds was characterized.

2.2. Insects

Cultures of rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and lesser grain borer, *Rhyzopetha dominica* (F.) (Coleoptera: Bostrychidae), were reared on whole wheat (*Triticium aestivum*) whereas adzuki bean weevil, *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae), were reared on whole green gram (*Phaseolus aureus*). Adult insects were used in the experiments carried out in the laboratory maintained at 27 ± 2°C and 70 ± 5% r.h.

2.3. Insecticidal activity

Toxicity of the purified compound against the stored product insects, S. *oryzae, R. dominica* and *C. chinensis* adults was studied using a residual contact bioassay method (Obeng-Ofori et al., 1998). One ml of solution (0.016- 0.156 mg/cm^2) was applied on Whatman N°1 (9cm) filter paper and placed in a glass Petri dish. The solvent was allowed to evaporate for 10 min prior to the release of 20 adults of the test insect in to each dish. Control filter paper discs were treated with solvent only. Each treatment consisted of four replicates. Insect mortality was recorded after 24 h and percent mortality was determined, while control mortality was corrected as suggested by Abbott (1925).

2.4. Insecticidal effects on the treated grain

The compound dissolved in 2 mL of methanol was applied to 50 g grain at 25, 50, 100 and 125 mg/kg. Controls were treated with solvent alone. Thirty unsexed adults of *C. chinensis, R. dominica* or *S. oryzae,* were introduced into the glass jars (250 mL) containing the 50 g grain. The glass jars were covered with cotton cloth held with rubber bands. Mortality was recorded after 24 h, and 168 h (7 d) post treatment storage period.

2.5. Effect on F_1 progeny

Grains were treated as described above and, after 7 days, the insects (dead and live) were removed and the grains were kept under the same experimental conditions until the emergence of F_1 progeny. Based on the life cycle of the insect species, the counting period of F_1 progeny was established so as to avoid an overlap of generations for each species. At weekly intervals, the F_1 progeny were recorded for 8 consecutive weeks. Percentage reduction in adult emergence of F_1 progeny or inhibition rate (% IR) was calculated as

$$\% \text{ IR} = (C_n - T_n)\ 100/C_n.$$

Where C_n is the number of newly emerged insects in the untreated jar and T_n is the number of insects in the treated jar (Tapondjou et al., 2002).

2.6. Statistical analysis

The data was analysed using one-way ANOVA ($p < 0.05$) and means were separated by Duncan's multiple range test using Statplus 2007 software. The data were expressed as means ± SE.

3. Results

3.1. Insecticidal activity

Contact toxicity of the compound by filter paper bioassay show that insect mortality was dose dependent at 24 h of exposure (Figure 1). Highest mortality (100%) was observed at dosage 0.156 mg/cm^2 to all the tested insect species. LC_{50} ranged from 0.33 mg to 0.43 mg/cm^2 (Fig. 1).

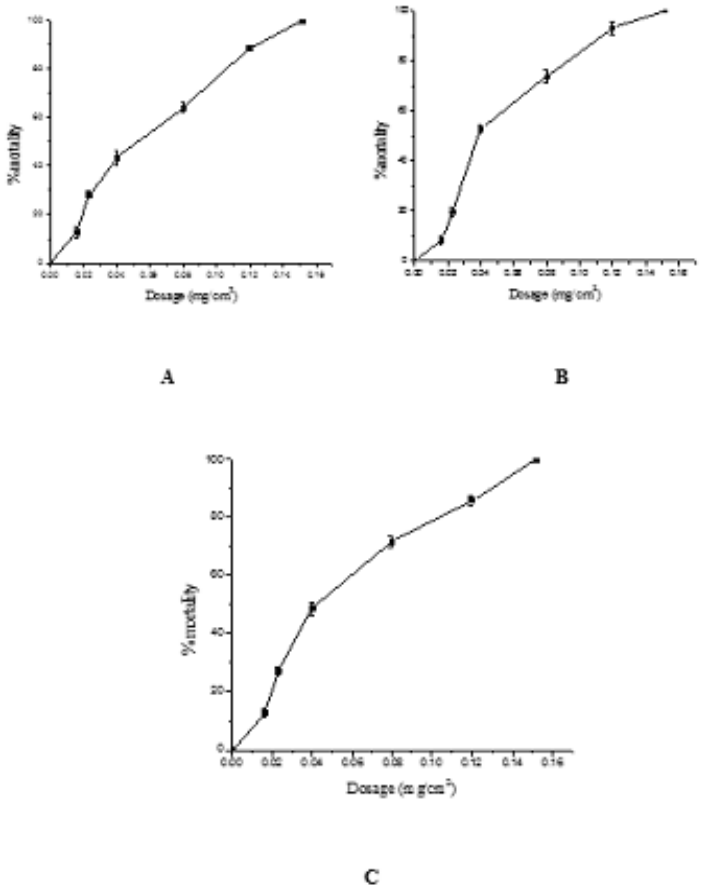

Figure 1 Insecticidal activity of Decaleside-I to **A.** *Sitophilus oryzae*; **B.** *Rhyzopertha dominica* and **C.** *Callosobruchus chinensis* in the contact bioassay. Values are averages of 4 replicates and represents the mean ± SE

3.2 Insecticidal effect on treated grain

Grains treated with the compound at 125 mg/kg (125ppm) produced 90% and 100% mortality at 24 h and 168 h respectively, in all the test species (Table 1).

Table 1 Mortality response of stored product insect pests on wheat grain and green gram treated with Decaleside- I.

Dosage (ppm)	% Mortality (Mean ± SE)					
	S. oryzae		*R. dominica*		*C. chinensis*	
	24h	7d	24h	7d	24h	7d
25	25.6 ± 1.4a	30.6 ± 1.5a	29.3 ± 1.4a	38.85 ± 1b	26.47 ± 1a	34.6 ± 0.9a
50	42.5 ± 1.7b	48.1 ± 1.7b	50.8 ± 1.4b	62.9 ± 0.8b	44.2 ± 0.9b	48.9 ± 1.6b
100	81.9 ± 1.5c	87.2 ± 2.3c	89.5 ± 1c	94.3 ± 1.6c	80.1 ± 0.9c	86.2 ± 1.2c
125	94.6 ± 1.01d	100d	98.5 ± 2d	100d	94.3 ± 1.5d	100d

Values followed by different letters within the vertical columns are significantly different ($P < 0.05$) by Duncan's multiple range test.

3.3 Effect on F_1 progeny

Significant reduction to total suppression of F_1 progeny of all the insect species in the treated grain was observed (Table 2).

Table 2 Effect of Decaleside I from on adult emergence in F_1 progeny of stored product insects.

Dosage (ppm)	% Reduction in F_1 adult emergence		
	S. oryzae	***R. dominica***	***C. chinensis***
10	12.9 ± 1.4a	10.2 ± 4.04a	15.9 ± 1.7a
25	24.1 ± 1.4b	38.12 ± 1.9b	27.4 ± 1.7b
50	53.3 ± 1.5c	67.6 ± 1.4c	59.4 ± 0.6c
75	77.3 ± 0.21d	75.3 ± 0.6d	84.3 ± 1.4d
100	85.05 ± 0.4e	88.5 ± 0.4e	94.05 ± 0.6e
125	100f	100f	100f

Values followed by different letters within the vertical columns are significantly different ($P < 0.05$) by Duncan's multiple range test.

4. Discussion

With limitations on the use of contact chemical insecticides and fumigants in stored products, and increasing public demand for wholesome and pest-free food products, there is a need for developing biorational pest management technologies in stored products.

Pyrethrin, extracted from flower of *Tanacetum cinerariaefolium,* was considered an almost ideal insecticide. Today, its use is limited because of its high cost. Many studies have reported bioactive compounds from plant extracts with repellent/antifeedant/insecticidal activity against stored-product insect pests (Upasani et al., 2003; Akhtar et al., 2008; Yao et al., 2008) The rhizomes of *Acorus calamus* and the active ingredient (β-asarone) have been investigated for their insecticidal properties but the effort to develop β-asarone as an insecticide received a severe setback with discovery of its mutagenic effect (Abel, 1987). The seeds of *Azadiraechta indica* have been shows insecticidal activity against a variety of insect species and, azadirachtin, the active principle, has exhibits insect antifeedant, moult inhibiting and anti-gonadotropic effects in insects (Schmutterer, 1990). However, its bitter taste and lack of contact toxicity restricts its use and unsuited on stored-products meant for human consumption.

Many plant products and their essential oils have been shown to exhibit insecticidal activity against stored product insect pests (Rajendran and Sriranjini, 2008). At dosage of LD_{50} of 0.21 and 0.17 mg/cm^2, Carvacrol, a compound from *Thujaopsis dolabrata* was toxic to adults of *S. oryzae* and *C. chinesis* (Ahn et al., 1998). Linalool, a bioactive molecule from *Ocimum canum,* killed adults of *S.oryzae* and *R. dominica*, with LD_{50} of 0.42 and 0.428 mg/cm^2 (Weaver et al., 1991). Other natural compounds such as estragole (LD_{50} of 0.066 mg/cm^2) and (+) - fenchone (LD_{50} of 0.092 mg/cm^2) from *Foeniculum vulgare* were toxic against adults of *S. oryzae* (Kim and Ahn, 2001).

Our compound isolated from the roots of *D. hamiltonii* is a novel bioactive molecule as evident from the spectroscopic characterization and shows insecticidal activity against stored product insects by contact bioassay. The residual toxicity on treated grain surface was retained even after several days. Persistence of this toxicity indicates that the compound is stable enough.

The treated grain was free from infestation at the highest dosage (125 ppm) due to cessation of F_1 progeny. Protection of the treated from infestation could be attributed to contact toxicity as well as ovicidal effect. Our study shows that the bioactive compound from *D. hamiltonii* could be a potential grain protectant, which acts by killing various life stages of stored grain insect pests and total suppression of emergence of progeny in treated grain. Furthermore, since the compound derived from an edible source with a long history of human use, it appears to be safe to mammals (Shereen, 2005). Hence, the compound from *D. hamiltonii* could belong to a new class of bioinsecticide and may serve as a promising grain protectant of natural origin

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Human behaviour and application of residual insecticides to control storage and food industry pests

Stejskal, V.*#, Aulický, R.
Crop Research Institute, Drnovska 507, Praha 6, CZ-16106, Czech Republic. Email: stejskal@vurv.cz

* Corresponding author
# Presenting author

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Abstract

We measured the individual variation of the area-estimate of simple geometrical patterns (circle, rectangle) in ten people. We found that they tended to underestimate (max. 5 x) the correct area of the tested geometrical patterns. Consequently, we explored how the insecticides Ficam 80WP, K-Othrine 25, and Actellic EC50 are robust or sensitive to the measured extent of over-dosage (2x) or under-dosage (5x). We also tested the effects of incorrect dosages of insecticides applied to porous filter paper nonporous glass and bioassayed with adult *Sitophilus oryzae* and *Tribolium castaneum*. We found that the tested insecticides were surprisingly robust to under-dosage on the glass surface but sensitive to under-dosage on the porous paper surface.

Keywords: Pesticides, Insecticides, Residual treatment, Dosage, Human behaviour

1. Introduction

The contemporary management of storage pests still relies primarily on chemical and physical control (Zettler and Arthur, 2000; Huang et al., 2004). Residual sprays are routinely employed as surface treatments (Zettler and Arthur, 2000, Hubert et al., 2007) to control dispersal of stored product pests from cracks and crevices (Arthur et al., 2006; Kucerova et al., 2003). Methods and strategies of pesticide application to control stored food product and public health pests have been reviewed by Zettler and Arthur (2000). Generally, pesticide application equipment should be designed to deliver the correct amount of active ingredient to the proper place in the most efficient and economical way. The prerequisite of good efficacy and safe use of any residual insecticide is also the ability of the human-applicator to deliver a proper (label) dose of active ingredient to a target surface. In contrast to mechanized and automated insecticide application equipment for crop fields and orchards, the application of insecticides to food industry premises relies mainly on human labour and behaviour. Notably, the proper dosage of insecticide largely depends on the human applicator and the individual's perception and estimate of the area to be treated.

The objectives of this research were to: 1) to estimate the extent of variation in individual perception and estimate of the simulated area (two simple geometrical patterns, a circle and rectangle) to be treated, and 2) explore how are various insecticides (Ficam 80WP, K-Othrine 25, Actellic EC50) are sensitive to over-dosage or under-dosing stemming from an incorrect estimate of the treated area.

2. Materials and methods

In the initial stage of the experiment we measured the extent of individual variation of the human area-estimate. We asked ten individuals, all of whom reached at least middle school education level, to estimate an area two simple geometrical patterns (a circle and rectangle) measuring 24.5 cm^2 printed on paper, without using any measurement tools. We tried to simulate the field situations when pest control persons frequently estimate the area to be treated by a residual insecticide without any measuring equipment. The aim of our experiment was to determine the maximal and minimal estimate-departures (i.e., extreme values) from the correct value of 24.5 cm^2. The overestimated and underestimated values were then used for the consequent experiments modelling biological effects of over-dosing or under-dosing of three insecticides.

As a reference, the following labelled doses were used: Actellic 25 EC-8% (1.0 g pirimiphos methyl m^{-2}), K-Othrine 1% (0.0125 g deltamethrin m^{-2}), Ficam W 0.3% (0.12 g bendiocarb m^{-2}). Concurrently, we tested the effects of incorrect dosages of insecticides on a porous and nonporous surface to control *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera:

Tenebrionidae): For maximal over-dosage (2x) or under-dosage (5x) we used data from Table 1. Plain glass Petri dishes (diameter, 150 mm) were used to simulate a non-porous surface. To simulate an extremely porous surface we used five layers of Whatman No. 4 filter paper (medium retention and flow rate) fixed to the bottom of Petri dishes. The diluted insecticides were evenly applied on the treated surfaces and left to dry for 24 h at 20°C. Diluted insecticide formulations were applied (10 mL) by a micro-syringe (Socorex Acura 825 autoclavable 100-1000 µL) evenly over a treated surface. In experiments, we used 20 insects in each paper/Petri dish in 10 repetitions for each treatment. A short term exposure of 10 min (Arthur, 1998; Stejskal et al., 2009) was used, after which beetles were added to new Petri dishes. We assessed mortality 102 h after exposure.

3. Results and discussion

We explored the influence of over dosage and underdosage on short exposure efficacy of Ficam 80WP, K-Othrine 25, Actellic EC50 to two stored-product beetles. We tested the potential influence of human applicator behaviour on the incorrect dosage of insecticides. The extent of incorrect dosage was derived from human incorrect estimate of the surface area. Table 1 shows the surface-area estimate of two geometrical patterns (circle and rectangle) printed on the paper, performed by ten experimental persons. We found that the experimental persons tended to underestimate (max. cca 5x) rather than to overestimate (2x) the correct area of the tested geometrical patterns. This small-scale (cm^2) estimate should be verified at larger scale in field conditions.

Table 1 The surface-area estimate of two geometrical patterns (circle and rectangle of 25.4 cm^2) printed on the paper performed by 10 experimental persons, maximum (Max.) and minimum (Min.) values.

Geometrical pattern	**Circle**		**Rectangle**	
	Max. value	**Min. value**	**Max. value**	**Min value**
Estimated area	50 cm^2	5 cm^2	50 cm^2	12 cm^2
Ratio to the correct value	2,1x higher	4,9x lower	2,1x higher	2,1x lower

Tables 2 and 3 show differential robustness of the tested insecticides to the simulated extent of over-dosage (2x) or under-dosage (5x) on porous and non-porous surfaces in *T. castaneum* (Table 2) and *Sitophilus oryzae* (Table 3). We found that the tested insecticides were surprisingly robust to under-dosage on the glass surface while they were sensitive to under-dosage on the porous paper surface. We also found site-specific differences: *Sitophilus oryzae* was more sensitive to the under-dosed insecticides compared to *T. castaneum*.

Table 2 Short-exposure (10 min) efficacy of three insecticides on *Tribolium castaneum,* when applied in correct and incorrect label-doses on porous (filter paper) and nonporous surface (glass).

	% Mortality ± SD after 102 h					
Dose	**Correct dose**		**Over-dosage 2x**		**Under-dosage 5x**	
Surface	**Paper**	**Glass**	**Paper**	**Glass**	**Paper**	**Glass**
K-Othrine	1.7±3.7	10±10	17±11	37±39	6.,7±9.4	12±17
Ficam	100±0	100±0	100±0	100±0	0±0	100±0
Actellic	1.7±3.7	100±0	3.3±4.7	100±0	0±0	100±0

Table 3 Short-exposure (10 min) efficacy of three insecticides on *Sitophilus oryzae*, when applied in correct and incorrect label-doses on porous (filter paper) and nonporous surface (glass).

	% Mortality ± SD after 102 h					
Dose	**Correct dose**		**Over-dosage**		**Under-dosage**	
Surface	**Paper**	**Glass**	**Paper**	**Glass**	**Paper**	**Glass**
K-Othrine	97±4.7	100±0	100±0	100±0	85±9.6	100±0
Ficam	100±0	100±0	100±0	100±0	63±22	100±0
Actellic	43±18	100±0	50±27	100±0	43±29	100±0

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Insecticidal action of the combined use of spinosad and deltamethrin against three stored-product pests in two stored hard-wheat varieties.

Vayias, B.J.*#[1], Kavallieratos, N.G.[2], Athanassiou, C.G.[3], Tatsi, G.[1]
[1] Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, 75 Iera Odos str., 11855, Athens, Greece. Email: bvayias@gmail.com
[2] Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta str., Kifissia, Attica, Greece
[3] Laboratory of Entomology and AgriculturalZoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Phytokou str., 38443, N. Ionia, Mangisia, Greece

* Corresponding author
# Presenting author

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Abstract

The combined use of spinosad with deltamethrin against adults of *Sitophilus oryzae*, *Sitophilus granarius* and *Tribolium confusum* was evaluated in a series of laboratory bioassays in two hard wheat varieties (Athos and Sifnos). Two groups of bioassays were carried out. In the first group of bioassays, spinosad or deltamethrin were applied alone at the tested wheat varieties at the doses of 0.01, 0.1 and 0.5 ppm for spinosad and 0.125 ppm for deltamethrin. In the second group of bioassays, the tested wheat varieties were treated with the combination of the above spinosad rates with 0.125 of deltamethrin. In both series of bioassays, mortality of the tested species was evaluated after 7 d of exposure on the treated wheat varieties at 25°C and 65% r.h. Mortality for all species was always significantly higher in Athos than Sifnos. The highest mortality of *S. oryzae* (73 and 40% for Athos and Sifnos respectively) or *S. granarius* (88% and 58% for Athos and Sifnos respectively) was recorded in the cases that spinosad was applied alone at 0.5 ppm. On the contrary, in the case of *T. confusum*, 0.125 ppm of deltamethrin was significantly more effective than any of the application rates of spinosad either when applied alone or in combination with deltamethrin. Despite the fact that the highest mortality of *S. granarius* adults was recorded after exposure on the wheat varieties treated with 0.1 ppm of spinosad x 0.125 ppm of deltamethrin, in light of the results of the present study, the combination of spinosad with deltmethrin requires further investigation since in most of the tested cases of the present study, single application of spinosad or deltamethrin was more effective or of equal effectiveness than the respective combination of spinosad with deltamethrin.

Keywords: Spinosad, Deltamethrin, *Tribolium, Sitophilus*, Wheat, Variety

1. Introduction

The consumers' growing demand for residue-free goods as well as the fact that many species have now developed resistance to the most commonly used grain protectants (Arthur, 1996) have made essential the evaluation of new insecticides for the control of stored-product pests. Spinosad can be considered as one promising alternative to the currently used grain protectants, as it has low mammalian toxicity (Sparks et al., 2001; Subramanyam et al., 2003) and also it is effective against many of the most important stored-product pests (Fang et al., 2002; Athanassiou et al., 2008; Vayias et al., 2009). Deltamethrin is a pyrethroid insecticide, registered in many parts of the world for stored grain protection. This insecticide is also effective against stored-product pests and can provide a long-term protection that lasts four months or more (Athanassiou et al., 2004).

Insecticides vary regarding efficacy against different target species. For instance, spinosad is moderately effective against *Tribolium* spp. (Fang et al., 2002, Vayias et al., 2009), while deltamethrin is generally more effective against these species (Athanassiou et al., 2004). Hence, the combined use of more than one pesticide is likely to moderate these differences, and provide a grain protectant with satisfactory protection against a wider range of species. In a recent study, Athanassiou et al. (2009) found that the combined use of deltamethrin with chlorpyriphos-methyl successfully controlled stored-grain psocids, which could not be controlled with spinosad or natural pyrethrum. In the present study, the combined use of low spinosad combined with low deltamethrin application rates was evaluated on two hard wheat varieties originating from Greece. This combination was assessed against three stored-product pests

which are very common to in bulk grains stored in Greece; two primary colonizers, the rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), and one secondary colonizer, the confused flour beetle *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae).

2. Materials and methods

Unsexed, <2 week-old adults of *S. oryzae, S. granarius* and *T. confusum,* obtained from laboratory cultures reared on hard wheat at 27 ± 1°C and 60 ± 5% relative humidity (r.h.) were used in the study. The tested wheat varieties were Athos and Sifnos obtained from Greek crops. The moisture content of the tested varieties at the beginning of the tests ranged between 10.9 and 11.5%. The grain characteristics of the tested varieties are given in Table 1. In the first group of tests four 1-kg lots of each variety were separately treated with three spinosad application rates (0.01, 0.1 and 0.5 ppm) and one deltamethrin rate (0.125 ppm). In the second group of experiments three 1-kg lots of each variety were separately treated with the combination of each spinosad rate x 0.125 ppm deltamethrin (e.g. 0.01 ppm spinosad x 0.125 ppm deltamethrin; 0.1 ppm spinosad x 0.125 ppm deltamethrin; 0.5 ppm spinosad x 0.125 ppm deltamethrin). In addition, a 1-kg lot of each grain was sprayed only with distilled water and served as the untreated control. From each treated (or untreated) 1-kg lot of grain, of a specific variety, three 30 g samples were obtained and used as a bioassay substrate with the above insect species at 25°C and 65% r.h. Mortality was assessed after 7 d of exposure of the tested species on the treated or untreated grains. The total procedure was repeated three times (3 x 3 vials per treatment) by preparing new 1-kg lots from each wheat variety each time. Data were analyzed separately for each species by a two way ANOVA with mortality as the response variable with grain variety and treatment as the main effects. For the separation of means the Tukey and Kramer HSD test was used at $P<0.05$ (Sokal and Rholf, 1995).

Table 1 Grain characteristics of the tested wheat varieties.

	Wheat variety	
Specifications	**Athos**	**Sifnos**
Brush length	Short	short
Kernel shape	Ovoid	semi elongate
Mean (± SE) weight of 100 kernels (g)	4.3 ± 0.1	5.2 ± 0.1
Mean (± SE) kernel size of 100 kernels (ml)	5.7 ± 0.1	6.9 ± 0.2
Protein content (%N x 5.7)	16.5	14.5
Gluten index	22.14	64.4
Mean (± SE) bulk density (g/lt)	742.5 ± 0.2	744.5 ± 0.6

3. Results

Mortality of the tested species on the untreated wheat varieties was negligible and did not exceed 2.5% in any of the tested cases. Irrespective of the treatment, susceptibility of all of the tested species was overall higher in treated Athos in comparison with treated Sifnos with the sole exception of the combination of 0.1 ppm spinosad x 0.125 ppm deltamethrin against *S. oryzae*, while the reverse was noted (Table 2). Among the tested species, *T. confusum* was the most tolerant to spinosad, deltamethrin or their combination. *Sitophilus granarius* was more susceptible than *S. oryzae* in all of the tested cases. Generally, the combination of spinosad with deltamethrin did not appear to be compatible for *S. oryzae* or *T. confusum* since the highest mortality ratio for those species was with 0.5 ppm of spinosad for the former and 0.125 ppm for the latter when these substances were applied alone rather than in combination. For instance, although 73% of the exposed *S. oryzae* adults were dead after exposure on Athos variety treated with 0.5 ppm of spinosad, mortality was only 39% on the same variety treated with 0.5 ppm spinosad x 0.125 ppm (Table 2). With *T. confusum*, mortality from exposure to spinosad was generally low and did not exceed 15% on both varieties, while it was slightly increased to 22% when 0.125 ppm of deltamethrin was applied to Athos prior to application of 0.5 ppm spinosad. The combination of deltamethrin with spinosad also gave low mortality levels with *T. confusum*. Mortality was only 28% on wheat treated with 0.125 ppm of deltamethrin alone, and was significantly higher or of equal effectiveness with the tested spinosad combinations (Table 2). An additive effect of deltamethrin with spinosad was observed only when *S. granarius* was exposed to 0.1 ppm spinosad x 0.125 ppm

deltamethrin. In the latter case, efficacy of 0.1 ppm spinosad x 0.125 ppm deltamethrin increased to 90% in Athos and 62% in Sifnos while efficacy of the respective spinosad or deltamethrin doses when applied alone did not exceed 54% in Athos or 26% in Sifnos. The combination of 0.5 ppm of spinosad x 0.125 ppm deltamethrin, slightly improved the performance of the respective spinosad dose in Athos against *S. granarius*, but did not demonstrate an additive effect in Sifnos against the same species (Table 2).

Table 2 Mean (±SE) mortality of adults of *S. oryzae*, *S. granarius* and *T. confusum* after 7 d exposure on two hard wheat varieties treated with spinosad alone, deltamethrin alone as well as with the combination of spinosad and deltamethrin. Within a given species, means followed by the same letter are not significantly different (lowercase letters for treatments; uppercase letters for varieties). For treatments *df*=6, 62; For varieties *df*=1, 17; Tukey and Kramer HSD test at *P<0.05*.

			Mortality (%)	
	Dose (ppm)		Wheat variety	
Species	**Spinosad**	**Deltamethrin**	**Athos**	**Sifnos**
S. oryzae	0	125	47.4 ± 3.1 Abc	25.9 ± 3.0 Bbc
	0.01	0	42.6 ± 3.9 Abc	20.0 ± 1.0 Bc
	0.1	0	53.3 ± 4.4 Ab	29.3 ± 3.1 Bb
	0.5	0	73.0 ± 3.7 Aa	39.6 ± 3.7 Ba
	0.01	0.125	15.2 ± 3.6 Ad	19.3 ± 2.9 Ac
	0.1	0.125	17.8 ± 3.1 Ad	24.4 ± 3.1 Abc
	0.5	0.125	35.9 ± 2.3 Ac	32.6 ± 2.7 Aab
S. granarius	0	125	66.3 ± 5.1 Ab	25.2 ± 1.4 Bc
	0.01	0	53.3 ± 2.4 Ab	17.8 ± 1.8 Bc
	0.1	0	57.4 ± 2.3 Ab	25.2 ± 2.2 Bc
	0.5	0	88.1 ± 2.1 Ab	58.1 ± 3.0 Bab
	0.01	0.125	61.1 ± 3.6 Aa	28.1 ± 1.9 Bc
	0.1	0.125	90.0 ±2.5 Aa	62.2 ± 3.3 Ba
	0.5	0.125	91.1 ± 3.0 Aa	49.6 ± 3.9 Bb
T. confusum	0	125	28.1 ± 2.2 Aa	23.3 ± 3.0 Aa
	0.01	0	1.9 ± 0.8 Ac	0.7 ± 0.5 Ac
	0.1	0	4.8 ± 1.0 Ac	3.0 ± 1.5 Ac
	0.5	0	14.4 ± 3.7 Ab	8.9 ± 1.6 Ab
	0.01	0.125	2.6 ± 1.3 Ac	1.9 ± 0.6 Ac
	0.1	0.125	3.7 ± 0.9 Ac	4.4 ± 1.4 Ac
	0.5	0.125	21.5 ± 5.4 Aa	18.6 ± 3.3 Aa

4. Discussion

In our study, the combined use of spinosad with deltamethrin at low application rates was not successful against *S. oryzae* or *T. confusum*, but was effective against *S. granarius*. With *S. granarius* only the specific combination of 0.1 ppm spinosad x 0.125 deltamethrin was highly effective since effectiveness of the remainding combinations against the same species was lower than or at least equal to the effectiveness of a single spinosad or deltamethrin application. It is possible that spinosad could synergize deltamethrin under specific conditions and for specific insect species; therefore more extensive research on this combination is needed. The fact that effectiveness of deltamethrin or spinosad varied between the tested varieties could be attributed to differences in physical or chemical characteristics of the grain, variations in insect behavior after contact with the treated kernels of a specific variety, or a combination of the above factors. Sifnos had more elongated, heavier and larger kernels compared to Athos. Also, gluten index was higher in Sifnos than in Athos. The above characteristics may have affected the efficacy of the tested formulations, as the treated species were overall less susceptible on Sifnos than in Athos, although in the case of *T. confusum* the differences were not significant. Kernel size is likely to play an important role in efficacy of insecticides, since better distribution of the toxicant is achieved on smaller kernels compared to larger ones (Huang and Subramanyam, 2007). As a result, insects may be able to more easily avoid the treated areas of larger kernels and consequently, avoid contact with the toxic substance (Athanassiou et al., 2003).Since distribution of deltamethrin or spinosad was better in the

smaller kernels of Athos compared to Sifnos, this could partially explain the higher mortality that occurred in Athos compared to Sifnos. Nevertheless, correlation of grain characteristics with efficacy of grain-insecticides is not always feasible. Fang et al. (2002), found that a significant variation in the performance of spinosad against several stored-product pests occurred among different wheat classes. The authors however, could not correlate this differential spinosad performance to grain kernel diameter, kernel weight, kernel hardness, protein content, dockage, or fiber. Hence, differential performance of insecticides among commodities or even varieties of a given commodity is a very complicated issue, and requires further experimental work.

Generally, pyrethroids provide quick mortality against insects, and this fact may influence their combination with other slow-acting insecticides. Le Patourel and Singh (1984) found that the combination of silica, which is a slow acting insecticide, with high doses of permethrin, cypermethrin and deltamethrin, did not exhibit any additive effect against the red flour beetle, *Tribolium castaneum* (Herbst) due to rapid knock down caused by the pyrethroids. In our study, additive effect was evident only in the case of the low doses of the insecticides tested, and this could be attributed to the delayed mortality, allowing both insecticides to act. Additional measurements at shorter intervals (e.g. 24 or 48 h) are necessary to clarify the basis of this hypothesis.

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Mortality and suppression of progeny production of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) in seven different grains treated with an enhanced diatomaceous earth formulation

Kavallieratos, N.G.[1], Athanassiou, C.G.[2], Vayias, B.J.*#[3], Boukouvala, M.[4]
[1] Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta str., Kifissia, Attica, Greece
[2] Laboratory of Entomology and AgriculturalZoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Phytokou str., 38443, N. Ionia, Mangisia, Greece
[3] Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens, 75 Iera Odos str., 11855, Athens, Greece. Email: bvayias@gmail.com
[4] Department of Plant Production, Technological Educational Institute of Larissa, 41110, Larissa, Greece

* Corresponding author
# Presenting author

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Abstract

DEBBM, an enhanced diatomaceous earth (DE) formulation consisting of a mixture of DE and the plant extract bitterbarkomycin, was applied to seven different grains (wheat, barley, oats, rye, triticale, paddy rice and maize) at two dose rates 50 ppm and 150 ppm. Unsexed, 7d old adults of *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Tribolium confusum* (Coleoptera: Tenebrionidae) were exposed to the DEBBM treated commodities and their mortality was assessed after 7d and 14d of exposure at 25 °C and 65% r.h. Furthermore, progeny production of the tested species per treated commodity was also assessed. *Sitophilus oryzae* appeared to be more susceptible than *T. confusum* to DEBBM. Performance of DEBBM was better in barley, wheat and oats compared to the remainder of the tested commodities. DEBBM performed better in rye and triticale than in paddy rice against both species although in many cases, significant differences among these grains were not recorded. Despite that DEBBM reached its highest efficacy levels on barley, wheat, and oats it did not suppress progeny production of the treated species in any of the grains. A significant reduction in progeny production of the treated species was recorded in the DEBBM treated grains in comparison with the untreated ones. This reduction in progeny production was expressed more vigorously to *S. oryzae* rather than *T. confusum*. In commodities with high DEBBM performance such as barley, oats or wheat, > 9-fold less progeny of *S. oryzae* were recorded at 150 ppm of DEBBM than in the untreated commodities. Although significantly less progeny of *T. confusum* were recorded in DEBBM treated grains than untreated grains, progeny suppression of this species was neither dose nor commodity dependant.

Keywords: Diatomaceous earth, Bitterbarkomycin, *Tribolium, Sitophilus*, Mortality, Commodity

1. Introduction

The use of diatomaceous earth (DE) for the control of stored product pests is one of the promising alternatives to the conventional insecticides and fumigants that are used in stored product protection. DE has low mammalian toxicity (Quarles, 1992) and is effective against a wide range of stored product insects (Korunic, 1998, Subramanyam and Roesli, 2000; Mewis and Urlichs, 2001; Athanassiou et al., 2005; 2006). Nevertheless, high DE application rates (400-1000 ppm) are required for a satisfactory control of stored product insects (Korunic, 1998; Athanassiou et al., 2005; 2006). At these application rates DEs negatively affect some of the physical properties of grains, which has limited the wide use of DE as grain protectant (Korunic, 1998). According to Arthur (2003) the combination of reduced rates of DE with other insecticides could alleviate some of the effects on physical properties.

Athanassiou et al., (2008a) evaluated a mixture of DE with the plant extract bitterbarkomycine (BBM) against adults of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). This enhanced DE formulation was very effective at application rates less than 150 ppm. BBM, known also as angulatin A, is a sesquiterpene polyol ester, extracted from the roots of the plant *Celastrus angulatus* Max. (Wang et al., 1991). In initial tests, Wang et al. (1991) found that BBM at low doses (25-50 ppm), had strong insecticidal and antifeedant effects against several insect species, especially aphids, while in a latter study Athanassiou et al. (2009) found that low doses of BBM (0.0375–0.0875 ppm) proved very effective

against several stored product insect pests including *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae).

The objective of the present study was to evaluate DEBBM as grain protectant against two major stored product insects *Sitophilus oryzae* (L.) (Coleopetra: Curculionidae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) as applied to wheat, maize, barley, rice, oats, rye and triticale. In addition, progeny production of the above species after exposure on the treated with DEBBM grains was also assessed.

2. Materials and methods

The tested insects were obtained from laboratory cultures. *Sitophilus oryzae* was reared on whole wheat kernels while *T. confusum* was reared on wheat flour plus 5% brewers yeast. Both species were reared at 27±1°C, 65 ± 5% r.h. in continuous darkness. All insects used in the tests were adults < 3 wk old. The DEBBM formulation that was used in the experiments contained 90% DE and 0.05% BBM. The examined commodities were hard wheat (variety Mexa), whole (raw) barley (variety Persephone), oats (variety Cassandra), rye (variety Danko), triticale (variety Vronti), paddy rice (variety Thaibonnet) and maize (variety Dias). All commodities used in the experiments were clean, untreated and infestation free. The moisture contents of the tested commodities, as determined prior to the experiments by a Dickey–John moisture meter (Dickey-JohnMultigrain CAC II, Dickey-John Co, USA), ranged between 10.9 and 11.5%. One kilogram lots were obtained from each commodity and each lot was separately treated with DEBBM at two dose levels (50 ppm and 150 ppm). In addition, 1 kg-lots from each commodity remained untreated and served as controls. Six samples of 60 g each were obtained from each treated or untreated commodity, placed inside small glass vials, and infested with 30 adults of *S. oryzae*. Mortality of adults was assessed 7 d and 14 d post exposure of the weevils on the treated or untreated commodities at 25°C and 65% r.h. After the 14 d bioassays, all dead or alive weevils were discarded and the vials were placed again at the above conditions for an additional period of 50 d. Next, the vials were opened and live adult progeny were counted. The same procedure described above was followed also for *T. confusum* by preparing new commodity lots. Control mortality in any of the exposure did not exceed 3%, and no correction for mortality values was done. Mortality data were subjected to one way ANOVA separately for each species and exposure interval, while the main effects were dose and commodity type. Prior to analysis of progeny production data, a Dunnnet's test for each species and commodity combination revealed that progeny production of the tested species was always significantly higher in untreated commodities than the respective treated ones. Hence, progeny production in the control groups was not included in the analysis of progeny production data. Similarly to mortality data, progeny data were subjected to one-way ANOVA separately for each species, with dose and commodity type the main effects. For the comparison of means for either mortality or progeny production data the Tukey and Kramer's HSD test at $P< 0.05$ was used (Sokal and Rholf, 1995).

3. Results

3.1. Sitophilus oryzae

Application rate and commodity type significantly affected mortality of S. oryzae adults on DEBBM treated commodities at both of the tested exposure intervals (7d exposure: application rate: $F_{2,125}$=51.1, P<0.001; commodity: $F_{6,125}$=13.6, P<0.001; 14 d exposure: $F_{2,125}$=30.1, P<0.001; commodity: $F_{6,125}$=17.0, P<0.001). After 7d or 14 d of exposure, efficacy of 50 ppm DEBBM did not exceed 17% in treated maize or rice and was always significantly lower than that recorded in the remainder of the treated grains (Fig.1). Weevil mortality was significantly increased following an increase of DEBBM dose to 150 ppm at both exposure intervals. Thus, after 7d of exposure of weevils on the DEBBM-treated grains, efficacy of 150 ppm increased to 88% in treated barley, wheat or oats while the same dose killed less than 50% of the exposed weevils in treated maize or rice (Fig.1). Seven days later, 93.6, 92.8 and 90.2% of the exposed weevils were dead on oats, barley and wheat treated with 150 ppm respectively, while mortality in treated rice or maize was 50.4 or 46.8% respectively (Fig. 1). As a result, significant differences in efficacy of 150 ppm of DEBBM against rice weevil after 7d or 14d of exposure were not recorded among barley, wheat or oats as well as between maize and rice, although a remarkable variation in performance of DEBBM between these two groups of grains (barley, wheat or oats and maize or rice) was always recorded (Fig. 1). Application rate and commodity type also significantly affected progeny

production of *S. oryzae* on DEBBM grains (application rate: $F_{2,125}$= 23.7; P<0.001; commodity: $F_{6,125}$= 2.6; P=0.01). Nevertheless, significant differences in weevil progeny between the tested DEBBM doses were recorded only in the case of barley or oats, while in the remainder of the grains, progeny production that was observed on DEBBM treated grains was dose independent (Fig. 2). Progeny production of rice weevil on untreated commodities was 36.5 ± 1.6, 18.9 ± 1.6, 29.5 ± 2.6, 16.3 ± 1.6, 23.7 ± 2.9, 22.3 ± 4.1 and 21.0 ± 1.8 weevils per 60g of barley, maize, oats, rice, rye, triticale and wheat, respectively. Even though the highest values of rice weevil progeny production were recorded in barley (17.3 weevils/60g) or maize (13.7 weevils/ 60g) treated with 50 ppm of DEBBM, progeny production on grains treated with 150 ppm of DEBBM ranged 4.3 - 6.1 weevils per 60 g of commodity and as a result, significant differences between the tested grains were not recorded (Fig. 2).

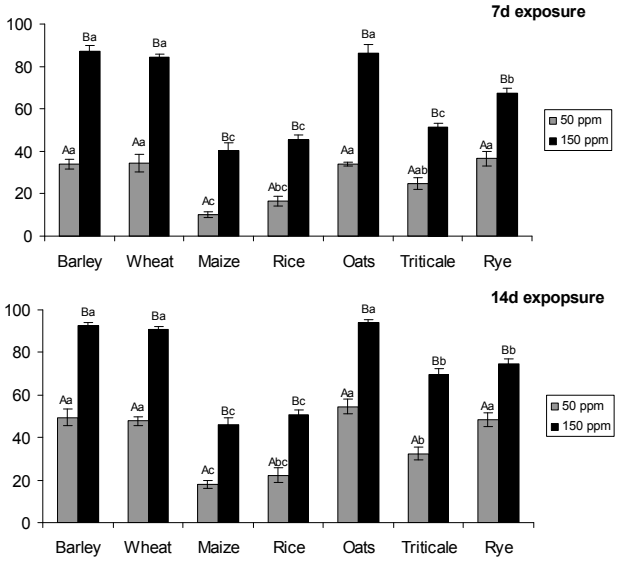

Figure 1 Mean (± SE) mortality of *S. oryzae* adults after 7 d and 14 d exposure on seven grains treated with two doses of DEBBM at 25°C and 65% r.h. Within each exposure interval and application rate means followed by the same lowercase letter are not significantly different. Within each exposure interval and grain, means followed by the same uppercase letter are not significantly different. (Uppercase letters for dose; Lowercase letters for grain. For the comparison of doses *df*=1, 11; For the comparison of grains *df*=6, 35; Tukey and Kramer HSD test at P<0.05).

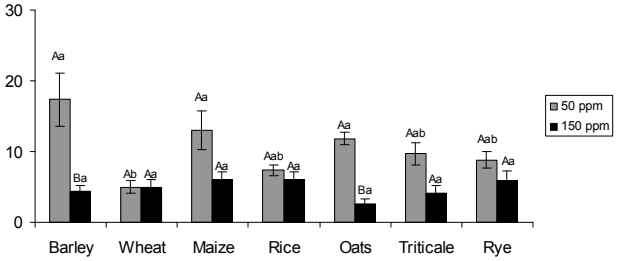

Figure 2 Mean progeny production (number of live weevils ± SE per 60 g of grain) of *S. oryzae* in grains treated with two doses of DEBBM at 25°C and 65% r.h. Within each dose means followed by the same lowercase letter are not significantly different. Within each grain, means followed by the same uppercase letter are not significantly different. (Uppercase letters for dose; Lowercase letters for grain. For the comparison of doses *df*=1, 11; For the comparison of grains *df*=6, 35; Tukey and Kramer HSD test at P<0.05).

3.2. Tribolium confusum

Mortality of *T. confusum* adults after exposure on DEBBM-treated grains was significantly for dose (7d exposure: $F_{2,125}$=47.2, P<0.001; 14 d exposure: $F_{2,125}$=11.5, P<0.001) and commodity type (7 d exposure: $F_{6,125}$=21.8, P<0.001; 14 d exposure: $F_{6,125}$=22.6, P<0.001) at both of the tested exposure intervals. After 7d of exposure, the maximum performance of 50 ppm of DEBBM was observed in treated oats (30%) and barley (22.2%) although 19.4% of exposed adults were killed in treated wheat (Fig.3). Although this DEBBM dose performed equally well on wheat and barley, significantly more beetles were killed in treated oats than treated wheat. A further increase of dose to 150 ppm resulted in a significant increase in DEBBM efficacy, which increased to 57.8 and 53.3% in treated barley and oats respectively (Fig. 3). The lowest DEBBM performance at 50 ppm was observed in maize or rice where it did not exceed 5%, while in the case of 150 ppm *T. confusum* appeared to be most susceptible in treated maize, rice and rye since mortality on these grains was lower than 35% (Fig. 3). Similar trends in DEBBM performance were observed 7 d later, although efficacy of both DEBBM doses was increased with the increase of exposure. Thus, the best DEBBM performance was observed in 150 ppm treated oats, barley, or wheat where DEBBM efficacy reached 80%, while approximately half of the exposed adults were killed on triticale treated with the same dose (Fig. 3). Even 14 d of exposure on 50 pmm treated maize, rice or oats, was not adequate to control *T. confusum* adults since very low (<12%) mortality was recorded in these grains, though mortalitiy did increase as the dose increased to 150 ppm (Fig.3). Progeny production of confused flour beetle on untreated commodities was 5.5 ± 0.8, 3.2 ± 0.6, 3.4 ± 0.6, 2.2 ± 0.4, 5.3 ± 0.7, 3.5 ± 0.9 and 5.8 ± 1.0 beetles per 60 g of barley, maize, oats, rice, rye, triticale and wheat respectively. In the case of treated commodities, the number of emerged beetles did not exceed 2.7 beetles per 60 g of treated commodity and thus, unlike mortality of this species, application rate ($F_{2,125}$= 1.7; P=0.091) or commodity type ($F_{6,125}$= 1.3; P=0.262) had not a significant impact on progeny production of this species on the DEBBM treated grains that were examined here (Fig. 4).

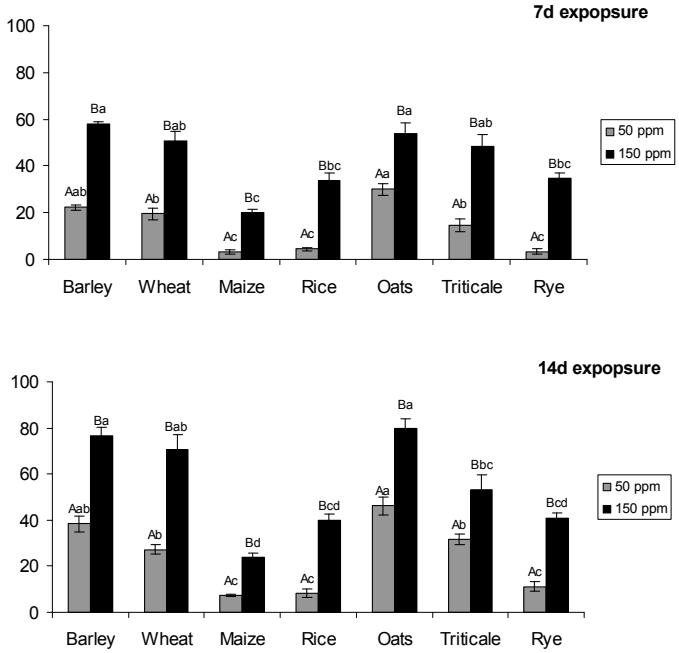

Figure 3 Mean (± SE) mortality of *T. confusum* adults after 7 d and 14 d exposure on seven grains treated with two doses of DEBBM at 25°C and 65% r.h. Within each exposure interval and application rate means followed by the same lowercase letter are not significantly different. Within each exposure interval and grain, means followed by the same uppercase letter are not significantly different. (Uppercase letters for dose; Lowercase letters for grain. For the comparison of doses *df*=1, 11; For the comparison of grains *df*=6, 35; Tukey and Kramer HSD test at P<0.05).

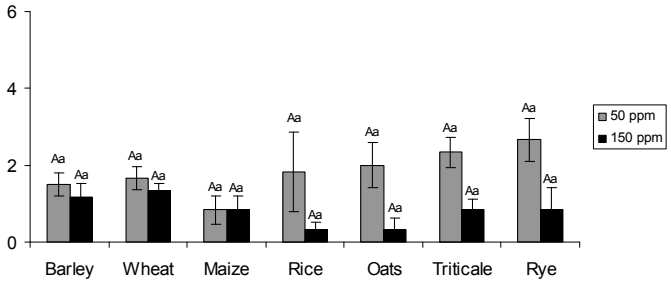

Figure 4 Mean progeny production (number of live beetles ± SE per 60 g of grain) of *T. confusum* in grains treated with two doses of DEBBM at 25°C and 65% r.h. Within each dose means followed by the same lowercase letter are not significantly different. Within each grain, means followed by the same uppercase letter are not significantly different. (Uppercase letters for dose; Lowercase letters for grain. For the comparison of doses df=1, 11; For the comparison of grains df=6, 35; Tukey and Kramer HSD test at P<0.05).

4. Discussion

Tribolium confusum is considered as one of the most tolerant species to DE (Korunic, 1998; Athanassiou et al., 2005) and the present study, this DE formulation appeared to be less efficacious against this species than *S. oryzae.* However, differential performance of DE does not occur only among target species but it can also occur among different commodities. Athanassiou et al. (2003) examined Silicosec, a commercially available pure DE formulation, in a variety of grain commodities and found high efficacy on some commodities but moderate or low effectiveness in others. This differential performance of DE among various commodities was observed also in the present study with the mixture of DE with BBM. Based on our results, the tested grains can be divided into three groups according to the descending order of DEBBM performance. The first group includes barley, wheat and oats, where DEBBM at the tested doses exhibited its highest efficacy levels, the second includes rice and maize where performance of DEBBM was low, while the third group includes triticale and rye (in some cases) where DEBBM exhibited intermediate efficacy levels. The fact that DE exhibits low performance in maize compared to other grains such as wheat or barley has been also reported by other researchers (Athanassiou et al., 2003; Kavallieratos et al., 2005). Athanassiou et al. (2003) found that effectiveness of Silicosec against *S. oryzae* was much lower in maize than in barley while this was also confirmed by a latter study of Kavallieratos et al. (2005) against *R. dominica* F. (Coleoptera: Bostrychidae). Kavallieratos et al. (2005) examined Silicosec and Insecto, which is also a pure DE formulation, in different grain commodities and found higher efficacy levels of these DEs in wheat or triticale than in barley against *R. dominica*. However, this was not the case with our results since DEBBM was either of equal or higher effectiveness compared to wheat against both *S. oryzae* and *T. confusum*. Although this contradictory finding requires further attention, the fact that different species were used in our study compared to Kavallieratos et al. (2005) may be considered as a possible explanation, since diet-preferences that vary among insect species (Baker, 1988; McGauchey et al., 1990) often determine their fitness and consequently their susceptibility to control methods (Athanassiou et al., 2008b).

Differences in morphological traits among kernels of different grains can often influence DE efficacy. The decreased performance of DEBBM in maize may be partially attributed to the wide spaces between the maize seeds that allow insects to crawl through them and thus avoid areas where DEBBM concentration was high (Athanassiou et al., 2003). Furthermore, differences in physicochemical properties of seed perikarp that exist among various grains may affect the retention of DE on kernel surface and thus lead to differential DE efficacy. Athanassiou and Kavalieratos (2005), reported that retention rate of DE on wheat, barley or rice was much higher than maize. The above result can explain the fact that DEBBM was not effective in maize but it can not explain the low performance of this formulation in rice. Although we did not examine retention rate of DEBBM to the different grains tested, it seems that in the case of rice kernels retention rate can not be used to explain efficacy of DEs. Although our findings in rice require further investigation the fact that DEs become gradually inactivated since they absorb oils from the perikarp of the rice kernels McGaughey (1972) could be considered as a

possible explanation for the decreased performance of DEBBM in this commodity. Contrary to maize or rice, DEBBM was shown to be effective in barley, wheat or oats and the fact that this high level of protection was achieved with low dose rates is very encouraging to also confirm the efficacy of this DE formulation under field conditions.

DEBBM was not able to suppress the progeny production of the tested species. Since DEs are slow acting insecticides they require a sufficient period of time to act (Subramanyam and Roesli, 2000; Vayias and Athanassiou, 2004; Athanassiou et al., 2005). It seems that during that lag period, treated parentals can mate or oviposit before they eventually die. However, despite that progeny production was not completely suppressed it was more than 9-fold lower in the treated grains than the untreated ones. Hence, we can estimate that DE and/or BBM negatively affected mating or oviposition of the parental individuals.

On conclusion, DEBBM applied at 150 ppm can provide satisfactory control against *S. oryzae* in wheat, barley or oats. However, in the case of *T. confusum* or in the case of rye, triticale, maize and rice, higher than 150 ppm application rates were required for a satisfactory performance of DEBBM. Thus, the labeled rate of such an enhanced DE formulation should be target species and commodity sensitive. In order for DEBBM to be effective, insects should come in contact with its particles. Hence, these low application rates of DEBBM should be also confirmed in field studies given that a significant proportion of grain mass, which remain untreated as long as DE formulations are applied at low levels, may provide shelter to insects that would have previously avoided the DEBBM particles and by this means application of DEBBM at low doses may be rendered ineffective.

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Influence of temperature and relative humidity on the efficacy of diatomaceous earth and *Metarhizium anisopliae* (Metschinkoff) Sorokin (Hyphomycetes: Deuteromycotina) against *Tyrophagus fatimii* F. (Astigmata: Acaridae)

Wakil, W.*#[1], Ghazanfar, M.U.[2], Mustafa, F.[1]
[1] Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan.
Email: waqaswakeel@hotmail.com
[2] Plant Pathology, College of Agriculture, D.G. Khan, Sub-Campus University of Agriculture, Faisalabad, Pakistan

* Corresponding author
# Presenting author

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Abstract

The combined as well as the alone effect of diatomaceous earth (DE) and entomopathogenic fungi were evaluated against *Tyrophagus fatimii* (Astigmata: Acaridae). Two different dose rates of DE (1 g and 1.5 g/kg of wheat) and three of the fungus *Metarhizium anisopliae* (Hyphomycetes: Deuteromycotina) (3.6 x 10^7, 3.6 x 10^8 and 3.6 x 10^9 conidia/kg of wheat) were taken and studied at 20°C and 25°C with 45% and 55% r.h. under three exposure intervals. It was found that the combined effect of DE diatomaceous earth and *M. anisopliae* was maximum at 25°C and 55% r.h. which gave 75% adult mortality at their highest dose rates, however, DE alone exhibited the highest mortality (61.3%) at 25°C and 45% r.h. On the other hand, *M. anisopliae* gave maximum mortality of mites (48.7%) at 20°C and 55% r.h. at 3.6 x 10^9 conidia/kg of wheat. It was concluded that the efficacy of both DE and *M. anisopliae* increased with the increase of the exposure interval. Moreover, the increase of dose increased the mortality. In addition, temperature and r.h. are the key factors for determining the effectiveness of both DE and *M. anisopliae*.

Keywords: Diatomaceous earth, *Tyrophagus fatimii*, *Metarhizium anisopliae*, Stored wheat.

1. Introduction

Stored products protection is of utmost importance to secure a continuous and safe food supply all over the world (Ferizli et al., 2005). Many chemicals are used as selective pesticides for different pests in stored grain which include insect growth regulator's (IGR's), organophosphates, pyrethroids and plant extracts (Sanchez-Ramos and Castanera, 2003). Organophosphates have been used since 1960's as one of the main sources of stored-product pest control, as admixture with grains or applied directly to the storage structures (Cook and Armitage, 2000). Nevertheless, today the control is focused on the use of pyrethroids as an alternative to some of the traditional organophosphates due to their quick action and low toxicity to humans (Hubert et al., 2007). On the other hand, injudicious use of chemicals leads to resistance development in stored grain mites (Zdarkova, 1994) and also increases residues in the stored product commodities.

Some new control strategies are under consideration for the last few decades which includes diatomaceous earth (DE) and entomopathogenic fungi, they are ecologically sound and may be used alone or combined for the control of stored grain insect pests (Wakefield et al., 2002; Ferizli et al., 2005; Athanassiou and Steenberg, 2007; Batta, 2008). DE is a naturally occurring substance that is mined from geological deposits of fossilized diatoms and is composed of mainly by silicon dioxide (Korunic, 1998; Cook et al., 2008) with low mammalian toxicity and provide grain protection with no toxic residues (Mahdi and Khalequzzaman, 2006). Entomopathogenic fungi are also valuable tools for IPM strategy (Sivasundaram et al., 2008) as many isolates have been shown effective against several insect pests (Batta, 2004; Hong et al., 2005; Cherry et al., 2007).

The idea of synergistic interaction between DE and entomopathogenic fungi is an interesting alternative to traditional pesticides in stored grain insect control (Michalaki et al., 2007). Synergistic interaction of DEs and entomopathogenic fungi has been observed against many species (Akbar et al., 2004; Kavallieratos et al., 2006; Athanassiou et al., 2008; Batta, 2008).

There are many data available for he combined use of DEs with fungi against stored-grain insects; however, there are few reports for the efficacy of this combination against stored-product mites. The present study investigates the sole and collective effect of DE and the entomopathogenic fungi

Metarhizium anisopliae (Metschinkoff) Sorokin (Hypomycetes: Deuteromycotina) in wheat against one stored-grain mite species.

2. Materials and methods

2.1. DE and fungal formulation

The DE used was Protect-It (Hedley Technologies, Mississauga, Ontario, Canada), which contains 83.7% SiO_2 with 10% silica aerogel. The *M. anisopliae* strain (WG01) was firstly isolated from *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) which was collected from a local rice storage structures in the periphery of District Faisalabad, Punjab, Pakistan and it was sub-cultured on synthetic media.

2.2. Commodity

Fresh, clean and infestation-free wheat was taken from the grain market of Faisalabad, Pakistan. The moisture content of the grain, as measured by a Dickey-John moisture meter, was 12.1%.

2.3. Mites

Infested samples of wheat grains were collected from different storage structures and flour mills of Faisalabad district and the stored grain mites were separated by using Berlese funnel. They were reared on the diet containing yeast and organic whole meal wheat flour at the ratio of 3:1. The mites were taken into a conical flask with 2-3 spatulas of diet. The top of the flask was closed by using cotton plug in such a way that the mites could not escape from the conical flask. When the culture was prepared, it was placed into the incubator at 20°C and 80% r.h. The culture was inspected after one week and shifting was carried out after 15 d in a new flask.

2.4. Bioassay

Three dose rates of *M. anisopliae* used in the experiment were 3.6×10^7, 3.6×10^8 and 3.6×10^9 conidia/kg of grain, while the DE doses were 1 and 1.5 g/kg. The wheat was treated with each dose rate of both DE and entomopathogenic fungi alone and also in their possible combinations. Samples of 60g of treated wheat for each dose rate were taken into three separate plastic jars (replicates) also three jars with untreated wheat were prepared which served as a control. Then, 30 adult mites of same age were added in each jar and the top of the jars were closed with muslin cloth to maintain the aeration. The treatments were kept into incubators and experiments were conducted at different sets of temperature (20 and 25°C) and r.h. (45 and 55%). The r.h. levels of 45 and 55% were kept constant in the incubator at 20°C by using the salts of K_2HPO_4 and $NiCl_2.6H_2O$ respectively; similarly, the conditions were maintained at 25°C by using the salt of Potassium Carbonate for 45% r.h. and Magnesium Nitrate for 55% r.h. (Winston and Bates, 1960; Greenspan, 1977). Mite mortality was assessed after 5, 10 and 15 d of exposure. The data for mortality was recorded by counting all the live and dead mites in each replication.

2.5. Statistical analysis

The mortality of the mites was tested statistically by using the GLM procedure through MINITAB software after correcting the mean mortalities by using Abbott's (1925) formula. Every exposure interval was subjected to one-way analysis of variance separately; the mortality for the control was very low so not included in the analysis. Means were compared by using Tukey-Kramer test at $P \leq 0.05$ (Sokal and Rohlf, 1995).

3. Results

3.1. Mortality (%) of T. fatimii after 5 days

Main effects and interactions were significant at $P<0.05$ (treatments df=10,131; F=69.4; temperature df=1,131; F=56.7; r.h. df=1,131; F=24.2; treatment x temperature df=10,131; F=5.7; treatment x r.h. df=10,131; F= 3.2) with the exception of temperature x r.h. (df=1,131; F=0.1; P=0.7) and treatment x temperature x r.h. (df=10,131; F=0.1; P=1.0). The highest mortality (66.7%) was obtained at 25°C and 55% r.h. with the higher dose rates of DE and *M. anisopliae*. However, in the case of DE alone the highest mortality obtained was 41.1% at 25°C and 45% r.h. combination, while in the case of *M. anisopliae*, the highest mortality was 38.9% at 20°C and 55% r.h. (Table 1).

Table 1 Mean mortality of *Tyrophagus fatimii* (% ± SE) after 5 days of exposure on wheat treated with *Metarhizium anisopliae* and DE at different temperatures (20ºC, 25ºC) and r.h. (45%, 55%) at different dose rates; DE_1=1g/kg DE_2=1.5g/kg Ma_1=3.6x10^7 Ma_2=3.6x10^8 Ma_3=3.6x10^9 (within each column, means followed by the same letters are not significantly different; Tukey and Kramer HSD test; df=10,32)

	Mortality (%) ± SE			
	45% r.h.		55% r.h.	
Treatments	**20º C**	**25º C**	**20º C**	**25º C**
DE_1	27.7 ± 4.01cd	38.8 ± 2.94cde	23.3 ± 1.92f	30.0 ± 1.92f
DE_2	30.0 ± 1.92cd	41.1 ± 2.94cd	26.6 ± 1.92ef	36.6 ± 1.92def
Ma_1	21.1 ± 2.94d	17.7 ± 2.94g	28.8 ± 2.94def	25.5 ± 2.94f
Ma_2	26.6 ± 1.92cd	22.2 ± 2.94fg	34.4 ± 2.94cdef	30.0 ± 1.92f
Ma_3	30.0 ± 1.92cd	26.6 ± 1.92efg	38.8 ± 2.94bcde	34.4 ± 4.01ef
DE_1 + Ma_1	24.4 ± 2.94d	33.3 ± 1.92def	27.7 ± 2.94def	38.8 ± 2.94def
DE_1 + Ma_2	28.8 ± 2.94cd	40.0 ± 1.92cde	34.4 ± 2.94cdef	44.4 ± 2.94cde
DE_1 + Ma_3	34.4 ± 4.01bcd	45.5 ± 2.94bcd	40.0 ± 1.92bcd	50.0 ± 1.92bcd
DE_2 + Ma_1	40.0 ± 1.92abc	48.8 ± 2.94abc	45.5 ± 2.94abc	54.4 ± 2.94abc
DE_2 + Ma_2	46.6 ± 1.92ab	55.5 ± 2.94ab	51.1 ± 2.94ab	61.1 ± 2.94ab
DE_2 + Ma_3	51.1 ± 2.94a	61.1 ± 2.94a	56.6 ± 1.92a	66.6 ± 1.92a
ANOVA	F = 11.4	F = 25.0	F = 16.5	F = 26.1
	P < 0.01	P < 0.01	P < 0.01	P < 0.01

3.2. Mortality (%) of T. fatimii after 10 days

Main effects and interactions were significant at P <0.05 (treatments df=10,131; F=30.9; temperature df=1,131; F=26.4; r.h. df=1,131; F=11.3; treatment x temperature df=10,131; F=2.8; treatment x r.h. df=10,131; F=1.8) with the exception of temperature x r.h. (df=1,131; F=0.1; P=0.7) and treatment x temperature x r.h. (df=10,131; F=0.1; P=1.0). The highest mortality (70.8%) was obtained at 25ºC and 55% r.h. with the collective highest dose rates of DE and *M. anisopliae*. In the case of DE alone, the highest mortality recorded was 49.5% at 25ºC and 45% r.h., while mortality for *M. anisopliae* alone was 43.9%, at 20ºC and 55% r.h., at 3.6 x 10^9 conidia/kg of wheat (Table 2).

Table 2 Mean mortality of *Tyrophagus fatimii* (% ± SE) after 10 days of exposure on wheat treated with *Metarhizium anisopliae* and DE at different temperatures (20°C, 25°C) and r.h. (45%, 55%) at different dose rates; DE_1=1g/kg DE_2=1.5g/kg Ma_1=3.6x10^7 Ma_2=3.6x10^8 Ma_3=3.6x10^9 (within each column, means followed by the same letters are not significantly different; Tukey and Kramer HSD test; df=10,32)

	Mortality (%) ± SE			
	45% r.h.		55% r.h.	
Treatments	**20°C**	**25º C**	**20° C**	**25° C**
DE_1	33.6 ± 2.17bcd	46.0 ± 6.93abcd	30.5 ± 2.90d	35.0 ± 2.51cd
DE_2	39.8 ± 2.64abcd	49.5 ± 6.35abcd	34.9 ± 2.32cd	43.7 ± 3.81bcd
Ma_1	25.2 ± 1.53d	21.7 ± 2.17e	34.3 ± 0.52cd	29.8 ± 0.99d
Ma_2	30.4 ± 2.21cd	27.0 ± 2.27de	38.9 ± 0.55bcd	34.9 ± 1.81cd
Ma_3	34.9 ± 0.88bcd	30.2 ± 2.32cde	43.9 ± 4.51bcd	39.2 ± 2.62bcd
DE_1 + Ma_1	29.7 ± 4.20cd	38.3 ± 0.92bcde	33.9 ± 1.79cd	43.5 ± 1.19bcd
DE_1 + Ma_2	34.3 ±0.52bcd	44.3 ± 1.79abcde	39.2 ± 3.23bcd	49.8 ± 1.81abcd
DE_1 + Ma_3	39.2 ± 2.62abcd	50.7 ± 2.59abc	44.4 ± 2.78bcd	55.8 ± 4.32abc
DE_2 + Ma_1	44.6 ± 4.25abc	54.6 ± 5.16ab	49.2 ± 2.59abc	58.4 ± 0.83ab
DE_2 + Ma_2	50.0 ± 3.13ab	60.8 ± 7.13ab	55.4 ± 7.43ab	67.3 ± 8.67a
DE2 + Ma3	55.3 ± 7.03a	66.2 ± 6.38a	61.3 ± 1.72a	70.8 ± 9.08a
ANOVA	F = 7.4	F = 9.3	F = 8.5	F = 9.5
	P < 0.01	P < 0.01	P < 0.01	P < 0.01

3.3. Mortality (%) of T. fatimii after 15 days

Main effects and interactions were significant at P<0.05 (treatments df=10,131; F=12.4; temperature df=1,131; F=13.3; r.h. df=1,131; F=4.4) with the exception of treatment x temperature (df=10,131; F=1.5; P=0.1); treatment x r.h. (df=10,131; F=0.7; P=0.7); temperature x r.h. (df=1,131; F=0.2; P=0.6); treatment x temperature x r.h. (df=10,131; F=0.0; P=1.0). The highest mortality due to the application of DE alone was 61.3% at 25°C and 45% r.h., while in the case of *M. anisopliae* alone the maximum mortality was 48.7% at 20°C and 55% r.h. at their highest dose rates. The results for the highest dose rate of DE and *M. anisopliae* exhibited the highest mean mortality (75%) at 25°C and 55% r.h. (Table 3).

Table 3 Mean mortality of *Tyrophagus fatimii* (% ± SE) after 15 days of exposure on wheat treated with *Metarhizium anisopliae* and DE at different temperatures (20°, 25°C) and r.h. (45%, 55%) at different dose rates; DE_1=1g/kg DE_2=1.5g/kg Ma_1=3.6x10^7 $_{Ma2}$=3.6x10^8 Ma_3=3.6x10^9 (within each column, means followed by the same letters are not significantly different; Tukey and Kramer HSD test; df=10,32)

Treatments	Mortality (%) ± SE			
	45% r.h.		55% r.h.	
	20°C	25°C	20°C	25°C
DE_1	39.6±5.20abc	57.0±1.78ab	35.1±1.85c	48.4±4.56abc
DE_2	45.2±4.80abc	61.3±6.21ab	39.6±1.65bc	53.3±8.82abc
Ma_1	30.1±1.65c	25.7±2.11b	39.9±6.85bc	34.2±3.09c
Ma_2	35.0±3.07bc	31.1±4.10ab	44.8±4.91abc	38.9±4.47bc
Ma_3	39.0±2.08abc	36.1±5.42ab	48.7±3.77abc	43.0±8.52abc
DE_1 + Ma_1	33.1 ± 1.31bc	43.3 ± 3.44ab	39.1 ± 4.19bc	48.1 ± 4.30abc
DE_1 + Ma_2	38.2 ± 2.75bc	50.0 ± 5.77ab	44.8 ± 2.60abc	56.0 ± 3.62abc
DE_1 + Ma_3	44.8 ± 2.60abc	54.1 ± 8.33ab	49.6 ± 2.92abc	59.8 ± 1.55abc
DE_2 + Ma_1	50.0 ± 2.62abc	61.6 ± 7.26ab	56.5 ± 3.62abc	65.5 ± 8.68abc
DE_2 + Ma_2	54.8 ± 9.80ab	66.6 ± 8.33ab	61.5 ± 9.63ab	71.1 ± 4.44ab
DE_2 + Ma_3	62.5 ± 7.22a	72.2 ± 20.03a	66.6 ± 6.67a	75.0 ± 14.43a
ANOVA	F = 4.4	F = 3.4	F = 4.0	F = 3.5
	P < 0.01	P = 0.007	P = 0.0031	P = 0.0069

4. Discussion

There are numerous studies available on the efficacy of DE and entomopathogenic fungi for the control of stored grain insect pests but there are few studies available for stored-grain mites (Cook and Armitage, 1999, 2000; Wakefield et al., 2002; Palyvos et al., 2006; Athanassiou and Palyvos, 2006; Cook et al., 2008). In the present experiment, the effectiveness of both DE and entomopathogenic fungi was evaluated against *T. fatimii* alone or in combination in various doses.

Mortality of *T. fatimii* was increased with the increase in the dose rate of *M. anisopliae.* However, the efficacy was increased by the addition of the lowest dose of DE at 25°C and 55% r.h. This was also confirmed by Michalaki et al. (2006) who suggested that the DE promoted the action of fungi when the conidial concentration was at a certain threshold level. In fact, the presence of the DE particles may damages the conidia due to their abrasive action, but by increasing the dose rate of fungi, the number of undamaged conidia can increased the insecticidal efficacy.

The temperature plays a key role in determining the effectiveness of DE and entomopathogenic fungi (Arthur, 2000; Michalaki et al., 2006). At high temperature water loss from the insect's body occurs quickly; also, the insects move faster, and more DE particles are attached with the cuticle (Fields and Korunic, 2000). Our results are contradictory to Michalaki et al. (2006) who studied the effectiveness of *M. anisopliae* against *T. confusum* and found that the increase of temperature increases the efficacy of *M. anisopliae*. Our results are in accordance with the results reported by Moore et al. (1996), where the increase of temperature negatively affected the germination of conidia. Moreover, Hedgecock et al. (1995) and Moore and Higgins (1997) reported the viability of the conidia was seriously reduced at temperature 30°C as compared to -10°C during storage. High temperature (35°C) reduces the fungal germination (Sun et al., 2003), while Ouedraogo et al. (1997) reported 25°C as optimum temperature for

M. anisopliae. In our case, 25°C was the best for the germination of conidia of *M. anisopliae*. The influence of temperature on the effectiveness has yet not been explored and needs additional attention.

Another abiotic factor, the relative humidity is also very important as our results indicated that the mortality of *T. fatimii* was maximum at 55% r.h. when the DE and *M. anisopliae* was used collectively. These results are in agreement with Michalaki et al. (2006) who proved that *M. anisopliae* was more effective at 55% r.h. against *T. castaneum*. The efficacy of DEs is also affected by the change of r.h. in the commodity as DE particles absorb moisture from the air and their ability to attach with the insect cuticle decreases (Stathers et al., 2004). Shi et al. (2008) reported that conidial germination depends on the r.h. and temperature as they proved that the fungal action was significant at 20-25°C but not at 30°C, also, the effect of temperature was pronounced at 51-74% r.h.

There are not many data available for the efficacy of DE and entomopathogenic fungi alone and in combination under different temperature and r.h. regimes against stored-grain mites. It was concluded that combined dose rates of both DE and *M. anisopliae* gave highest mortality at 25°C and 55% r.h. after 15 d of exposure. It was also observed that mite mortality increased with the increase of temperature and r.h. The results may provide the basic information for a reduced-risk control of stored-grain mites but more focused research is needed to exploit the best alternatives for a successful IPM program.

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Efficacy assessment of diatomaceous earth against *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) on gram at different temperature and relative humidity regimes

Wakil, W.*[1], Ghazanfar, M.U.#[2], Ashfaq, M.[1], Ali, K.[1], Riasat, T.[1]
[1] Department Of Agricultural Entomology, University Of Agriculture, Faisalabad, Pakistan.
Email: waqaswakeel@hotmail.com
[2] Plant Pathology, College Of Agriculture, D.G. Khan, Sub-Campus University Of Agriculture, Faisalabad, Pakistan

* Corresponding author
# Presenting author

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Abstract

The efficacy of diatomaceous earth against *Callosobruchus maculatus* (Coleoptera: Bruchidae) was evaluated on stored gram under laboratory conditions. The bioassay was conducted at 25 and 30°C in combination with 50 and 60% r.h. Diatomaceous earth (DE) formulation (Diafil 610), at the dose rates of 200, 400, 600 and 800 ppm was admixed with gram grains. Fifty unsexed adults of *C. maculatus* were released in each jar and treatments replicated thrice. Mortality data was recorded after 2, 3 and 5 days of exposure intervals and after every count the dead individuals were removed, and the commodity was maintained for an additional period of 25 d, in order to record the emergence of F1 adults. The results showed that all treatments were highly effective against the bruchids; however, the highest mortality (100%) was observed at 30°C and 50% relative humidity at 800 ppm of DE with minimal progeny production.

Keywords: Diatomaceous earth, *Callosobruchus maculatus*, Temperature, relative humidity, Gram

1. Introduction

The pulses are considered to be an important source for fulfilling the protein needs for low income groups of the population in many regions of Asia. *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) is a primary grain beetle (Cherry et al., 2007) which is widely distributed throughout the world and also causes considerable damage in stored gram. The losses in seed weight due to this beetle was estimated at 55-60% (Gujar and Yadav, 1978) and also 45.5-66.3% in protein content in India; similarly, 60% losses due to *C. maculatus* were reported in Nigeria during three months of cowpea storage (Caswell, 1981). Moreover, weight losses up to 30% occurred after six month of storage which made 70% of the grain unfit for human consumption (Singh and Jackai, 1985). Also, in Benin, 100% losses was inflicted due to *C. maculatus* and *Bruchidus atrolineatua* (F.) after few months of storage (Kossou et al., 2001). This situation makes necessary the application of control measures, in order to minimize the losses caused by this pest.

Admixture of diatomaceous earth (DE) formulations with dry grain is an excellent method of protecting stored products as they can be used as replacement to traditional chemicals. They have very low mammalian toxicity, are non reactive, leave no residues on grains (Cook and Armitage, 2000); control the insecticide resistant pests and long-lasting (Vayias et al., 2006). DEs absorb the wax layers of the insect's cuticle, causing desiccation and mortality due to water loss (Subramanyam and Roesli, 2000; Korunic and Fields, 2006). There are several commercially available DE formulations which have been successfully evaluated as stored grain protectants against a wide range of insect species (Korunic, 1998; Arthur, 2000; Fields and Korunic, 2000; Subramanyam and Roesli, 2000; Athanassiou et al., 2003; 2004; 2005; Stathers et al., 2008).

In Australia, Germany, USA, UK and other European countries DEs are used with success against different stored grain insect pests; however, in the Asian sub-continent DEs are not in use for stored-product protection. The purpose of this study is to evaluate the efficacy of DE at different concentrations, temperature and relative humidity levels against *C. maculatus*. The capacity for progeny production in the treated substrate was also recorded.

2. Materials and methods

2.1. Test insect

Callosobruchus maculatus was reared in the IPM laboratory in the Department of Agric. Entomology, University of Agriculture, Faisalabad (Pakistan) on the mung beans (*Vigna radiata* L.) in plastic jars, at 28 ± 2°C and 55-60% r.h. with photoperiod 14:10 L:D.

2.2. DE formulation

The DE used was DiaFil 610 (Celite Corporation, USA), which is a white fresh water DE containing 89% amorphous silicon dioxide, 4.0% Al_2O_3, 1.7% Fe_2O_3, 1.4% CaO, less than 1% of MgO and K_2O and 3% moisture. The median particle size is 10 microns, specific gravity is 2.2, surface area is 35.7 m2/g, pH is 8 and crystalline silica is >0.1%.

2.3. Bioassay

The study was conducted under two different temperatures (25 and 30°C) in combination with 50 and 60% r.h. The dose rates applied were 200, 400, 600 and 800 ppm. DE was mixed thoroughly for 2-3 minutes with the gram grains separately. Then, for each dose, the jars were kept undisturbed for 30 minutes so as to allow the dust to settle down. A lot of 150 grams of treated grains was divided into three parts of 50 g for each dose rate, so 15 cups (replicates) including untreated control were prepared for each set of temperature and relative humidity. Fifty unsexed adults of *C. maculatus* per treatment were placed into each jar and the opening of the jars was tightly closed with muslin cloth to avoid beetle escaping. The mortality data were recorded after three exposure intervals (2, 3 and 5 d) and after every count the dead adults were removed from the jars. After the last count the jars were kept for 25 d more in order to record progeny production of *C. maculatus*.

2.4. Statistical analysis

The mortality of *C. maculatus* was corrected by using Abbott's (1925) formula, and then the mortality data was subjected to the statistical analysis (MINITAB) and the means were separated by Tukey-Kramer test at $P = 0.05$. The control mortality was not included in the analysis as there was less than 3% mortality in the control jars. The same procedure was followed for the analysis of progeny data, but in this case control progeny was also included.

3. Results

3.1. Mortality of C. maculatus

The analysis of variance showed that most of the main effects were significant; however, their interactions were non significant (Table 1). The highest beetle mortality after 2 d of exposure (72.6%) was recorded where the highest dose rate of DE was applied at 30°C and 50% r.h. (Fig. 1). Similarly, after 3 d of exposure at the same conditions, the highest dose of DE gave 92.6% mortality (Fig. 2). The DE treated grains which were kept for 5 d exhibited 81.1% mortality at 25°C and 60% r.h. (Fig. 3) which was lower than the mortality at 25°C and 50% r.h. The highest mortality (100%) was recorded at 30°C and 50% r.h. and the lowest (91.7%) at 30°C and 60% of r.h.

Table 1 ANOVA for main effects and their associated interactions for mortality of *C. maculatus* after 2, 3 and 5 d of exposure of Diafil 610 (total df = 47; blank spaces in columns of *P* values are non-significant).

Parameters	**df**	**2 d**		**3 d**		**5 d**	
Temperature	**1**	**42.71**	**<0.001**	**18.28**	**<0.001**	**3.35**	**0.07**
Relative humidity	1	86.63	<0.001	13.82	0.001	3.90	0.05
Dose	3	137.59	<0.001	35.29	<0.001	6.12	0.002
Temperature x relative humidity	1	0.63	0.43	0.12	0.73	0.00	0.98
Temperature x dose	3	1.32	0.28	1.03	0.39	0.08	0.96
Relative humidity x dose	3	0.38	0.76	0.02	0.99	0.01	0.99
Temperature x relative humidity x dose	3	0.08	0.97	0.03	0.99	0.00	1.00
Error	32	-	-	-	-	-	-

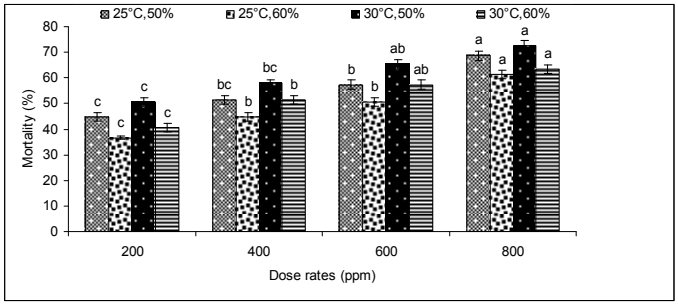

Figure 1 Means comparison of the data regarding the mortality (% ± SE) of *Callosobruchus maculatus* at different dose rates of diatomaceous earth in stored gram after 2 d of exposure (means followed by the same letters are not significantly different with each other in the temperature and relative humidity).

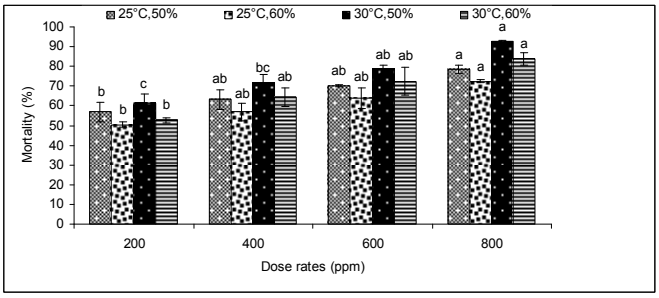

Figure 2 Means comparison of the data regarding the mortality (% ± SE) of *Callosobruchus maculatus* at different dose rates of diatomaceous earth in stored gram after 3 d of exposure (means followed by the same letters are not significantly different with each other in the temperature and relative humidity).

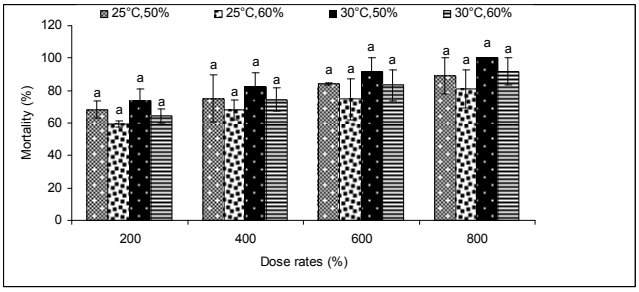

Figure 3 Means comparison of the data regarding the mortality (% ± SE) of *Callosobruchus maculatus* at different dose rates of diatomaceous earth in stored gram after 5 d of exposure (means followed by the same letters are not significantly different with each other in the temperature and relative humidity).

3.2. Production of F1

The analysis of variance showed that all the main effects were significant (temperature $F_{1,59}$ = 685.9, P <0.001; relative humidity $F_{1,59}$ = 182.4, P <0.001; dose rates $F_{4,59}$ = 1498.6, P <0.001) and their interactions were not significant. The maximum numbers of offspring were in the control jars (94.3 adults per jar) which were significantly different from all other treatments (Fig. 4). Maximum dose (800 ppm) of DE showed minimum development of the bruchids (5.3 adults per jar) as compared to minimum dose rate of DE.

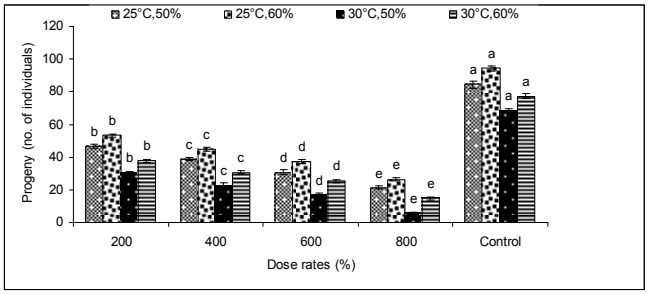

Figure 4 Means comparison of the data regarding the production of progeny (number of live adults ± SE per jar) of *Callosobruchus maculatus* at different dose rates of diatomaceous earth in stored gram (means followed by the same letters are not significantly different with each other in the temperature and relative humidity).

4. Discussion

The diatomaceous earths have proved successful for the management of different stored grain insect pests and can be replaced with the conventional insecticides (Islam et al., 2009) but their overall efficacy depends upon different factors, such as type and concentration of DE, grain moisture content, temperature, insect species, insect density and type of grain commodity (Korunic, 1997; Rigaux et al. 2001; Fields et al., 2003; Korunic and Fields, 2006). Among these factors the temperature and relative humidity plays an important role in determining the efficacy of DE against stored grain insect pests (White and Loschiavo, 1989). We have concluded in the present study that the DE used (Diafil 610) proved effective at high temperature and low relative humidity for the control of *C. maculatus*. These findings are in agreement with findings from previous studies (Arthur, 2000; Fields and Korunic, 2000; Mewis and Ulrichs, 2001; Vayias and Athanassiou, 2004; Vayias and Stephou, 2009). Generally, water loss in the insect's body may be increased when humidity or moisture is low (Fields and Korunic, 2000; Stathers et al., 2004). However, Akbar et al. (2004) reported that there was no significant effect of relative humidity on the efficacy of DE against the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae).

High temperature enhances the effectiveness of DE (Korunic, 1996; Mewis and Ulrichs, 2001) as mortality is increased with the increase of temperature (Arthur, 2000; Athanassiou et al. 2004). Our results for *C. maculatus* also demonstrated similar findings on the effect of temperature on DE efficacy. The possible reason of higher mortality at higher temperature would be due to the fact that at high temperature the mobility of the insect increases which provide more chance for the attachment of DE with the cuticle (Fields and Korunic, 2000). Also, there is enhanced metabolic activity which eventually increases the loss of water from the body of an insect (Ceruti et al. 2008). However, Athanassiou et al. (2007) showed that there was high mortality of the insect pest at 20ºC in comparison with 30ºC.

Longer exposure interval suppresses the progeny emergence in the treated substrate provided the dry conditions prevails (Athanassiou et al., 2003; 2005). Also, also the higher dose rate was negatively correlated with the production of F1 of *Sitophilus* spp. (Paula, 2001). The present trial undoubtedly supports the statements of other researchers as there was less progeny at high dose rates and longer exposure intervals.

In storage structures, DEs may prove good alternative to the traditional fumigants and synthetic insecticides, in an effort to provide residue-free commodities for the consumers. The results clearly indicated that the *C. maculatus* was controlled at higher temperature and lower relative humidity with less production of progeny at the higher dose rates of DE. The utmost effort should be focused on storing the grain commodities at higher temperature and lower r.h. combinations in order to benefit for the application of a DE-based strategy.

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Effects of methoprene on extreme temperature tolerance and reproduction of *Tribolium castaneum* (Coleoptera: Tenebrionidae)

Wijayaratne, L.K.W.#[1,2], Fields, P.G.*[2]
[1] Department of Entomology, University of Manitoba, 12 Dafoe Road, Winnipeg, Manitoba, Canada R3T 2N2
[2] Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba, Canada R3T 2M9.
Email: paul.fields@agr.gc.ca

* Corresponding author
# Presenting author

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Abstract

The juvenile hormone analogue methoprene is a reduced-risk insecticide. It disrupts insect development of immature stages preventing the emergence of adults. Several studies have shown that lower concentrations that permit the emergence of adults also have sub-lethal effects. Exposure to methoprene (Diacon II) at 3.33 ppm reduced the heat tolerance of *Tribolium castaneum* (Herbst) adults. However, it did not affect the heat tolerance of larvae at 0.07 ppm. Higher concentrations of methoprene were lethal to larvae without heat treatment. Methoprene (67 ppm) had no effect on the cold tolerance of adults. Furthermore, methoprene (0.03 ppm) did not alter cold tolerance of larvae. Exposure to 15°C for 2 weeks increased the cold tolerance of adults from 4 d to 7 d, and larvae 3 d to 5 d; however, methoprene concentrations had no effect on cold tolerance. *Tribolium castaneum* larvae exposed to methoprene (0.001 ppm) had lower fecundity as adults. Males were more affected than females in reducing the offspring when paired with untreated mates. These results show the potential of methoprene as an emerging insecticide and a viable alternative to currently used synthetic insecticides. The data on the effect of methoprene on extreme temperature tolerance of *T. castaneum* have been submitted to the Journal of Stored Products Research.

Keywords: Methoprene, Extreme temperature tolerance, Reproduction, Larvae, Adults

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Evaluation of inert dusts against phosphine resistant strains of *Cryptolestes ferrugineus*

Cao, Y. *#, Li, Y., Li, G.
Academy of State Administration of Grain, P.R. China, Beijing 100037. Email: lyy@chinagrain.org

* Corresponding author
# Presenting author

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Abstract

The relative phosphine resistance of two strains of *Cryptolestes ferrugineus* was measured by the method of knockdown at 2 mg/L of phosphine. The efficacy of five inert dusts (Puliangtai, G-1, G-2, G-3 and G-4) against the adults of two strains (JXCF and YYCF) of *C. ferrugineus* was assessed. The insects were exposed to the five inert dust on filter paper inside petri dishes for 1 day at 30±1°C and 65±1% r.h. Then, the adults were held for 4 days with food at the same conditions without inert dust in surface bioassay. Also, they were placed in 25±1 °C and 65±1% r.h. in grain bioassay. In surface bioassay, the two strains of *C. ferrugineus* were susceptible to the five inert dusts at 0.08 g/m^2 and 0.1 g/m^2. G-3 appeared the most effective against both strains, since its efficacy was 3-6 times higher than the other four dusts tested. YYCF strain was more susceptible than JXCF, with 1-4 times higher mortality level. The five inert dusts were also effective in grain bioassay. This research indicated that inert dusts were effective on phosphine-resistant *C. ferrugineus* populations.

Keywords: Inert dust, *Cryptolestes ferrugineus,* Phosphine resistance

1. Introduction

In the recent years, many relevant reports have been published on phosphine resistance of *Crytolestes. ferrugineus* (Stephens) (Jiang et al., 1995; Liu et al., 2003 ;Lin et al., 2004; Liu, 2004; Wang et al., 2004; Yan. et al., 2004). The efficacy of phosphine is not satisfactory to keep *C. ferrugineus* under control in the high temperature and humidity conditions, where *C. ferrugineus* can survive at phosphine concentrations below 200 mL/m (Lu et al., 2005). Some resistance strains could be killed effectively only when phosphine concentration reaches 550 ppm for 45 days (Pang et al., 2002). Under these circumstances phosphine concentration has to be increased. However, higher concentrations of phosphine causes the insect pests narcosis (Lu et al., 2005); also, the higher concentration of phosphine will increase the risk of phosphine residues in grains. Thus, it is essential to develop alternative methods to phosphine for the control of this pest. This research assessed the efficacy of the inert dust against *C. ferrugineus* as the possible alternative control treatment.

2. Materials and methods

2.1.Insects

Two strains of *C. ferrugineus* collected from Jiangxi Wannianzhongshen State Grain Reserve, JXCF Strain, and from Yiyang Depot, YYCF strain, directly under the Central Grain Reserves.

The insects from the two strains were reared in a mixture of oatmeal, wheat flour and yeast (at ratio of 6:3:1-w/w/w) at 30±1°C and 75±5% r.h. Three days later the adult insects were removed into clean feeding bottle and maintained, and the eggs were maintained in the original bottle until they grew up into adults; then, we chose the adults 2 –weeks after expression as test insects. The strains mentioned above have been maintained for generations in the Stored Product Insect Pests Controlling Laboratory of the Academy of State Administration of Grain.

2.2. Test chemicals

Puliangtai, G-1, G-2, G-3 and G-4 were five inert dusts there were selected by the laboratory mentioned above.

2.3. Test for phosphine resistance

We placed 10 test insects into the fumigated knockdown test bottle of 100-500 mL airtight with a rubber plug. A certain amount of PH_3 gas was discharged into the bottle until the concentration of PH_3 was 2

mg/L. The insects were considered to be in the condition of knockdown when they were in spasms or paralysis. The paralysis duration of each test insects (the value of KT) was recorded. The experiments of each strain were repeated three times (Cao and Zhang, 2000).

2.4. Inert dust surface bioassay

The clean culture dish (the bottom with 70 mm diameter, upper lid of 75mm diameter) was dried and sterilized at 100°C for 2 hours. After cooling, the dish internal bottom was pasted with the round filter paper with the same diameter as the bottom without any gaps. Moreover, the wall of the dish was coated with polytetrafluoroethylene (PTFE) with brush to make the internal wall smooth to prevent the insects from escaping. The dish was dried at 60 °C for 30 minutes before use. The test insecticide was weighed and placed into the dishes. The dish was covered with lid and shaken manually for several times on the test-bed. After the suspending insecticide settling down the lid was removed carefully. The test inert dust was distributed as evenly as possible.

The test insects were taken on the day before the experiment. The insects were placed into the clean petri dish with the filter paper without food in order to remove their foreign substance as their crawling. The insects were placed into two desiccators randomly and exposed in the conditions of 30 ± 1°C and 65 ± 1% r.h. for 12 h. During the test, 20 insects were placed in the prepared inert dust in surface bioassay, and a compared group (three replicates) was put into each desiccators. After treated under the experiment conditional for 1 day, the insects were removed gently to another prepared clean Petri dish without the insecticide with a small brush. With adequate amount of food added, the insects were maintained and observed at the same temperature and humidity for 4 following days. The mortality of the insects was checked and recorded every day.

2.5. The method of inert dust in grain bioassay

The glass can bottle of with 70 mm diameter × 150 mm was dried and sterilized at 100°C for 2 hours, and cooled for use. A circular belt of PTFE of about 2 cm width on the internal wall of the bottle to prevent the insects from climbing up. Then, the bottle was dried and sterilized at 60°C for 30 min and taken out for use. We weighed 100 g wheat (adding 0.5% cracked wheat) and put it into the prepared bottle, then weighed the experimental chemicals by the method of weighing by difference and put it into the bottle with wheat. The bottle was covered with cloth and sealed with rubber band. After this procedure, the bottle was shaken manually for 5 minutes. Each dosage series was repeated three times.

The test insects were taken on the day before the test, and then placed in the clean Petri dish with the filter paper with no food in order to remove the foreign materials as their crawling. The insects were exposed in the environmental conditions of 30 ± 1°C and 65 ± 1% r.h. for 12 hours. During the experiment 20 insects were taken and placed into the bottle with the inert dust in grain, and placed to the desiccators as noted above. Every day the mixed grain was gently poured onto another prepared tray, and the death of the test insects was checked with a brush. The mortality were checked and recorded for the 6 following days.

Toxicity results were analyzed by probit analysis method (Finney, 1971). The calculation of the mean, standard error and multiple comparisons were analyzed by Microsoft ® Excel 2000, DPS data processing software (Tang and Feng, 1996)

3. Results

3.1. Phosphine knockdown test

Table 1 indicated the knockdown duration of 50% insects of each strain of *C. ferrugineus*. KT_{50} was far higher than 30 min. KT_{50} value of the susceptible to phosphine strains was basically correspondingly low by the method recommended by the FAO (1975) while KT_{50} value of phosphine resistance strains was basically large (Wang et al., 2004), which indicated that phosphine resistance of the two strains was very high.

Table 1 Knockdown duration of 50% of two strains of *C. ferrugineus* (KT_{50}).

Insect strains	KT_{50}
YYCF	5 days
JXCF	3 days

3.2. Inert dust surface bioassay

Table 2 indicated that the low concentration of the inert dust was effective against phosphine resistance *C. ferrugineus*. Especially for G-3, its concentration below 0.08 mg/m^2 can completely kill adults of YYCF strain. The effects of inert dusts against pests are better than Puliangtai with the concentration of mg/m^2 except the G-4 against JXCF. Under the same experiment conditions, the mortality was significantly increased, and almost reached 100% when the two strains were treated with dust concentration of 0.1 mg/m^2. *Crytolestes ferrugineus* was very sensitive to the inert dusts, which further proved the results by Liu (2005).

Table 2 Efficacy of inert dusts in surface bioassay against *Cryptolestes ferrugineus.*

Strains	Concentration (mg/m^2)	Inert dust	Mortality rate (%)
#	0.08	Puliangtai	18.0±16.0
		G-1	33.0±18.3
		G-2	31.0±16.8
		G-3	100.0±0.0
		G-4	38.0±27.5
JXCF		Puliangtai	13.0±8.1
		G-1	14.0±9.1
		G-2	29.0±13.1
		G-3	79.0±12.0
		G-4	10.0±6.9
YYCF	0.1	Puliangtai	98.3±1.7
		G-1	96.7±1.7
		G-2	98.3±1.7
		G-3	100.0±0.0
		G-4	96.7±1.7
JXCF		Puliangtai	98.3±1.7
		G-1	100.0±0.0
		G-2	100.0±0.0
		G-3	100.0±0.0
		G-4	100.0±0.0

Compared with result from the same concentration of 0.08 mg/m^2 and the same pesticide, the sensitivity of two strains to the inert dust was different. When the two strains were treated with G-1, G-3 and G-4, the efficacy was significantly different. YYCF strain was more susceptible than JXCF, with 1-4 times higher mortality level

Table 3 The efficacy of inert dusts in 13% m.c. grain at 50 mg/kg, against *Cryptolestes Ferrugineus*, JXCF strain.

Inert dust	Mortality rate of test insects (%)
Puliangtai	40.0±15.3b
G-1	96.7±3.3a
G-2	96.7±3.3a
G-3	100.0±0.0a

3.3. Inert dust in grain bioassay

Table 3 showed the efficacy of inert dust in grain bioassay against *C. ferrugineus*. As can be seen in Table 3, under the condition of 13% moisture, application of inert dusts at 50 mg/kg could control this species, especially G-1, G-2, G-3 and G-4. The efficacy of G-1, G-2, G-3 and G-4 was by far higher than the respective levels reported by Liu (2005). Under the same conditions, G-3 could provide 100 % mortality of *C. ferrugineus* adults.

4. Discussion

Reichmuth (1991) proposed that, when treated with phosphine at 1 mg/L, the insect pests could still act normally, which indicated the insect pests possessed the relative phosphine resistance. When knocked

down within 30 min, the insect pests were determined as the ones susceptible to the phosphine. This essay adopted phosphine of 2 mg / L against YYCF and JXCF, and the KT_{50} values were far higher than 30 minutes proposed by the reference assay, which indicated that the two strains of *C. ferrugineus* have a strong phosphine resistance that could cause fumigation failure.

In the experiment of the inert dust in surface bioassay, under the same conditions, when the concentration was slightly increased from 0.08 mg/m^2 to 0.1 mg/m^2, mortality of *C. ferrugineus* had greatly increased, and reached, in some cases, 100%. Choosing the adequate concentration was very crucial, and inert dust was an effective insecticide as disinfectation line and empty depot disinfectants. In addition, the experiment with the inert dusts in surface bioassay and in grain bioassay indicated that G-3 was the best of the five inert dusts, and should be tested more thoroughly.

Inert dust is effective, broad-spectrum, safe and natural insecticide, that does not leave residues on the final product. Inert dusts can be also used in the case of organic products, and can provide long term protection. The experimental results showed that inert dusts can be used as an alternative to phosphine in a rotation strategy to alleviate the development of the phosphine resistance.

Low concentration and low amount of inert dust could inhibit over 90% of the populations of phosphine-resistant strains of *C. ferrugineus*. The treatment amount for surfaces and grains can be 0.1 mg/m^2 and 50 mg/kg, respectively.

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Study on the distribution of deltamethrin residues in stored wheat using sequential fractionation procedure

Zhang, Y.#, Wang, S.*
Academy of State Administration of Grain, No.11 Baiwangzhuang Street, Beijing, 100037, P. R. China.
Email: wsxtdo@foxmail.com

* Corresponding author
# Presenting author

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Abstract

The distribution of deltamethrin residues in different fractions of stored wheat were investigated by sequential fractionation procedure, which modified the plant cell wall fractionation procedure established by Langebartels and Hams (1985) to conform to the property of deltamethrin. In this procedure, deltamethrin - treated wheat were firstly extracted with pH 7.0 buffers and organic solvents to remove the extractable deltamethrnin. Subsequently, the treated wheat was fractionated into six macromolecular components using various enzymatic or chemical reagents. With the quantification of deltamethrin by gas chromatography, a majority of the released deltamethrin residues was found in organic solvents, and the unextractable residues were mainly distributed in starch, protein and pectin components of grain. Control incubations in the absence of enzyme or chemical reagents were further performed, which indicated that the interactions between unextractable deltamethrin and six macromolecular components may be different.

Keywords: Deltamethrin, Residues, Fractionation, Stored grain

Section: Integrated Pest Management

Optimizing heat treatments for management of stored-product insects in food-processing facilities

Subramanyam, B.*#
Department of Grain Science and IndustryKansas State University, Manhattan, KS 66506, U.S.A.
Email: sbhadrir@k-state.edu

* Corresponding author
# Presenting author

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Abstract

Stored-product insects associated with food-processing facilities are managed with fumigants (methyl bromide and sulfuryl fluoride), aerosols, residual products and heat. Heat treatment is safe, effective and an environmentally friendly technology for managing insects in food-processing facilities. Heat is a viable alternative to methyl bromide, a structural fumigant that was phased out in the United States, except for certain critical uses, as of 2005. Heat treatment involves raising the temperature of the whole structure or a portion of it to temperatures between 50 and 60^{o}C, and maintaining these high temperatures for at least 24 h. Optimizing heat treatments requires determining the right amount of heat energy to raise and hold the temperatures for effective disinfestation, predicting insect mortality in "real time" during a heat treatment so that corrective action can be taken to improve efficacy, and stopping the heat treatment when all insects have died.

At Kansas State University, Heat Treatment Calculator software was developed to estimate the heat energy required as well as costs of doing a heat treatment using various energy/fuel sources. The calculator also allows the user to explore "what if" scenarios (alter ambient and threshold temperatures, select a fuel type) of a heat treatment. The calculator was validated during heat treatment of a large pasta facility and the calculator heat energy estimates were compared with company heat energy values based on amount of natural gas consumed. The calculator estimates were within 4% of the actual energy values and cost.

A novel thermal death kinetic model was developed and validated using the heat tolerant stages of the confused flour beetle (old larvae) and red flour beetle (young larvae). The model was based on a logarithmic decrease in insect numbers as a function of time at specific temperatures, and a logarithmic decrease in insect numbers as a function of temperature. The model accurately predicting insect survival as a function of time, and the decrease in survival during a heat treatment was faster at higher heating rates (3-5^{o}C/hour). The model predicts insect survival based only on time-dependent temperature data. This model has been used to show survival curves for data collected from numerous facilities subjected to heat treatments. However, the limitation is that these curves can be generated using a Microsoft® Excel program after data are collected. In most cases, plotting survival curves as a function of time-dependent temperature data revealed that heat treatments can be effectively conducted in 24 h or less.

In order to provide "real time" insect survival estimates based on temperature data during a heat treatment, another software called E.A.R.T.H. (Efficacy Assessment in Real Time during Heat Treatment) was developed. This program requires a base station connected to a computer that sits outside a heated facility. Wireless sensors are placed throughout the facility in designated areas, and during heat treatment these sensors communicate with the base station and transfer "real time" temperature data. The acquired data are used by the thermal death kinetic model to predict insect survival in "real time". The temperature data and the survival curves are displayed graphically for each wireless sensor and the user can view these curves during a heat treatment. This allows the user to determine if certain areas are not heating properly, enabling corrective action to be taken in these locations to improve heat treatment efficacy, such as moving a fan, placing an additional heater, or moving a heating duct. The software has been validated at Kansas State University pilot flour mill and at a commercial facility in 2009. The use

of this software should allow the users to stop a heat treatment when predictions show that all of the insects, in locations where temperatures are being measured, are dead.

A major ready-to-eat breakfast cereal company does heat treatments for 34 h in their large facility at approximately monthly intervals using old steam heaters. Our research at this facility using the tools described here showed that all insects, including the heat tolerant stages, were completely killed within 12 h. As a result the company currently does heat treatments for only 24 h with cost savings of $28,000 per year. The use of the Heat Treatment Calculator software, thermal death kinetic model, and the E.A.R.T.H. software are recent developments, and should be used to improve and gauge facility heat treatments. The use of these tools will improve heat treatment efficacy while at the same time reducing costs to the users.

Keywords**:** Heat treatment, Optimizing treatments, Software programs, Models, Real time data acquisition

Fluorescent non-toxic bait as a new method for black rat (*Rattus rattus*) monitoring

Aulický, R.#[1], Fraňková, M.[1,2], Rödl, P.[3], Eliášová, B.[2], Frynta, D.*[2], Stejskal, V.[1]
[1] Crop Research Institute, Drnovská 507, Prague 6, Czech Republic.
[2] Departments of Zoology, Faculty of Science, Charles University, Viničná 7, Prague 2, Czech Republic.
Email: frynta@centrum.cz
3 National Institute of Public Health, Šrobárova 48, Prague 10, Czech Republic

* Corresponding author
# Presenting author

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Abstract

The detection of synathropic rodents may be difficult since they are animals with nocturnal activity. Methods of their detection and monitoring rely mostly on indirect signs of their activity such as the presence of faeces, urine, consumed foods and damaged materials. Our experimental hypothesis was that the production of fluorescent faeces - following consumption of fluorescent bait - may be used for rodent monitoring. For this purpose we studied the production of fluorescent faeces, temporal dynamics and detectability in wild black rat (*Rattus rattus*). Wild black rats were individually housed in experimental cages with the wire-mesh grid floor and faeces were collected in short-time intervals. The peak of fluorescent activity in faeces was detected 10-20 hours after bait ingestion. We found that there is only relatively short delay between bait consumption and defecation and fluorescent faeces are easily detectable at distance using an ultraviolet hand lamp. Thus, this method can contribute to effective monitoring of rodent pests.

Keywords: *Rattus rattus*, Fluorescent bait, Monitoring, Rodent control

1. Introduction

Black rat (*Rattus rattus*) belongs to the three most important rodent species which cause serious damages in agricultural and urban environment in Europe (Meyer et al., 1993). Rats are rodents with a nocturnal activity and most of the day is spent in hidden shelters or nests. They are good climbers, prefer dry areas above ground and are well know for their behavioural response to novel objects (neophobia) (Battersby et al., 2008), which complicates an effective control (Leung & Clark, 2005). Their presence is not easily detected by direct observation but rather according to the signs of their activity - faeces, urine, consumed foods or damaged material. Research is traditionally focused on trapping and poisoning (Shafi et al., 1992; Prakash et al., 2003, Selvaraj and Archunan, 2006). Nevertheless the precise knowledge of rodent spatial activity is also an important prerequisite for their effective control. Indirect monitoring of rodent movements was traditionally realised by administration of the marking substances into the bait and its subsequent detection in rodent bodies and tissues (Savarie et al., 1992).

In the present study, we focused on a new method of monitoring rodent pests by non-toxic fluorescent bait. This bait enables detection of rodent movements via fluorescent faecal pellets without contact with the target animals. In laboratory test, we offered the bait to wild black rats and monitored temporal dynamics of production of fluorescent faeces.

2. Materials and methods

Fluorescent bait- pellets were formulated by ICB Pharma Poland using encapsulation in a thermoset melamine (formaldehyde) sulphonamide resin complex. Orange fluorescence agent was composed from 2 fluorescent pigments: 2.0 % -orange and 0.4 % yellow (CIBA Specialty Chemicals).

The experimental animals included wild black rat, *Rattus rattus*. Rats were kept solitary in cages with a wire mesh bottom. Standard food (ssniff, Germany) was removed from the feeders the day before experiment (20:00 h). The following day (at 16:00 h) 20 g of fluorescent pellets were offered to each rat. At 18:00 h fluorescent pellets were removed and replaced by standard pellets. All remaining fluorescent pellets and their fragments were collected and weighed; this enabled to estimate the amount (weight) of consumed fluorescent pellets.

All faecal pellets were collected every two hours for 38 hours after administration of the fluorescent pellets. The collected faecal pellets were counted, inspected under the ultraviolet illumination (ICB Pharma, a 21 LED flashlight which emit UV-A light, 390 nm) and classified into the following three groups: (i) highly fluorescent, (ii) poorly fluorescent, and (iii) exhibiting no sign of fluorescence. Finally, all collected pellets were dried and weighed.

3. Results

Rats produced on the average 109 faecal pellets per 38 h; ranging from 76 to 155. The dry weight of the produced faeces ranged from 3.4 to 9.0 g (mean = 5.2 g). The experimental subjects consumed 1.1 – 13.2 g of the pellets (mean = 6.5 g). The first fluorescent faeces were recorded 2-4 h after the introduction of fluorescent pellets into the cages. Total weight of detectable fluorescent faeces (WDFF) produced during the experiment increased with weight of consumed fluorescent pellets (WCFP) following equation: WDFF = WCFP * 0.110 - 0.221.

Figure 1 shows that the production peak of highly detectable fluorescent faeces was 6-16 hours after bait introduction. The last detectable faeces were recorded 28-30 h after bait introduction in most of the experimental animals. However, one individual produced fluorescent faecal pellets even 34 and 38 hours after bait introduction.

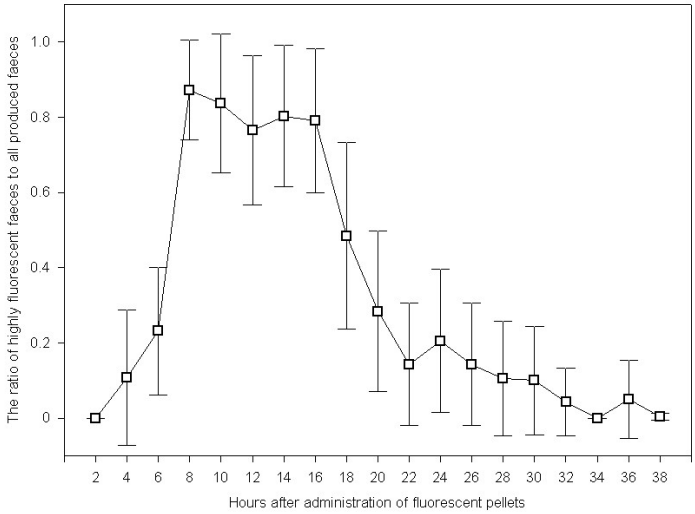

Figure 1 Proportion of the number of highly fluorescent faeces to all produced faeces by black rat after the consumption of fluorescent pellets. Data are given as means and 95% confidence intervals.

4. Discussion

We confirmed that fluorescent pigment is easily detectable with the UV flashlight in black rat faeces after consumption of fluorescent bait. The fluorescent faeces were highly visible from a distance of several meters even when relatively poor flashlight ultraviolet illumination was used. The low overall production of faecal pellets by black rats may be considered as a limiting factor for rat monitoring under field condition, since some proportion of faecal pellets is usually deposited in inaccessible sites (shelters etc.). However, the high visibility of even a single faecal pellet in UV light may help to overcome this limitation.

The production peak of detectable fluorescent faeces was 6-16 hours after administration of fluorescent pellets into the rat cages. This delay corresponds with those reported for Norway rat (Bungay et al. 1981). The rate of bait conversion into highly fluorescent faeces was 11%. It means that nine times more bait should be administered and consumed to produce a unit weight of detectable faeces. Hence, high palatability and an appropriate placement of the bait are requested for effective monitoring (Clappperton, 2006). Fluorescent bait was offered to rats in non-choice experiment and some individuals, although food

motivated, did not accept the food immediately. It confirms behavioural neophobia in black rats which should be taken into account in the application of the method. This phenomenon is going to be studied in our future research.

Acknowledgments

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Simulation model of the red flour beetle in flour mills

Flinn, P.W.*#[1], Campbell, J.F.[1], Throne, J.E.[1], Subramanyam, B.[2]
[1] USDA-ARS, CGAHR, 1515 College Ave, Manhattan, Kansas 66502 USA. E-mail: paul.flinn@ars.usda.gov
[2] Department of Grain Science and Engineering, 201 Shellenberger Hall, Kansas State University, Manhattan, Kansas 66502 USA

* Corresponding author
# Presenting author

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Abstract

Red flour beetle (*Tribolium castaneum*) is one of the most common insect pests infesting wheat flour mills (Toews et al. 2006). Structural treatments such as methyl bromide, sulfuryl fluoride and heat, are used to control the red flour beetle. The structural treatments do not provide any residual action and thus, any surviving insect stages can rebound rapidly depending on the temperature. In addition, the distribution of the fumigant or heat-treatment in the facility can result in different mortality rates for different floors or regions of the mill. Simulation models can be used to develop optimal integrated pest management strategies (Thorpe et al., 1982; Longstaff, 1988; Hagstrum and Throne, 1989; Flinn et al., 1997). Models developed for the flour beetle in stored grain have been useful for predicting population trends (Hagstrum and Flinn, 1990). Our objective was to develop a model for the red flour beetle in wheat flour mills that could be used to predict the effects of various structural treatments and subsequent population rebound. Currently, heat-treatment is not included in the model - it will be added in a future version.

A distributed-delay model was used to predict population growth of red flour beetle as a function of inside air temperature. The model uses a distributed delay to simulate variation in developmental time, manage survivorship, and move insects through stages. The model predicts mean insect density for each floor of the mill based on historical hourly inside air temperature for each floor (we may add relative humidity to the model if studies show this to be an important variable). The model consists of four parts: 1) an equation for rate of insect development as a function of temperature and RH; 2) a delay process for moving insects through stages; 3) an equation for age-specific fecundity as a function of temperature; 4) stage-specific mortality for methyl bromide or sulfuryl fluoride fumigation (stage-specific mortality for heat-treatment will be added in a later version of the model). Starting numbers are entered for each insect stage and for each floor of the mill. The date of each fumigation, type of fumigant used (methyl bromide or sulfuryl fluoride), and the percent of the population that will not be exposed to the fumigant can be specified (refugia). Validation data was obtained by monitoring a flour mill from 2002 to 2004 (Campbell et al., 2010ab). Pheromone/food-oil baited pitfall traps were placed throughout the building with eleven traps on each floor. The traps were inspected and replaced every 2 weeks (pheromones were replaced every 6 weeks). Hobo temperature recorders were placed at one location on each floor.

Trap catch field data (2002-2003) showed that the two methyl bromide fumigations knocked down the red flour beetle population (Fig. 1). The simulated numbers of adult red flour beetle tended to follow trap catch fairly well. Both the actual population and the simulated red flour beetle numbers increased rapidly in August following the first fumigation. The rapid increase was probably due to warmer temperatures. Population growth following the second fumigation was slower; this was probably due to lower temperatures inside the building during the fall and winter. Figure 2 shows trap catch data (2003-2004) for the same flour mill, but fumigated with sulfuryl fluoride on 19 June 2004. Both the simulated number of red flour beetle and the actual trap catch increased relatively slowly from September until April. The sulfuryl fluoride fumigation in June reduced the population; however the population quickly rebounded a few weeks later. The warmer air temperatures in June may have contributed to the rapid increase in adult numbers following fumigation. We plan to conduct additional validation studies with the model in other flour mills. Future enhancements to the model will include the ability to simulate facility heat treatments and fogging with aerosols. The model should be a valuable tool that can be used to develop optimal IPM strategies for the red flour beetle in flour mills.

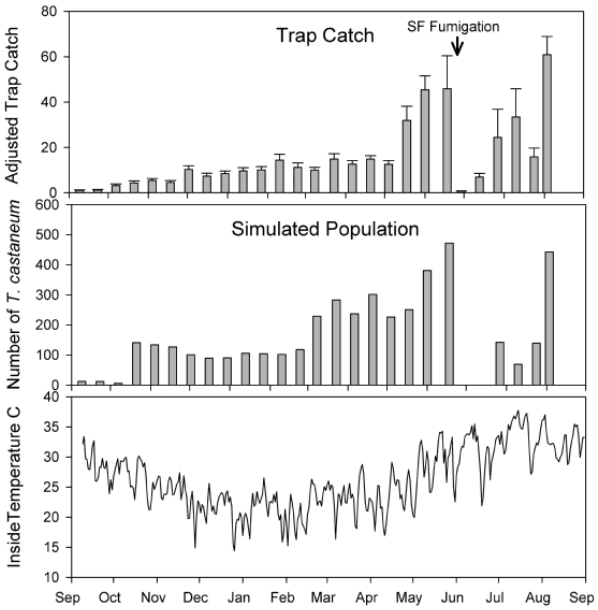

Figure 1 Mean red flour beetle trap catch (2-week duration), model predicted insect numbers and hourly inside building temperatures for a flour mill located in central Kansas USA. The flour mill was fumigated with methyl bromide (20 g for 24 h) on 28 June 2002 and 23 August 2002.

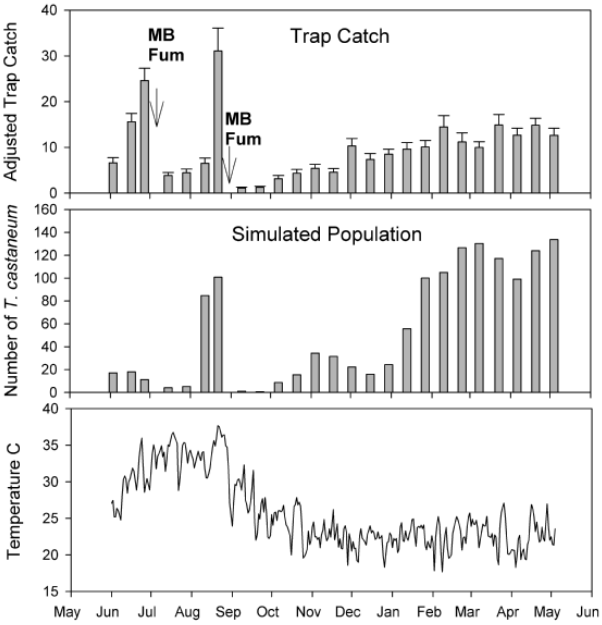

Figure 2 Mean red flour beetle trap catch (2-week duration), model predicted insect numbers and hourly inside building temperatures for a flour mill located in central Kansas USA. The flour mill was fumigated with sulfuryl fluoride (111 g for 18 h) on 19 June 2004.

❖ This abstract reports the results of research only. Mention of a proprietary product or trade name does not constitute a recommendation or endorsement by the US Department of Agriculture or Kansas State University.

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African Postharvest Losses Information System – a network for the estimation of cereal weight losses

Hodges, R.J.*#[1], Bernard, M.[2], Knipschild, H.[2], Rembold, F.[3]
[1] Natural Resources Institute, Chatham Maritime, Kent, ME4 4TB, UK. Email: R.J.Hodges@gre.ac.uk
[2] BLE / ISICAD, Federal Agency for Agriculture and Food (BLE), 53168 Bonn, Germany
[3] Joint Research Centre of the European Commission, 21027 Ispra, via Fermi 2749, Italy

* Corresponding author
# Presenting author

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Abstract

Soaring food prices during 2007/2008, and the realisation that sporadic food shortages are likely to continue long into the future, has reawakened interest in the benefits of reducing food losses. As a means of making more accurate estimates of how much food is lost, the Joint Research Centre (European Commission) proposed the development of the African Postharvest Losses Information System (APhLIS) (http://www.phlosses.net). APhLIS estimates postharvest losses (PHLs) by cereal crop, by country and by province in East and Southern Africa. The system went live in March 2009 and combines a loss calculator, a free access database of key information, and a network of local experts who contribute the latest data and verify loss estimates. The loss calculator works of loss figures contributed from the literature and by local experts but also takes account of the prevailing climate, scale of farming (small/large), damp weather at harvest, larger grain borer (in the case of maize), proportion of grain held in farm storage or marketed, and multiple harvests. Before the introduction of APhLIS, the origin and justification of PHL estimates were not well founded. Now PHL estimates are available that are

- Transparent in the way they are calculated
- Based on a complete screening of available research and literature
- Contributed (in part) and verified by local experts
- Based on the primary national unit (i.e. province not just country level, so estimates are more relevant)
- Upgradeable as more (reliable) data become available, so that there is the opportunity for increasing accuracy in loss estimation over time.
- Supported by a downloadable loss calculator that can be used to make loss calculations at a geographical scale below primary national unit.

In the future, APhLIS may be expanded in technical scope (crops) and geographical range (countries) and used to help prioritize and justify loss reduction strategies including those for grain storage.

Keywords: Weight loss, Loss calculator, Postharvest operations, Cereal supply

1. Introduction

The grain storage community has had a long standing interest in the assessment of postharvest losses (PHLs), especially since the food crisis of the 1970s. Estimates of PHLs became both a justification, and an objective measure, for the subsequent Prevention of Food Losses (PFL) programme led by FAO (UN Food and Agriculture Organization). The PFL programme continued into the 1990s but drew to a close with declining food prices. Soaring food prices during 2007/2008, and the realisation that sporadic food shortages are likely to continue long into the future, has reawakened interest in the benefits of reducing food losses, especially as these may offer better use of natural resources than equivalent increases in food production.

PHLs have negative impacts on hunger, poverty alleviation, income generation and economic growth. PHLs are crop/product specific and occur at many stages in the supply chain (harvesting, drying, storage, market, transport, etc.). They are evident as loss of weight and loss of quality and are compounded by subsequent losses of market opportunity and lost production resources such as land, water, labour, agricultural inputs and soil fertility. Yet, the magnitude and location of PHLs are poorly understood because PHL figures are still frequently guesstimates, are relatively difficult to trace for both logic and

information source, and the sources themselves may not be very reliable. By improving PHL estimates it will be possible to target loss-reduction interventions at the most affected areas (geographically), the most affected links in the postharvest chain or those links that would be most cost effective to address. A further use for PHL figures is in the calculation of the cereal supply/demand balances of developing countries. An estimate of how much grain may be available to consumers emerges when national cereal production/import figures are corrected for PHLs. National cereal supply is usually determined through a food balance sheet, by institutions such as the Ministry of Agriculture or the Statistical Service, while in highly food-insecure countries, so called Crop and Food Supply Assessment Missions (CFSAMs) are often requested by the country and implemented under the supervision of FAO and WFP (UN World Food Programme). Examples of national cereal balance calculations, including the PHL figures applied, can be seen in the CFSAM reports on the website of FAO's Global Information and Early Warning System (GIEWS). These reports will influence food aid policy on the donors' side, so the availability of reliable PHL data is essential for good decision making.

In response to the need for better PHL estimates, the Joint Research Centre and EuropeAID (European Commission) proposed the development of the African Postharvest Losses Information System (APhLIS) (http://www.phlosses.net). APhLIS estimates the cumulative weight losses that occur along the postharvest chain, including harvesting, drying in the field and/or on platforms, threshing and winnowing, transport to store and then farm storage (large or small scale), transport to market and market storage. Excluded are losses due to cereal processing, e.g., milling and consumer wastage. The main features of APhLIS are presented below; a separate scientific publication is planned to describe it more fully in the future.

2. Development of APhLIS

In its first phase of development, APhLIS has included the countries of East and Southern Africa. Estimates of PHLs for each of the major types of cereals are made for the first administrative sub-divisions (e.g. provinces) of these countries. This makes loss estimation more useful than if it was done on a country-wide basis and conforms to the administrative requirements for making cereal supply calculations. APhLIS consists of four components:

1. A PHL calculator that determines a cumulative weight loss from production for a given cereal grain type using loss figures for each link in the postharvest chain (harvest through to market storage). This set of loss figures is called a PHL profile and such profiles are the basis to loss calculation.
2. A database, accessible by the Network, which stores key data.
3. The Network of experts in East and Southern Africa that provide data relevant to the calculation of PHLs and verify PHL estimates.
4. A web site to display the PHL data in the form of tables and interactive maps (Fig. 1).

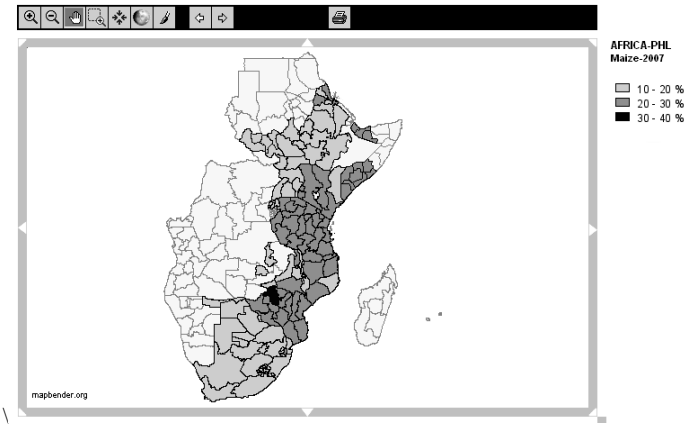

Figure 1 APhLIS interactive map showing maize PHL estimates throughout East and Southern Africa by province, for the year 2007.

For Maize in Malawi: Provinces of Malawi Click on a loss figure in the table below to see in detail how the figure was derived: Send us your comments if you have the feeling that the underlying data and assumptions could be improved. Please sent your comments to R.J.Hodges(at)gre.ac.uk.

Back						
Province	2003	2004	2005	2006	2007	2008
Area under National Administration	-	20.5	20.5	20.6	19.7	-
Central Region	-	20	20	20.1	19.3	-
Northern Region	-	20.8	20.9	21	20.6	-
Southern Region	-	20.5	20.5	20.6	19.8	-

Figure 2 APhLIS website - Maize PHL estimates, by year, for the provinces of Malawi. Loss figures may be 'clicked' to reveal tables displaying the data used for their calculation and origin (see Figs 3 to 5).

Calculation matrix documenting the PH loss calculation, quality of data sources and references to sources Country Malawi: Province: Area under National Administration; Climate: Humid subtropical climate (Cwa); Year: 2007; Crop: Maize

Annual production and losses	**Tonne**	**%**
Production	3,444,655	100
Grain remaining	2,767,401	80.3
Lost grain	677,254	19.7

Seasonal production and losses

Season	**Farm Type**	**Production (t)**	**Remaining (t)**	**Losses (t)**	**Production (%)**	**Remaining (%)**	**Losses (%)**
1	small	2,856,698	2,284,102	572,596	92.9	74.3	18.6
1	large	218,237	191,737	26,500	7.1	6.2	0.9
Seasonal:		3,074,935	2,475,839	599,096	100.0	80.5	19.5
2	small	369,720	291,563	78,157	100.0	78.9	21.1
2	large	0	0	0	0.0	0.0	0.0
Seasonal:		369,720	291,563	78,157	100.0	78.9	21.1
Annual:		3,444,655	2,767,401	677,254	100.0	80.3	19.7

NB Annual averages are a weighted average of the seasons

Figure 3 APhLIS website - Details of production and losses of maize grain for two harvesting seasons of large and small farms in the Area of National Administration in Malawi (follows from Fig. 2 after 'clicking' on the PHL figure for 2007 – 19.7%).

PHL (%) Calculation: Season: 1 Farm Type: small		
Marketed at harvest (%)	20	If data is missing **(no data)** it is assumed that for subsistence farmers all grain is stored but for commercial farmers all grain is marketed. Note: Figures in this table are farm type specific (small or large farms). The value **Marketed at harvest (%)** is used to determine the percentage of total production that is stored and marketed by this type of farm in this particular season (Season 1, Season 2 etc). The calculation only considers the portion that is produced by this type of farm. Consequently, the figures below for **Stored (%)** and **Marketed (%)** will only add up to 100% if all grain in a particular season is produced on this farm type. Otherwise the corresponding percent figures for the other farm type, in the same season, must be included to arrive at a sum of 100%.
Rain at harvest	no data	If weather is damp at harvest, leading to exceptional mould damage to the crop, then the value is **yes** and the **Harvesting/field drying** losses figure in the PHL profile is replaced by **16.3%**.
Storage duration (months)	no data	Effect of storage duration: 0-3 months % figure for storage is 0 (zero) 4-6 months the % figure of the PHL profile is divided by 2 more then 6 months or in case of missing data (no data) the % figure in the general profile is used

PHL (%) Calculation: Season: 1 Farm Type: small					
Larger Grain Borer	yes	If the crop is **maize** and the value is **yes** then the **Farm storage** loss figure in the PHL profile is multiplied by **2**.			
	Destination	Stored (%)		Marketed (%)	
		74.3		18.6	
Stages	PHL profile (adjusted)	Remaining grain	Loss increment	Remaining grain	Loss increment
Harvesting/field drying	6.4	69.5	4.8	17.4	1.2
Platform drying	4	66.8	2.8	16.7	0.7
Shelling	1.2	66	0.8	16.5	0.2
Winnowing	-	66	0	16.5	0
Transport to farm	2.3	64.4	1.5	16.5	0
Farm storage	9	58.6	5.8	16.5	0
Transport to market	1	58.6	0	16.3	0.2
Market storage	4	58.6	0	15.7	0.7
Total		58.6	15.7	15.7	2.9

Figure 4 APhLIS website - Details of the PHL profile and modifying factors used for the maize PHL estimate for 2007 for the Area under National Administration in Malawi (follows from Fig. 3).

PHL profile: Data quality display and references to sources: PHL profiles are used to calculate losses, each profile consists of a series of values, one for each link in the postharvest chain. Each value in the PHL profile is formed from the average of several figures drawn from the available literature. All these figures are shown individually in the tables below. Separate PHL profiles are given for small farms and large (commercial) farms. The reliability of each datum contributing to the calculation of each PHL profile value is displayed in the table below. The assessment is based on how specific the figure is to the situation in which it is being used. To do this, each figure is assessed according to whether it is from the same Cereal type (maize, rice etc), same Climate type (is from same Koeppen code), same Farm type (from a small farm or large commercial farm), and if the Method of loss assessment was an actual measurement of loss or was a questionnaire survey or guesstimate. The result of the assessment is indicated using the red/0 and green/1 system as follows –

0 - A datum used in the calculation of a PHL profile value is not specific to this situation or is from a questionnaire survey or a guesstimate, i.e. is not measured.
1 - A datum used in the calculation of a PHL profile value is specific to this situation or is measured.

References and individual loss figures % for small farms

Stages	**Loss figure**	**Reference**	**Origin of figure**			
			Cereal	**Climate**	**Farm type**	**Method**
	2.0	Boxall R.A. - 1998	0	0	1	0
	9.9	Grolleaud M. - 1997	1	0	1	0
	5.8	Mvumi B.M. - 1995	1	1	1	0
	9.5	Odogola W.R. - 1991	1	1	1	0
	5.0	Vervroegen D. - 1990	0	1	1	0
Harvesting/field drying	6.4		1	1	1	0

Figure 5 APhLIS website -The data quality display and derivation of the first element of the PHL profile (Harvesting and field drying – 6.4%) for maize produced on small farms in the Area under National Administration in Malawi (follows from Fig. 4). On the actual web page each 'Reference' can be 'clicked' to reveal the full bibliographical details.

PHL profile figures based on more 'green/1' data are considered to be more reliable than those based on more 'red/0' data. Against each PHL profile value the number of 'red/0' and 'green/1' assessments is averaged, and displayed in bold, to give a general assessment of the value. Frequently some parts of the

profile are more reliable than others, especially those where more loss data are available from the literature.

3. Loss estimation methodology and assumptions

When APhLIS estimates a PHL for a particular cereal crop, in a particular province, the pre-determined PHL profile is automatically loaded into the PHL Calculator. Examples of PHL profiles are shown in Table 1. A problem faced in seeking to provide PHL profiles is that for most of the many provinces of East and Southern Africa there are no PHL data specific to them. It is therefore inevitable that many different provinces have to share the same data. This sharing was achieved by clustering together the provinces of many countries that are basically similar with respect to the factors that influence PHLs; the most convenient method of clustering was found to be based on climate classification. The climates of East and Southern Africa are classified by the Köppen system (Peel et al., 2007), into one of three basic types, tropical savannah/forest, arid/desert or warm temperate. For each crop there is a PHL profile for each climate type, so with seven crops (maize, sorghum, millet, wheat, barley, rice and teff) there is a total of 21 (3 x 7) profiles.

In establishing PHL profiles it is necessary to create a generalized loss figure for each link in the postharvest chain. The basic data on which these were derived came from two sources, the scientific literature and figures supplied by the PHL Network. These figures were refined by 1) removing 'outliers', 2) avoiding the use of 'questionnaire/guesstimate' data where there are sufficient measured loss estimates and 3) averaging what data remained. In the case of storage loss figures, where these are taken from the literature, they are standardized to a nine-month storage period. If not already adjusted for farmers consumption patterns, then they are adjusted assuming an even consumption across the entire storage period. The PHL profile alone does not determine the magnitude of the cumulative loss as the loss estimates in the PHL profile are adjusted for:

Table 1 Examples of PHL profiles for three cereal crops, showing % weight loss from production, under different climates and scales of farming.

Climate type	**A**	**C**	**B**	**B**	**A**
Crop	**Maize**	**Maize**	**Sorghum**	**Millet**	**Rice**
Scale of farming	**Small**	**Large**	**Small**	**Small**	**Small**
Harvesting/field drying	6.4	2	4.9	3.5	4.3
Drying	4	3.5	-	-	-
Shelling/threshing	1.2	2.3	4	2.5	2.6
Winnowing	-	-	-	-	2.5
Transport to store	2.3	1.9	2.1	2.5	1.3
Storage	5.3	2.1	2.2	1.1	1.2
Transport to market	1	1	1	1	1
Market storage	4	4	4	4	4

1. Whether or not there is wet/damp weather at time of harvest – damp weather increases losses and the % weight loss figure in the profile is set to 16.3% (as this is currently the only measured figure in the literature for this type of loss).
2. The length of the farm-storage period. If the duration is less then 3 months then the % storage loss figure is set to zero. If the duration is 4 to 6 months then the standard % weight loss for storage is divided by 2 (i.e., is only half the annual figure). If grain is stored for 7 or more months then the annual % storage loss figure is used.
3. In the case of maize, whether or not lesser grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) is expected to be a significant pest. If a significant pest, then this has the effect of doubling the storage weight loss. From experience in the use of APhLIS it has been found that these factors have a much greater influence on the magnitude of the final PHL estimate than the initial choice of PHL profile for a crop according to climate type. Furthermore, the Calculator takes into account:
4. The proportion of grain that is marketed directly. This affects the proportion of harvest for which farm-storage losses are considered. Furthermore, it is assumed that subsistence farmers eventually

consume all grain that is not marketed so this stock suffers no transport to market or trader-store losses.

5. Whether or not the crop is harvested in one, two or three seasons – each season is a separate calculation and losses are computed as a weighted average.

4. What the website shows

The APhLIS website displays the basic data submitted by the Network used in the estimation of PHLs. This data includes crop production, rainfall, temperature, extreme climatic events, etc., by crop and by year for country and province.

PHL estimates are presented as interactive maps (Fig. 1) or as a series of tables. In the tables, PHLs are first presented by crop, then by country and then by province. PHLs for maize in the four provinces of Malawi are shown in Figure 2. By 'clicking' on one of the provincial PHL figures shown in Figure 2, details of the loss calculation are presented (Figs. 3 and 4).

The PHL profiles is shown in Figures 4 and 5 present details of how specific, to the situation in question, is each of the loss estimates that comprise this particular PHL profile. Each loss figure in the profile is itself derived from a series of estimates from the literature or the experts of the APhLIS Network. The table shows each of these contributed figures and gives it a rating according to whether it is specific to the cereal crop, to the prevailing climate and to the farm type. It also rates the 'Method' by which the estimates were derived, i.e., by actual measurement or from questionnaires/guesstimates. Figures are rated as '1/green' if they are 'specific' or 'measured' or rated as '0/red' if 'not specific' or 'questionnaire/guesstimate'.

Furthermore, a source is quoted for each and every figure. In most cases, some of the estimates applied in a PHL profiles are generalized, i.e., are derived partly or completely of figures that are not specific to the cereal type, climate or farm type in question. In the example of Figure 5, none of the component estimates for the 'Harvesting and field drying' figure were 'measured' estimates, they were all 'questionnaire/guesstimates', consequently, they are all scored as '0/red' for 'Method'.

5. The downloadable PHL calculator

Besides offering PHL estimates on the web site, a downloadable version of the PHL calculator is available, on an Excel spreadsheet. This offers users several advantages. They can substitute the most up-to-date or most relevant data and at a chosen geographical scale within east and southern Africa. To be able to choose the geographical scale is important when the political boundaries of provinces do not match natural agro-climatic boundaries. In this case, the estimates presented on the website may hide considerable heterogeneity. Like the web site, the downloadable PHL calculator also presents ratings of specificity for the figures in the PHL profile. In this way, users can determine the suitability of the PHL estimates for their purposes.

6. Conclusions

There have often been demands for simplified loss figures. This for example has lead to the postharvest losses of maize for a country or region being reduced to just a single figure representative of many years. However, such an approach is likely to be misleading since Tyler (1982) noted "postharvest losses may be due to a variety of factors, the importance of which varies from commodity to commodity, from season to season, and to the enormous variety of circumstances under which commodities are grown, harvested, stored, processed and marketed." Therefore, it is important not only to work with figures that are good estimates at the time and in the situation they are taken, but to be aware that at other times and situations the figures will differ. This necessitates regular recalculation of loss estimates with the best figures available. APhLIS addresses this task.

APhLIS was launched in March 2009 and in its early stages may or may not provide loss estimates that are different from those used previously. If they are different, there will be no solid evidence that they are more accurate. However, APhLIS generates estimates that are

- Transparent in the way they are calculated
- Contributed (in part) and verified by local experts
- Based on the primary national unit (i.e., province not just country level, so estimates are more relevant)
- Upgradeable as more (reliable) data become available, so that there is the opportunity for increasing accuracy in loss estimation over time.
- Supported by a downloadable loss calculator that can be used to make loss calculations at a geographical scale below primary national unit.

APhLIS estimates will be tested during CFSAM missions and compared with estimates derived by other means. In the medium term, APhLIS would benefit greatly from the supply of additional loss figures. To strengthen the ability of target countries to collect relevant data, in the format needed by the system, requires suitable initiatives underpinned by modern rapid approaches to loss assessment.

In the future, the use of the APhLIS is expected to benefit agricultural development and food security by

1. highlighting missing data and helping identify which gaps in our knowledge would be the most cost-effective ones to fill, and
2. acting as a model system to explore loss scenarios so that opportunities for loss reduction can be identified. Furthermore, as climates become increasingly less stable it may be used to suggest how climate change could impact on PHLs.

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Effect of storage management on free fatty acid content in dry cocoa beans

Jonfia-Essien, W.A.*#[1], Navarro, S.[2]
[1] Research Department, Quality Control Company Ltd (COCOBOD), P. O. Box CO 247, Tema, Ghana.
Email: wajonfiaessien@gmail.com
[2] Senior Consultant, Food Technology International Consultancy Ltd., Israel

* Corresponding author
# Presenting author

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Abstract

Though not a quality parameter, it is expected that the free fatty acids (FFA) content must be less than 1.0% to meet the acceptable level of 1.75% in cocoa butter extracted from the dry cocoa beans. This study therefore investigates the FFA content of stored dry cocoa beans from Ghana that was generally low compared to that of Côte d'Ivoire. The FFA content of dry cocoa beans increases with storage time and this was evident for both countries. The mean FFA of Ghana's cocoa beans was 2.03% in 1999 and 0.90% in 2008 while that of Cote d'Ivoire's cocoa beans was 2.57% in 2002 and 1.43% in 2008. The low mean moisture content of 6.5% of Ghana cocoa beans and the mean moisture content 8.0% of Côte d'Ivoire cocoa beans might have influenced the differences in mean FFA levels. To evaluate the effect of insect infestation on increase of FFA, dry cocoa beans were infested with ten young adults of *Lasioderma serricorne* (Fabricus), *Tribolium castaneum* (Herbst), *Cryptolestes ferrugineus* (Stephens) and stored for 9 mo under dry condition at 30±2°C. The mean FFA of the insect-infested dry cocoa beans increased from 0.76% at the time of storage to 1.81% after 9 mo of storage. However, the mean FFA of the control dry cocoa beans increased from 0.79% at the time of storage to 0.93% after 9 mo of storage. It could therefore be inferred conclusively that FFA content in dry cocoa beans increases with insect infestation.

Keywords: Cocoa beans, Free fatty acids, Storage management, Quality preservation, Insect infestation.

1. Introduction

Quality of dry cocoa beans in international trade is assessed on the percentage level of total mould, slaty, purple, insect infested, flat, and germinated beans. Recent cocoa trade has assumed a scientific dimension and emphasis is placed on the content of free fatty acids (FFA) which is influenced by many factors such as humidity, oxygen and insect infestation. For these reasons hermetic storage has been considered as a successful storage method for the management of FFA, insect control and quality preservation.

Ghana is the world's second biggest producer of cocoa *Theobroma cacao* L. after neighbouring Côte d'Ivoire (Sarpong, 2002). Under the climatic conditions of these countries, output is sometimes affected significantly by infestation. Infestation of dry cocoa beans in the post harvest sector starts from the drying mats and continues during storage. At the farm, insects in drying mats are an important source of infestation. At the end of the season they are usually rolled up and stored under the eaves but they often carry pupae from which *Ephestia cautella* (Walker) may emerge to infest the new crop. Similarly, the area around mechanical dryers can provide a breeding ground for pests (Wood and Lass 1985, Jonfia-Essien, 2004). This incidence of pests is a worldwide phenomenon which cannot be completely eradicated but can be curbed through pragmatic measures. Unless storage is properly carried out there is a risk of dry cocoa beans becoming damaged from insect infestation, mould and foreign odours (Jonfia-Essien, 2001).

1.1. Infestation of cocoa

All cocoa is susceptible to insect infestation and both beetles and moths infest cocoa beans. Some of the common beetles are *Lasioderma serricorne* (F.) (the cigarette beetle) and *Araecerus fasciculatus* (Degeer) (the coffee bean weevil), which can pierce the shell of the bean thereby providing an entrance for moths such as tropical warehouse moth (*Ephestia cautella*) (Walker) and for moulds (Jonfia-Essien, 2001; 2004). In Ghana, dry cocoa beans were monitored for insect pests associated with the cocoa in storage from 1995 to 2000 and eleven species identified including *Tribolium castaneum* (Herbst),

Cryptolestes ferrugineus (Stephens)*, E. cautella, L. serricorne* and *A. fasciculatus* (Jonfia-Essien, 2001; 2004).

Insect pests inflict their damage on stored products mainly by direct feeding, but their very presence in foodstuffs is a nuisance. In many species both larvae and adults cause damage, which may be devastating at times. Several factors may be responsible for insect infestation in storage and of special importance is the number of flying insects searching for food (Hodges et al., 2002). Studies in Ghana, with experimental and real stores, have shown that the number of flying beetles during the period of storage is directly correlated with the probability that the produce in any given store will become infested (Birkinshaw et al., 2002).

1.2. Mould infection of dry cocoa

It is a known fact that high moisture content result in mould infection. Microflora, particularly moulds, has been associated with FFA occurrence in stored cocoa beans (Wood and Lass, 1985; Pontillon, 1998). Increase in FFA during storage could be attributed to the activities of the enzyme lipase, which is naturally present in raw cocoa (Minifie, 1989). The enzymes become active due to the changes in moisture content of the beans and high temperatures of storage environment.

1.3. Standards of cocoa trade

Various cocoa standards that apply relate to flavour and purity or wholesomeness. The most important of these standards are the ISO standards (ISO, 1973) and those in the contracts of various trade associations such as Cocoa Association of London, the Association Française du Commerce des Cacao, and the US Cocoa Merchants Association (Jonfia-Essien, 2004). The grade standards are based on the cut test that allows certain gross flavour defects to be identified by cutting open the beans to reveal the colour of the dried nib (Anon., 1996).

1.4. New dimension of cocoa standards

Recently, the cocoa trade has assumed a more scientific position and a lot of emphasis is placed on the content of free fatty acids (FFA) which is influenced by many factors including humidity, moulds and oxygen. Though the use of pesticide in the control of insect infestation in cocoa beans has been associated with residue and Maximum Residue Levels (MRLs) determination has now become a requirement, FFA level has ushered in an additional standard that will enhance the production of superior quality of cocoa beans, which should have a positive impact on cocoa trade in the future.

1.5. Justification

It was observed that some cocoa beans with low moisture content of 6.5% were found to contain high FFA levels. Incidentally, the cocoa beans were infested with insects. However there is no scientific data on the relationship between insect infestation and FFA. This study therefore, aims at determining the impact of insect infestation on FFA formation in raw cocoa beans in storage and the role of storage management on FFA content.

2. Materials and methods

A total of 195 samples of dry cocoa beans were used for this study out of which 54 were Cote d'Ivoire's beans and the rest (141) from Ghana. 24 samples of Ghana's cocoa beans were used for the categorization studies in storage, 9 for insect studies in storage and the rest (108) for yearly comparison studies. All the 54 samples from Cote d'Ivoire were used for the yearly comparison studies. Data collected from the studies were subjected to statistical analysis using GenStat 7.22 DE (Discovery Edition).

2.1. Infestation experiments

Laboratory reared storage insect pests of the species *L. serricorne*, *T. castaneum*, and *C. ferrugineus* were used to investigate the level of insect infestation on 500 g each of cocoa bean samples of different categories (super main crop, main crop, super light crop, light crop, small beans, type 4 and remnant) from Ghana in triplicates. Adult insect pests (10 unsexed each) were introduced into the dry cocoa beans using miniature prototype jute sacks and inserted into cotton cloth sacks to prevent insect migration from the cocoa beans and stored for 9 mo in a controlled environment at 30□2°C and relative humidity of 70□2%, based on the prevailing conditions at the cocoa warehouses in Ghana. A single insect species

per sample was used in the insect studies whereas all three species were used per sample for the categorization studies. The number of larvae, pupae and adults were assessed once every 3 mo for the period of 9 mo. The cocoa beans were also analysed for FFA content.

2.2. Sampling and preparation

Ghana exportable dry cocoa beans shipped in 1999, 2004, 2006 and 2008 were sampled from three different sites at SITOS warehouse in Amsterdam. Sampled from the same warehouse were Côte d'Ivoire exportable cocoa beans shipped in 2002 and 2008. All sampling was done in triplicates from each site. The cocoa beans were sieved and moisture content determined before subjecting the beans to any analysis.

Cocoa beans were bulked and reduced to the laboratory sample size before coding for analysis. Each sample (50 g) of cocoa beans was weighed in a Petri dish. The samples were roasted for about 2 h in the oven at 105°C, cooled and de-shelled to separate the nib from the shell. The de-shelled samples were milled (test samples) and moisture content of each was determined.

2.3. Free fatty acid analysis

Federation of Cocoa Commerce (FCC) recommended method was used for the FFA analysis and double extraction was carried out. Round bottomed flasks of 250 mL were dried in the oven at 105°C, cooled in the desiccator, weighed and 180 mL of hexane was measured into the round bottomed flasks. Each test sample of 10 g was measured into a thimble and was set up for extraction for two hours using the Soxhlet apparatus. The set up was allowed to cool and the solvent drained into the round bottomed flask.

Each sample was ground with sand and set up for two 2 h again. The solvent was concentrated into fat by evaporating the hexane using the rotary evaporator. The fat content was dried in the oven for 2 h and cooled in the desiccator. Weight of the extract and the flask were taken and recorded.

The weighed fat extract was dissolved in 50 mL ethanol/diethyl ether solution, 1/1 [v/v/]. About three drops of phenolphthalein indicator were added to the fat in 50 mL ethanol/diethyl ether. The mixture was titrated against 0.1 M sodium hydroxide in ethanol solution and the end point taken and recorded for the FFA calculation as follows.

$FFA = (282 \times V \times C) / 10xM$
$M = (M_2 - M_1)$
Where
282 = molecular mass of oleic acid
V = volume (mL) of standardised sodium hydroxide used for titration.
C = concentration (mol L^{-1}) of the standardised sodium hydroxide used for titration.
$C = Wp / (Mp \times Vp)$
Where
Mp = molecular weight of hydrogen phthalate
Vp = volume of sodium hydroxide solution
Wp = weight of sodium hydroxide phthalate
M = mass of extracted fat
M_1 = mass of conical flask and pumice stones before extraction
M_2 = mass of conical flask after extraction

3. Results

The mean moisture content of cocoa beans from Ghana was 6.5% and that of Côte d'Ivoire 8.0%. All the cocoa beans from both countries were infested with *T. castaneum, C. ferrugineus, E. cautella* and *A. fasciculatus*.

All three insect species that were introduced on the cocoa beans multiplied significantly ($P < 0.01$) over the storage period (Fig. 1) causing severe damage to the cocoa beans. The insect activity resulted in a significant ($P < 0.01$) increase in FFA content (Fig. 2) over the storage period. However, the control samples which were not infested with insects maintained a low FFA level throughout the storage period. The FFA content of insect infested cocoa beans increased by 138.2% whereas that of the control without insect infestation increased by 17.7% over the 9 mo storage period. In comparison, the FFA content of

cocoa beans from Ghana was significantly lower than that of Côte d'Ivoire (Fig. 3). The insect population in cocoa beans from Côte d'Ivoire was slightly higher than that of Ghana although not significant and the 8.0% moisture content of Côte d'Ivoire cocoa beans is just on the border line of FCC threshold. However, all the cocoa beans from Côte d'Ivoire contained more than 1.4% FFA while that of Ghana had FFA levels lower than 1.0%. Ghanaian cocoa beans stored between one year (2008 crop) and three years (2006 crop) had a FFA content lower than 1.0% while the cocoa beans stored for five years (2004 crop) higher than 1.0% though lower than the 1.75% threshold level (Table 1). The cocoa beans stored for ten years (1999 crop) contained more than 1.75% FFA. Differences in FFA content between the cocoa beans stored over the storage period and samples taken from different sites were significant ($P < 0.01$). Cocoa beans from Côte d'Ivoire stored for one year (2008 crop) had FFA content more than 1.4% and cocoa beans stored for seven years (2002 crop) contained more than 2.5% FFA (Table 2) which was higher than the internationally accepted level of 1.75%. The FFA content of 2008 crop differed significantly ($P < 0.01$) from that of 2002 crop although samples taken from the three sites were not significantly different ($P > 0.05$).

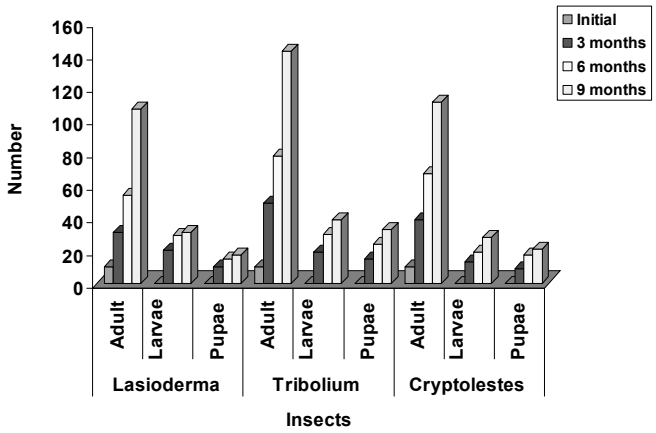

Figure 1 Number of insects found on 500 g of dry cocoa beans from Ghana after various months of storage (s.e. = 0.044).

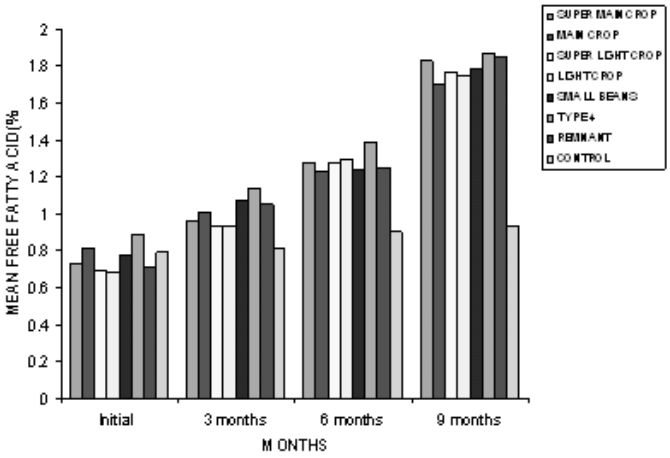

Figure 2 Free Fatty Acid (FFA) contents of various categories of dry cocoa beans from Ghana artificially infested with stored product insects and stored over different periods of time (s.e. = 0.006).

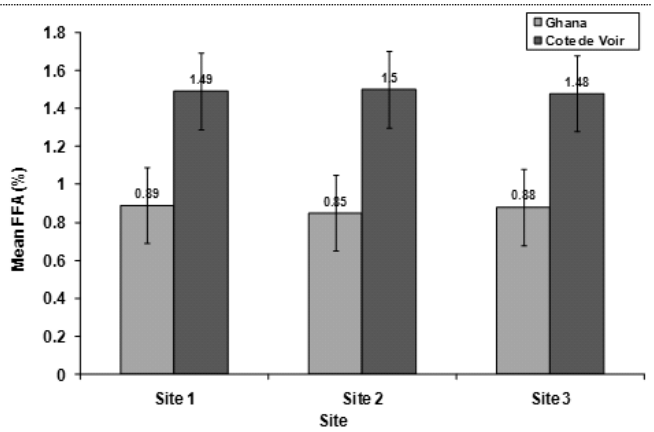

Figure 3 Free fatty acid (FFA) contents of 2008 crop year cocoa beans from Ghana and Cote d'Ivoire.

Table 1 FFA analysis of dry cocoa beans from Ghana stored over period of time (s.e. = 0.004).

	Mean FFA (%)							
	Sampling site 1			Sampling site 2			Sampling site 3	
Crop year	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2
1999	2.08	2.06	2.09	2.06	2.04	2.03	1.96	1.94
2004	1.40	1.49	1.45	1.40	1.43	1.41	1.37	1.35
2006	1.25	1.24	1.27	1.18	1.17	1.15	1.11	1.14
2008	1.10	1.09	1.07	0.91	0.93	0.90	0.70	0.69

Table 2 FFA analysis of dry cocoa beans from Cote d' Ivoire stored over period of time (s.e. = 0.005).

	Mean FFA (%)							
	Sampling site 1			Sampling site 2			Sampling site 3	
Crop year	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2
2002	2.63	2.60	2.58	2.53	2.51	2.55	2.57	2.60
2008	1.42	1.47	1.45	1.44	1.39	1.41	1.40	1.43

4. Discussion

4.1. Insect pest infestation

Storage management plays a vital role in maintaining the quality of cocoa beans in storage. Particularly dry and cool conditions should be preferred. Lower temperatures would result in maintaining better quality cocoa beans by inhibiting FFA and insect development. However, since cocoa beans are hygroscopic, sudden removal of the cocoa beans from cool to warm areas without sufficient temperature equilibration would cause moisture migration. A well managed cocoa bean warehouse would be free from insect infestation which causes devastating effect on the beans.

Some insects have been found not to be attracted to stored products at long range but to locate their food by making holes into (Hodges, 1994; Hodges et al., 1999) the cotyledon, causing much damage (Hodges, 1994; Hodges et al., 1999). The insects inflicted damage on the cocoa beans by direct feeding. Their multiplication on the cocoa beans is a nuisance, for example, members of the genus *T. castaneum* are known to produce toxic quinones (Mills and White, 1994).

4.2. Effect of insect pests on FFA content

Insect damage to the cocoa beans in storage resulted in mustiness, leading to mould formation and the breakdown of fat to free fatty acids in the beans. The significant increase in the level of FFA during storage suggests that insect infestation is one of the factors other than biochemical factors that may be

responsible for the increases in FFA levels in stored cocoa beans. Low moisture content can limit the increase in FFA, which are carboxylic acids released from triglycerides (Selamat et al., 1996) facilitated by a lipase (E.C. 3.1.1.3) or oxidation. Also the risks of oxidation are negligible in cocoa butter due to its low unsaturated fatty acid content (Whitefield, 2005) and high content of polyphenols, natural antioxidants, in cocoa beans (Nickless,1996). Under negligible biochemical activity, insect activity will breakdown cocoa butter, increasing the FFA level in the cocoa beans (Anon, 1970). The increase in the amount of FFA therefore has a direct impact on the fat content and causes a negative change in cocoa flavour (BCCCA, 1996). For reasons of quality therefore, the directive 73/241/EEC (EEC, 1973) limits the maximum FFA content to 1.75% oleic acid equivalent in cocoa butter.

In conclusion, this study showed that poor storage management resulting in insect infestation resulted in increased FFA content. Good storage management must be practiced to control insect pests and sustain the quality of the cocoa beans.

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Spatial distribution of stored grain insects in a rice storage and processing facility in Brazil

Lazzari, F.N.*[1], Lazzari, F.A.[2], Lazzari, S.M.N.#[1], Ceruti, F.C.[1]
[1] Department of Zoology, Universidade Federal do Paraná, Caixa Postal 19020, 81531-980, Curitiba, Paraná, Brazil. Email: ferlazzari@gmail.com
[2] Researcher, Private Consultant

* Corresponding author
# Presenting author

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Abstract

This study describes the spatial distribution of stored product insects captured biweekly using food-baited cage traps in a large rice storage and processing facility, in the state of Rio Grande do Sul, Brazil. Monitoring started in August 2009 and will be carried out for 1 year, the first 5 months of sampling being presented in this study. From end of August 2009 until the end of December 2009, a total of 9893 insects were captured in the 99 cage traps. The most abundant species were: *Carpophilus* spp. (76%), *Typhaea stercorea* (8.6%), *Ahasverus advena* (5.5%), *Tribolium castaneum* (2.3%), *Sitophilus oryzae* (2%), *Sitophilus zeamais* (1.5%), *Ephestia* spp. (1.2 %), *Cryptolestes ferrugineus* (1%), *Rhyzopertha dominica* (0.64%), *Oryzaephilus surinamensis* (0.6%), *Anthicus floralis* (0.4%), *Lasioderma serricorne* (0.25%). The first two species, which make up for 84.6% of the insects collected, are not considered pests in stored grain, rather are attracted by moldy material present in residues or even in the bait material. The other insects, including primary and secondary species, comprised about 15% of the total trapped. The spatial distribution of the most important species infesting rice grain and of the total insect number was analyzed using Surfer 6.04 (Golden software, Golden, CO, USA) and contour maps were constructed to target areas for sanitation. Except for trap 66, located by the rice hulk storage box, the spatial distribution we observed using the contour maps showed that the greatest number of insects was mostly captured in cages placed in the receiving area, around the dryers, as well as outside of the structure where grain residues frequently accumulate. As indicated on the maps for total number of insects, a few isolated infested spots were detected. The parboiled rice area had the least amount of insects, except for trap 61, placed outside the structure. The population of primary and the most important secondary insect species, as well as the overall number of insects, decreased after sanitation and physical control measures were applied. Our observations confirm that insect monitoring is an essential tool for targeting and evaluating the control measures adopted in the quality program of rice storage and processing facilities.

Keywords: Insect monitoring; Spatial distribution; Stored grain pests; Stored rice

1. Introduction

Rice, *Oryza sativa* (Poaceae), is one of the main food sources for people all over the world. According to FAO (2010), in 2008 the world produced 685 million tons (mt) of rice, 622 mt in Asia alone. Brazil was the 10th largest producer with 12 mt in 2008. The state of Rio Grande do Sul was the largest rice producer in Brazil with 7905 thousand t and a productivity of 7.15 Kg/ha in 2008, the highest in the country (FAO, 2009; CONAB, 2010).

The control of insect and mite pests in stored rice using fumigation and residual insecticides is still the most common practice in Brazil, these methods, however, may not be the most cost-effective. In addition, the residues of the active ingredients can cause contamination of the environment and health concerns with workers that are exposed to these chemical insecticides (Lorini, 1997; Fields and White, 2002). In addition, the resistance of insect populations to chemical insecticides has been documented in many countries (Subramanyam and Hagstrum, 1995; Collins et al., 2000). In fact, insect resistance has become a grave concern in some parts of the world, where only a few commercial insecticides are available.

Instead of scheduled pesticide applications, integrated pest management (IPM) uses a cost-benefit analysis to make decisions on when and how to perform pest control (Hagstrum and Flinn, 1996; Hagstrum and Subramanyam, 2000). These decisions, when possible, resort to alternative methods such

as the use of resistant varieties (Throne et al., 2000), aeration (Reed and Arthur, 2000), low and high temperatures (Evans, 1986; Fields, 1992; Pinto Jr., 1999; Ceruti and Lazzari, 2005; Fields, 2006; Lazzari et al., 2006; Beckett et al., 2007), inert dusts (Jayas, 1995; Subramanyam and Roesli, 2000; Lorini et al., 2002; Athanassiou, 2005; Lazzari and Ribeiro-Costa, 2006), natural enemies (Kistler, 1985), and other integrated measures of managing pests. The issue is that the management of stored product pests, especially insects and mites, using these non-chemical techniques, requires greater knowledge and training in pest biology, behavior, ecology, population dynamics, spatial distribution compared to conventional chemical insecticides (Hagstrum and Subramanyam, 2009).

Post harvest IPM focuses primarily on structural modifications, sanitation of the facilities, and targeted pest control. Clean and sanitized structures are less likely to be favorable to pest establishment (Subramanyam et al., 2005; Campbell et al., 2006).

In IPM programs, monitoring is one of the most important approaches used when making decisions on pest control tactics. Monitoring usually requires trapping, not for the purpose of catching as many pests as possible, but to accurately monitor the population levels and to obtain data on the pest populations and their spatial-temporal dynamics (Arbogast et al., 1998). At first, it might seem like a costly and time consuming operation, however, applying pesticide treatments when they are unneeded, may add unnecessary to the cost to the pest management operation.

Targeting pest management to the places where the pests are located, in or outside a structure, increases the probability of suppressing that population and it is usually less costly and risky (Brenner et al., 2006).

As well as sanitation and monitoring insect activity and presence with traps, contour mapping can also be a very important tool in IPM. Techniques for spatial analysis applied to entomology provide a powerful tool and can impart crucial information to assist in biological interpretation of trap captures. In contour analysis, data are first entered on a map as a series of sample points. A denser grid of data points is then generated by interpolation, what can be done using several different algorithms. Subsequently, lines are drawn between points of equal value. These lines are called contours and are used to estimate insect population density in areas that have not been sampled (Arbogast et al., 1998; Trematerra et al., 2004).

The objective of this study was to monitor insect infestations in and around a rice facility and thus determine areas of risk that need more emphasis on cleaning and other safe and efficient insect control methods.

2. Materials and methods

The study was conducted in the largest rice storage, processing and packaging facility in Brazil, located in a single location in the city of São Borja, State of Rio Grande do Sul, southern Brazil. This rice processing plant is located at 28°39'38"S; 56°00'16"W; and 96 m asl. The climate, according to the Köppen-Geiger climate classification system, is Cfa subtropical humid. The facility contains several holding silos for wet rice, 56 metallic silos for storage and a white rice and a parboil rice plant.

A total of 99 food-baited cage traps, similar to those used by Throne and Cline (1991), and adapted by Pereira (1999), were placed around the structure, of which 44 were placed around the silos, 34 in the white rice plant and 21 in the parboil rice plant (Fig. 1). The bait consisted of 2 parts of whole corn kernels, 2 parts of broken corn kernels, 1 part of whole rice, 2 parts of broken rice and 1 part of wheat germ, previously sifted and frozen for 7 d at -18°C to kill any insects present in the raw material. About 150 g of bait were placed in a foil pan on the bottom of each cage and removed, whenever possible, every 15 days and the captured insects were counted and identified. Monitoring started on August 2009 and will be continued for one year; however, for this paper only the first 5 months of monitoring are presented for the overall insect captures and 6 months for key rice pests.

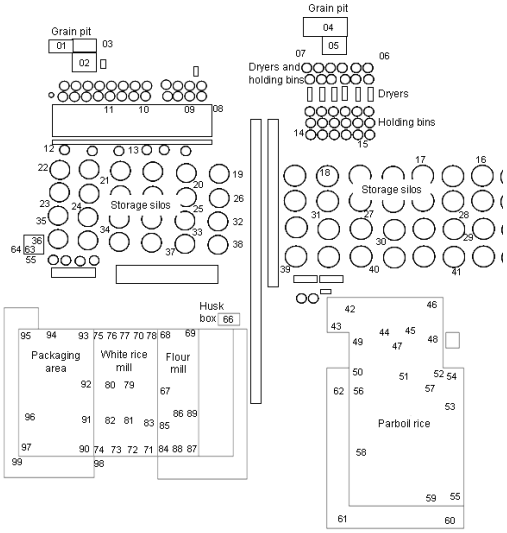

Figure 1 Food-baited cage trap distribution in the storage and processing areas of a rice facility in São Borja, Rio Grande do Sul, Brazil, from August 2009 to January 2010.

The spatial analysis was carried out using the program Surfer version 6.4 (Golden software, Golden, CO, USA). The x and y coordinates represent the position of the traps and z representing the total number of insects captured in a particular trap over a 15 d interval. By interpolating the z values using the interpolation algorithm linear Kriging with a zero nugget, Surfer produces a grid of values. The interpolation grid obtained is used to produce a contour map, which shows the configuration of the surface by means of isolines representing equal z values. Average temperature and relative humidity were recorded.

3. Results

A total of 9893 insects were captured with the food-baited cage traps from August until December 2009 (Table 1 and Fig. 2). The most abundant species were the sap beetles *Carpophilus* spp. (7527 specimens) and about 50% of them were collected in trap 66 on November 16. The second most abundant species was *Typhaea stercorea*, commonly known as hairy fungus beetle (857 specimens). These first two species represented about 85% of all captured insects until December 2009. Both pests are not direct pests of stored rice but may be associated with stored products, feeding mostly on molds and other decaying material. Several other insect species were captured, but in smaller numbers: *Ahasverus advena* (5.5%), *Tribolium castaneum* (2.3%), *Sitophilus oryzae* (2%), *Sitophilus zeamais* (1.5%), *Ephestia* spp. (1.2 %), *Cryptolestes ferrugineus* (1%), *Rhyzopertha dominica* (0.64%), *Oryzaephilus surinamensis* (0.6%), *Anthicus floralis* (0.4%), *Lasioderma serricorne* (0.25%).

Table 1 Species and number of insects captured with food-baited cage traps from August 2009 to December 2009 in a rice processing facility in São Borja, Rio Grande do Sul, Brazil.

	Date of collect							
Order/Family/Species	**Aug 27**	**Sep 10**	**Oct 02**	**Oct 13**	**Nov 16**	**Dec 14**	**Dec 26**	**Total**
Coleoptera								
Anobiidae								
Lasioderma serricorne (F., 1792)	1	3	4	3	56	0	31	117
Anthicidae								
Anthicus floralis (L., 1758)	10	11	15	1	2	3	1	43
Bostrichidae								
Rhyzopertha dominica (F., 1792)	10	2	8	4	10	8	21	63
Cucujidae								

Order/Family/Species	Date of collect							
	Aug 27	Sep 10	Oct 02	Oct 13	Nov 16	Dec 14	Dec 26	Total
Cryptolestes ferrugineus (Stephens, 1831)	1	1	14	23	41	17	5	102
Curculionidae								
Sitophilus oryzae (L., 1763)	78	61	22	26	5	6	2	200
Sitophilus zeamais Motschulsky, 1855	42	60	16	15	13	4	1	151
Mycetophagidae								
Typhaea stercorea (L., 1785)	62	154	139	86	145	156	95	837
Nitidulidae								
Carpophilus spp.	245	700	1005	443	3706	634	794	7527
Silvanidae								
Ahasverus advena (Waltl, 1834)	27	85	90	15	244	59	23	543
Oryzaephilus surinamensis (L., 1758)	13	4	0	0	39	0	0	56
Tenebrionidae								
Tribolium castaneum (Herbst, 1797)	115	28	8	1	70	4	4	229
Lepidoptera								
Pyralidae								
Ephestia spp.	20	3	4	3	56	0	31	117
Total	623	1112	1322	621	4341	897	977	9893

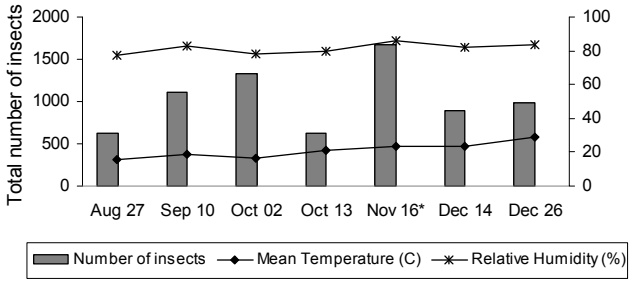

Figure 2 Total number of insects captured in food-baited cage traps compared to mean temperature and relative humidity in a rice facility in São Borja, Rio Grande do Sul, Brazil, from August 2009 to December 2009.

The most important insect pests that infest the rice grain, *S. oryzae*, *S. zeamais*, *R. dominica*, *T. castaneum* and *Ephestia* spp., added up to 7.6% of all insects captured. The population fluctuation of these species along the trapping period shows that in the first two collection dates, late August and early September, most of these species occurred in numbers higher than in the subsequent samplings (Fig. 3). After grain treatment and sanitation measures adopted in late September there was a decrease in the number of these insect species. In November, high population peaks of *T. castaneum* were recorded in trap 3 in the receiving area and of *Ephestia* spp. in traps 83 and 96 in the white rice milling and packaging areas (Fig. 3 and 4). In November, trap 61 captured a few *S. oryzae* on the outside of the parboil rice plant (Fig. 4).

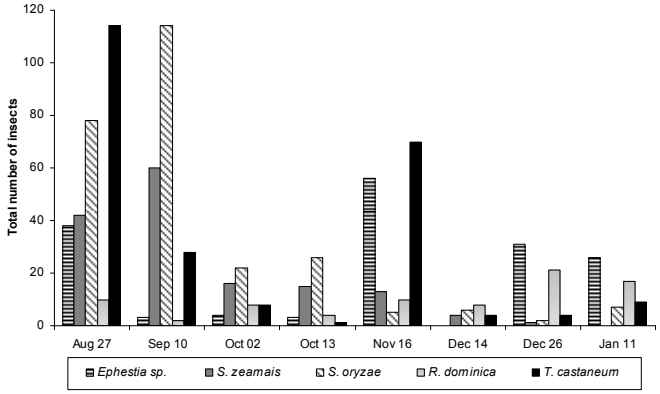

Figure 3 Total number of key insect pests for stored rice (*S. oryzae*, *S. zeamais*, *R. dominica*, *T. castaneum* and *Ephestia* spp) captured in food-baited cage traps in São Borja, Rio Grande do Sul, Brazil, from August 2009 to January 2010.

Even with increasing temperatures from December through January, the populations were maintained under control as result of sanitation and control measures.

Contour maps for the months of August, November and January were designed for these key species (Fig. 4). Trap 97 located in the white rice plant presented a high number of *T. castaneum* and trap 93 had a few *Ephestia* spp. in the August capture. For November and January there were no captures of *T. castaneum* in trap 97 and a decrease in captures of *Ephestia* spp. In the parboil rice plant, the November capture presented the largest number of insects. Trap 46 had a large number of *T. castaneum* and trap 61 had a large number of *Ephestia* spp. In January, trap 46 had no insects and trap 61 had only one *S. oryzae*. In the silo area, the capture for these key species was low in all three months. In November, there was an infestation point of *T. castaneum* in trap 3, near where the grain is received and dried. In January there was a large capture of *R. dominica* in trap 16.

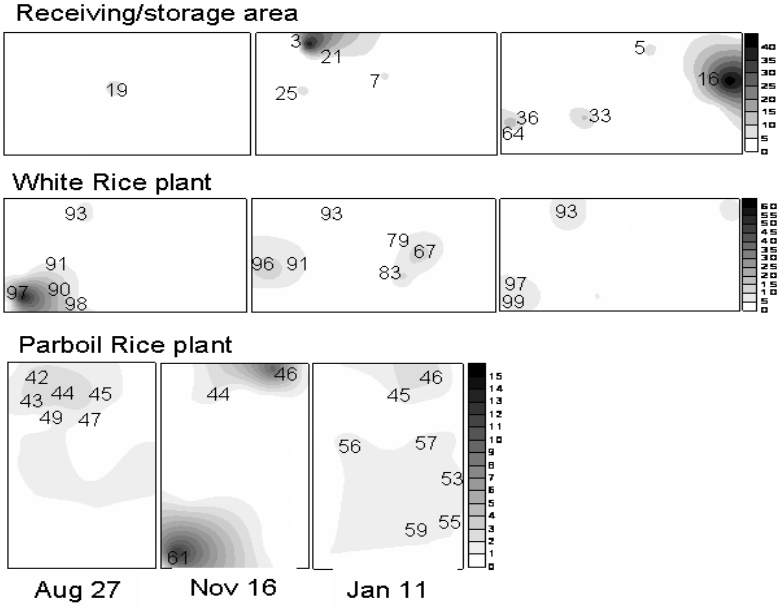

Figure 4 Spatial distribution of key pests for stored rice (*S. oryzae*, *S. zeamais*, *R. dominica*, *T. castaneum* and *Ephestia* spp.) captured in food-baited cage traps in the months of August, November and January, in São Borja, Rio Grande do Sul, Brazil. See Figure 1 for details of the rice plant.

The traps collected November 16th had the highest number of all captured insect species, but this was probably due to the long period of time that the traps remained in place without replacement (over 1 month).

Comparing the trap display in the receiving, drying and storage area (Fig. 1) to the contour map of the same area for the total number of captured insects (Fig. 5a), traps number 7, 14, 15 16, 22, 33 and 40 were the most infested, presenting a total of over 200 insects each. Traps 7, 14 and 15 are close to a pre-cleaning machine and the dryers and may represent a focus of infestation to the other traps nearby.

The contour map of the white rice plant (Fig. 5b) shows that this area had the largest number of insects, including traps 67 and 89, that are placed right in the rice flour milling area, and trap 98, which is placed just outside the plant (Fig. 1). Trap 66, on the outside north east corner of the white rice plant, was the trap that had the overall largest number of insects. On the November 16th collection alone, 2670 *Carpophilus* spp. were captured in that trap. Those insects were not plotted on Figure 1 to avoid overestimation of the data set.

Trap number 46 was the trap that had the largest amount of insects inside the parboil rice plant (Fig. 1 and 5c) and traps 61 and 62 had the largest amount of insects collected on the outside walls of the parboil rice structure, probably due to spillage.

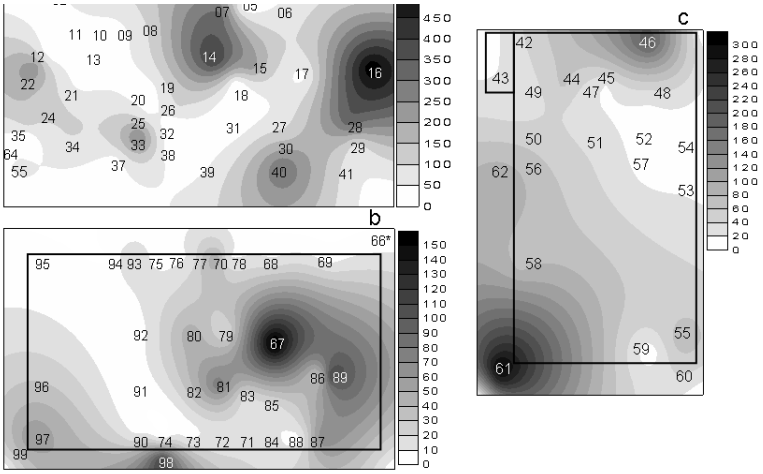

Figure 5 Spatial distribution of the total number of insects captured in 99 food-baited cage traps in the reception, drying and storage areas (a); white rice processing area (b); and parboil rice processing area (c), in São Borja, Rio Grande do Sul, Brazil, from August 2009 to December 2009. See Figure 1 for details of the rice plant.

4. Discussion

Monitoring with food-baited traps and the use of spatial maps of population distribution are two very important tools to detect insects in and around the structures to predict insect infestation and to target pest management. In this study key insect species infesting rice, as well as all insects occurring in rice storage and processing were monitored. Some of these insects, such as *Carpophilus* species, may not inflict direct damage to the grain, but they do indicate unsanitary conditions and present contamination if found in the final product.

The spatial distributions we observed through the contour maps indicate that the greatest number of insects were captured in places where the grain is received and dried, as well as where residues are stored and outside of the structure where grain spillage is common. Our results are similar to what Paula (2002), Pereira (1999), and Trematerra et al. (2004) reported for insect trapping in grain storage and processing facilities in southern Brazil. The different areas of the facility appear to have different species, populations, and infestation size. The parboil rice plants tend to have less infestation than the white rice plants and silos.

The results differ from what Trematerra et al. (2004) found in another rice storage facility in Massaranduba, State of Santa Catarina, as the various species did not show variable distribution in the same areas, and the populations seemed to remain in distinct parts of the structure. However, this characteristic might change for captures made during the coolest months of the year.

Even with the rising temperatures from August to December, the overall population of insects did not increase. This was due to the rigorous cleaning and sanitation measures that have been adopted since mid-September (Fig. 2). The entire structure, including silos and walls, were washed with high pressure water spray. The silos (3,500 t each) were thoroughly sprayed outside with a formulation of diatomaceous earth and deltamethrin before the new grain was added. The lower and upper 60 t of grain were dusted with powder DE plus deltamethrin during grain loading. Artificial chilled aeration was applied for about three days after the silo was loaded. The chilled air was introduced into the bin at 6 to 8ºC, and aeration continued until the grain mass reached 12 to 14ºC.

Basic cleaning measures, such as elimination of piles of old sacks, grain residues, garbage, other materials, and the cleaning of floors, machineries and silos before filling, as well as the control measures adopted resulted in an improvement in lowering the insect population. The real effects of these measures will be evaluated after a year of trapping. Samples of paddy rice have been taken periodically from the silos, but no insects have been observed in those samples.

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The potential of transgenic legumes in integrated bruchid management: assessing the impact on bruchid parasitoids

Lüthi, C.*#, Álvarez-Alfageme, F., Romeis, J.
Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstrasse 191, 8046 Zurich, Switzerland.
Email: christoph.luethi@art.admin.ch

* Corresponding author
# Presenting author

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Abstract

Leguminous seeds are an important staple food and source of nutrition in many countries. Bruchid beetles (Coleoptera: Bruchidae) are responsible for the greatest post-harvest losses to stored legumes. A powerful strategy to control bruchid infestations is the combination of plant resistance factors and biological control provided by parasitoids. Potent resistance factors are α-amylase inhibitors (αAI) which inhibit the starch metabolism in sensitive insects. Genetic engineering has been used to transfer αAI-1 from the common bean (*Phaseolus vulgaris*) to other leguminous plants which are subsequently protected from the attack by several bruchid species. However, there are concerns regarding the effects that the expressed insecticidal protein might have on non-target organisms. Here, we present an approach to assess the impact of αAI-1 genetically modified legumes on bruchid parasitoids.

Keywords: Risk assessment, Genetically modified plants, Non-target organisms; α-amylase inhibitor; αAI-1

1. Introduction

Legume seeds are an important source of nutrition for both humans and livestock. Their seeds are rich in proteins, carbohydrates and lipids, and they can be stored over extended periods. Additionally, the nitrogen-fixing abilities of the plants are important for the management of soil fertility. All these properties match perfectly with the requirements of small-scale, low-income farmers in developing countries.

Bruchid beetles (Coleoptera: Bruchidae) are responsible for the largest post-harvest losses to stored seeds, directly through consumption of the resource and, secondarily, through the qualitative deterioration of the commodity or the reduced stock viability. The females lay their eggs on the seed surface and the larvae burrow into the seed, where they feed and complete their development (Southgate, 1979). The beetles usually continue to multiply during seed storage, which can lead to extensive or even total losses, especially if the seeds are stored for long periods. Surface and fumigant chemical applications are thought to be the most effective methods for managing bruchid infestations. However, prohibitive costs, which limit their application to large scale or extended storage, and the risks of adverse secondary effects from such treatments, have driven the exploration of alternative strategies to manage bruchid infestations. These include biological control (Sanon et al., 1998; Gauthier et al., 1999; Schmale et al., 2006) and plant resistance factors (Ignacimuthu et al., 2000; Schmale et al., 2003; Appleby and Credland, 2004).

A crop protection tool with high potential for small-scale farmers is genetic engineering. Although the largest areas of genetically modified (GM) crop production have been in industrial countries, it's the small-scale farmers in developing countries that might benefit the most from this technology (Wambugu, 1999; Thomson, 2008). In 2008, 90% of the farmers planting GM crops were in developing countries (James, 2008). To date, all commercially cultivated insect-resistant GM crops are expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Berliner) (so called *Bt* crops). The potential of insects to evolve resistance against the deployed *Bt* Cry toxins, their narrow spectrum of activity and the risk of infringing existing patents have driven the development of alternative insecticidal traits for genetic engineering, including inhibitors of digestive enzymes (Malone et al., 2008).

2. Using a Phaseolus vulgaris resistance factor for genetic engineering

The common bean, *Phaseolus vulgaris* L. (Fabaceae) and other *Phaseolus* species possess a family of evolutionary related defence proteins including phytohemagglutinin (PHA), arcelin (Arc) and α-amylase inhibitors (αAI) (Chrispeels and Raikhel, 1991). The genes of these three proteins are encoded by a single locus in the *P. vulgaris* genome (Nodari et al., 1993) and it is likely that these homologous genes have arisen by duplication of an ancestral gene. The proteins feature different modes of action and insecticidal properties against bruchids (Leavitt et al., 1977; Liener, 1986; Osborni et al., 1988; Janarthan et al., 2008; Velten et al., 2007b). αAI can strongly inhibit the activity of α-amylases. These enzymes hydrolyse starch or glycogen and play a key role in the carbohydrate metabolism of microorganisms, plants and animals. Several insects, especially those feeding on starchy seeds during any period of their life cycle like bruchid beetles, depend on α-amylases for survival (Grossi de Sa et al., 1997; Franco et al., 2002). This feature has attracted notice to αAIs, making them a promising candidate for genetic engineering. The αAI of *P. vulgaris* exists in at least two different allelic variants. The isoform found in cultivated beans is called αAI-1 (Moreno and Chrispeels, 1989); a second variant, αAI-2, is found in some wild accessions of the common bean that contain Arc as the major storage protein instead of phaseolin (Suzuki et al., 1993). Of particular interest is that the two isoforms differ in their specificity towards α-amylases. With the exception of one major storage pest, *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), one or both αAI from the common bean have been shown to inhibit midgut amylases of major bruchid pests found worldwide (Ishimoto and Kitamura, 1992; Franco et al., 2002) (Table 1). The successful transfer of common bean αAI into tobacco plants (Altabella and Chrispeels, 1990) indicated that they could also be engineered to other plant species. Subsequently, genes encoding αAI-1 and/or αAI-2 from *P. vulgaris* were introduced by methods of genetic engineering into peas (*Pisum sativum* L.), cowpeas (*Vigna unguiculata* L.), azuki beans (*Vigna angularis* (Wildenow)) and chickpeas (*Cicer arietinum* L.) (Shade et al., 1994; Shroeder et al., 1995; Ishimoto et al., 1996; Morton et al., 2000; Solleti et al., 2008). The *α*AI gene construct introduced in these plants is regulated by flanking sequences from the seed-specific bean phytohemagglutinin PHA (*dlec*) gene. This promoter regulates the expression of the αAI restrictively to the cotyledon and embryonic axis of the developing seed (Schroeder et al., 1995), targeting exclusively seed-feeding herbivores like bruchids.

The potential of GM legumes expressing αAI to control bruchids has been confirmed in several studies. In peas, levels of expression of αAI ranged between 1.5 and 3.5% of total soluble protein, providing 100% control of *Bruchus pisorum* (L.) under glasshouse (Schroeder et al., 1995) and field conditions (Morton et al., 2000). Azuki beans expressing 0.9% αAI-1 (per dry weight) provided 100% control of the two bruchids, *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F., both important pest species in East Asia (Ishimoto et al., 1996). Similarly, αAI-1-GM cowpeas and chickpeas have been reported to strongly inhibit the development of *C. chinensis* and *C. maculatus* (Sarmah et al., 2004; Solleti et al., 2008).

3. Compatibility of αAI with biocontrol agents

The potential of combining plant resistance factors together with biological control agents, especially parasitoids, has been shown to be a powerful method to control storage pests like bruchids (Schmale et al., 2003; Velten et al., 2008). One major concern regarding the use of insect-resistant GM plants is the effect that the expressed insecticidal protein might have on non-target organisms. This is especially relevant for traits with a broad spectrum of activity such as αAIs where any organism relying on α-amylases for carbohydrate digestion is potentially affected by the inhibitor. The potential impact of αAIs on bruchid parasitoids has never been evaluated. Kluh et al. (2005) extensively screened the inhibitory activity of αAI-1 against 24 insect species from eight different orders. The most sensitive species belonged to the orders of Coleoptera, Diptera and Hymenoptera. The Hymenoptera tested included an endoparasitic wasp, *Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae). However, no bruchid parasitoids were included and whether they rely on α-amylases is still unknown.

4. Assessing the impact of legumes expressing αAI-1 on bruchid parasitoids

Environmental risk assessment for non-target organisms is a required step in the evaluation process of new GM plants. This is particularly true for GM crops expressing insecticidal proteins. The assessment typically follows a tiered framework which is conceptually similar to that of pesticides (Hill and Sendashonga, 2003; Romeis et al., 2008).

In an initial step one has to define what we do not want to see harmed by the GM trait. In the case of αAI-1 expressing legume seeds this is the biological control function provided by parasitic wasps that contribute to the control of bruchid species which are not or insufficiently affected by the introduced insecticidal trait. We can then develop a conceptual model describing a pathway how the presence of αAI-1 in the GM seeds can cause harm to biological control (Fig. 1). Following the different steps of the conceptual model allows a comprehensive evaluation of the risk for the parasitoids.

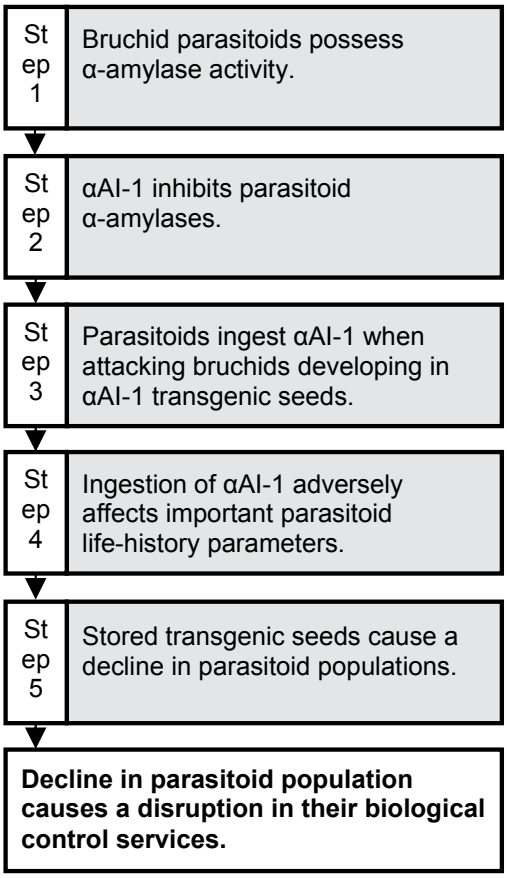

Figure 1 Pathway on how αAI-1 from *P. vulgaris* expressed in transgenic legume seeds could harm the biological control function provided by bruchid parasitoids.

The most basic requirement to make bruchid parasitoids a potential target of αAI-1 is the presence of α-amylases. Therefore the α-amylase activity was first characterized in five different parasitic wasps which are commonly used to control bruchids (Step 1): *Anisopteromalus calandrae* (Howard) (Pteromalidae), *Dinarmus basalis* (Rondani) (Pteromalidae), *Lariophagus distinguendus* (Förster) (Pteromalidae), *Eupelmus vuilleti* (Crawford) (Eupelmidae) and *Heterospilus prosopidis* (Viereck) (Braconidae). The characterization of such enzyme activity was based on the *in vitro* characteristics of complete insect extracts. Larval and female extracts of all species were able to hydrolyze the specific substrate potato starch although a higher activity was observed in the latter. Moreover, all extracts were highly susceptible to the specific inhibitors acarbose and wheat αAI. Taken all together, these results suggest that both larvae and females rely on α-amylase activity for carbohydrate digestion. Once α-amylase activity was detected, we went to the second step. The *in vitro* susceptibility to αAI-1 was determined by using different concentrations of the inhibitor in order to construct dose-response curves for all species. The α-amylase activities from all larval and female extracts were highly susceptible to αAI-1. This finding necessitates testing the impact of αAI-1 on the parasitoids *in vivo*.

The subsequent steps of the assessment will include tritrophic experiments using different plant-host-parasitoid systems. We selected αAI-1 expressing GM cowpea and chickpea lines and three bruchid species as hosts for the parasitoids, namely *C. maculatus*, *C. chinensis* and *A. obtectus*. The two former are reported being susceptible, the latter being resistant to αAI-1 (Table 1). Bioassays with bruchids developing in GM cowpea and chickpea lines, their non-transformed isolines and other non-GM varieties will be performed to assess the variance of resistance. Measuring different life-history traits will allow verifying the susceptibility of the bruchids to αAI-1 reported in the literature. The exposure to the inhibitor at the third trophic level will also be investigated; using Western Blotting or ELISA tests, the presence of the insecticidal compound in the body of collected bruchid larvae as well as parasitoid larvae and adult females of host-feeding parasitoids will be analyzed. These data allow retracing the path of αAI-1 through the food chain and help to understand the cause of possible impacts on the non-target organisms (Step 3).

Table 1 Present known distribution of major bruchid pests and their sensitivity to αAI-1 and αAI-2 from *P. vulgaris*.

	Distribution Worldwide						Inhibition of midgut α-amylase	
Species	**North America**	**South America**	**Africa**	**Asia**	**Australia**	**Europe**	**αAI-1**	**αAI-2**
Acanthoscelides obtectus	(+)	(+)	+	+	+	+	n[3]	n[3]
Bruchus pisorum	+	+	+	(+)	+	+	y[2]	p[2]
Callosobruchus analis	+		+	(+)			y[2]	
Callosobruchus chinensis	+	+	(+)	(+)	+	+	y[1,2,3]	n[1,3]
Callosobruchus maculatus	+	+	(+)	(+)	+	+	y[1,2]	y[3]
Zabrotes subfasciatus	(+)	(+)	+	+		+	n[1,2,3]	y[1,2,3]

(+) indigenous; + established; y = yes; n = no; p = partial; [1] artificial diet; [2] transgenic seeds; [3] amylase inhibitory activity

In the fourth step, tritrophic studies will be conducted to establish the host-mediated effect of αAI-1 on the selected susceptible parasitoids. Parasitoid females will be provided GM seeds expressing αAI-1 or untransformed control seeds infested with larvae of susceptible or tolerant bruchid species and parasitoid performance (e.g., survival, fecundity) will be assessed according to Velten et al. (2007a). If detrimental effects at the third trophic level are observed, bruchid-parasitoid population dynamic experiments will be conducted in the final step to assess long-term effects on the non-target population (Wäckers, 2003).

5. Conclusions

GM legumes expressing αAI-1 from *P. vulgaris* are protected from the attack by major bruchid pests. Some species, including *A. obtectus* and *Zabrotes subfasciatus* (Boheman), however, remain unaffected by this resistant factor. It is thus important that the biological control of these species is not disrupted by the insecticidal trait expressed in the GM legumes. However, the broad range of activity of this inhibitor and its possible transfer through the food-chain necessitate a detailed analysis of the possible impacts on beneficial non-target organisms. *In vitro* inhibition studies showed that αAI-1 inhibits α-amylase activity in larvae and females of several species of bruchid parasitoids. Consequently, parasitoids might be adversely affected when developing in or feeding on bruchid pests that are not controlled by the GM trait. The assessment of these non-target effects is necessary to ensure the compatible use of biological control and the GM host plant resistance trait for a sustainable control of bruchids.

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Influence of Sanitation on Post-Fumigation Pest Rebound

Mason, L.*#, Tsai, W., Ileleji, K.
1158 Entomology Hall, West Lafayette, IN 47907-1158, USA. Email: linda_mason@entm.purdue.edu

* Corresponding author
# Presenting author

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Abstract

Food processing plants often conduct thorough cleanings prior to fumigation, but sanitation practices post fumigation are quite variable. We conducted a study in several real-world fumigations in commercial flour mills to examine the influence of post-fumigation sanitation practices as well as other factors such as facility age, construction material, fumigant type, CT, door policy, and exterior pest pressure on the rate at which pest problems rebound to pre-fumigation levels. We found that although facility age, construction material and door policy were important, the most important factor was facility sanitation. Regardless of fumigant type, or time of year the fumigation occurred the facilities that maintain the highest sanitation levels, achieved the longest rebound time and thus received the maximum fumigation benefit. Those facilities that had poor sanitation practices, rebounded very quickly, sometimes within months, to pre-fumigation levels. Our findings support the use of sanitation as a pest management tool in flour mills and points out the importance of an IPM program.

Post-harvest technology transfer to reduce on farm grain losses in Kitui district, Kenya

Mutambuki, K. #, Ngatia, C.M.*, Mbugua, J.N.
Kenya Agricultural Research Institute National Agricultural Research Centre, P.O. Box 14733 00800 Nairobi.
Email: mutambukikimo@yahoo.com

* Corresponding author
# Presenting author

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Abstract

Training and demonstrations on post harvest technology transfer to reduce grain losses at farm level were conducted in five divisions of Kitui district with the overall objective of consolidating what the farmers already knew about storage. It was also to assist them select appropriate storage methods with emphasis on proper application of pest control products. A total of 163 participants were trained in storage pest management covering pest infestation cycle, use of chemical and non-chemical methods of control, storage practices and identification of major pests using specimen and pictures, dangers of mycotoxins on maize followed by on demonstrations.

Farmers demonstrated traditional practices of determining grain moisture content, and shovel mixing, both on the tarpaulin and wheelbarrows. Sticks were other tools used to mix chemical dust with grain on tarpaulins and in the bags. The research team from Kenya Agricultural Research Institute (KARI) demonstrated proper use of the shovel and the "fuffle", a device that is faster and more efficient in mixing grain with chemical dusts.

Treated grain and a control were stored on site in 90-kg bags. Evaluation, based on the level of damage and live infestation was done after 3 months. Despite anomalies like lack of uniformity of grain in different bags depending on source, KARI methods appeared better than farmers' methods in most instances. Farmers were able to make informed decisions based on the mixing methods which gave better results. The fuffle was an effective tool for mixing grain with chemical dusts and farmers were keen to have it fabricated by local artisans. Farmers appreciated the training and demonstration and promised to adopt proper grain preservation techniques as demonstrated to improve grain quality.

Keywords: Post harvest, Technology transfer, Grain losses, Farmers

1. Introduction

Maize is the most important food crop in Kenya with annual maize production between of 2.2-2.7 million tons and forms the bulk of stored grain (Anonymous, 2001). Storage insect infestation causes enormous losses reducing the investment made to its production and perpetuating famine and food insecurity. Efforts are therefore needed to address problems associated with maintaining acceptable quality stored grain.

During the 2001 survey in Kitui district, farmer clusters interviewed considered insect pests as number one constraints to maize storage (Mutambuki et al., 2001). A subsequent study established weight loss in Kitui to be 30% due to the presence of larger grain borer (LGB) *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) which had been there for 20 years (Mutambuki and Ngatia, 2003, 2006). Previously, the Ministry of Agriculture (MoA) responded by launching chemical treatment campaigns including store spraying whenever LGB outbreak was reported. However, Sumicombi (fenitrothion / fenvalerate), the chemical used was not readily available at the stockist shops in the villages since it was a donation. As one of the outputs from the above study, farmer training was seen as a key factor to successful pest management at farm level. Farmer training and demonstration took place between 4-8 June 2007 while evaluation was carried out between 27-31 August 2007.

The aim was to consolidate what farmers already know about storage and assist them practice appropriate pesticide application methods. The general objective of training was to trace the origin of problems in the storage of maize and highlight the best-suited methods of reducing them. Specifically, the training was:

- To highlight problem areas in the post harvest food pipeline (harvest - consumption).
- To point out improvement areas needed in storage methods.
- To demonstrate effective chemical dust application methods.
- To emphasise timeliness in the chosen IPM strategy applicable in storage pest control.
- To evaluate with farmers the chosen pesticide application methods.

2. Materials and methods

2.1. Venue selection

Pre-training planning meetings were held at the District Agricultural Office (DAO), Kitui and in five selected divisions including Mutomo, which has since been elevated into a new district to identify existing structures that could be utilised (Fig. 1). Five bags of 90-kg untreated maize were purchased from each of the identified Farmer Field School (FFs) who also provided a secure place to store prior and after treatment. The maize used was harvested during March 2007 and infestation had build up to moderate levels due to the 3 months in store without any protection.

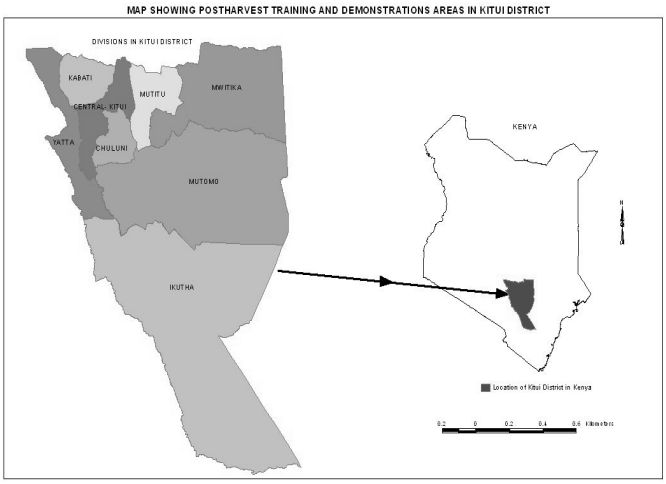

Figure 1 Map-showing postharvest training and demonstration areas in Kitu district in Kenya.

2.2. Farmer field school approach

Farmer field school approach was adopted to make use of the existing structures which guaranteed attendance. A discussion preceded each demonstration and efforts were made to standardise the delivery of topics by incorporating farmer participation using participatory methods adopted from a previous training program. Carefully formulated questions like "Whose maize was this?" ensured active farmer participation and ownership to the demonstration. A question like "What do you think is the problem with the maize?" led farmers to examine the grain and identified the various damage categories such as insect damaged and mouldy grains.

When asked to rate the level of insect pest damage, most farmers reported few insect damaged grains. Farmers were asked where they thought insects originated from. Most said that the origin of insect infestation was the farms probably due to the initial infestation often found on cobs during harvesting time. A question on whether there were insects found on maize but which were not harmful to the crop was puzzling. Farmers could not comprehend the concept of beneficial insects in the storage probably due to the fact that those deemed to be of benefit would ultimately contaminate after they die or due to their waste matter.

2.3. Effect of grain moisture and foreign matter on storage

To determine whether the maize for demonstration was dry, farmers demonstrated their varied ways of assessing produce dryness whose verdict was that the maize was dry. A Dickey-john moisture meter

(Dickey john Corporation, Auburn. IL, USA) confirmed their verdict. Many farmers stored grain with high levels of dust and foreign matter and they learned that apart from harbouring pests, dust can also reduce the effectiveness of the chemical powders applied on maize. Participants then used a sack sieve to remove appreciable amounts of dust, broken grain pieces, foreign matter and to their amazement, many free living insect pests, mainly the maize weevil.

2.4. Baseline damage levels and maize treatment methods evaluated

A kilogram of maize was taken from one bag and divided into 4 using coning and quartering method. Four groups of farmers were each given a portion of the sample maize to sort into undamaged, weevil damaged, mouldy grains, immature grains and broken pieces. Grains in each category were weighed and counted. To determine which methods to include in the demonstration, farmers described how they mixed stored grain with chemical dusts. Shovel mixing was common in all divisions and other methods included hand mixing on tarpaulin, in-bag mixing with a stick and shovel mixing in a wheelbarrow. The research demonstrated better ways of using the shovel and the "fuffle", equipment that can mix 20-kg grain load in about 10 sec. For comparison, a control was included to show farmers the increased level of infestation and damage if one did not treat stored maize.

2.5. Evaluation procedure

All the treated maize were stored on site and assessed after 12 wk, which would be 24 wk since the maize was harvested. A 1-kg sample from each treatment was analysed on site and another 500 g taken to the laboratory for thorough checking. Grains in the sample were analysed for the same parameters as in farmer-analysed samples. For reporting, each farmer-group constituted a replicate, hence the data for each treatment is the average of the four replicates. In laboratory, grains in each category were counted and weighed but only numbers are used for comparison. All the six parameters were evaluated to see which increased or declined over the storage period. Any change from the baseline was then linked to the maize treatment method used.

3. Results

3.1. Attendance

Out of a total of 163 that attended, the lowest, 22, were from Chuluni and 60, the highest from Matinyani divisions. The ratio of women to men varied from 1.5 to 2.3 in four divisions, while in Chuluni it was 0.6.

3.2. Grain damage levels during baseline and at evaluation time

The parameters in which farmers had strong interest were the declining undamaged portion, insect damaged grain, mouldy discolouration, broken pieces of grain, dust and foreign matter and grain moisture. Tables 1-5 shows what farmers did, but only the laboratory figures are used for reporting. The moisture content of maize used varied from 12.4% to 13.3%, which is considered safe for storage. Dust and foreign matter (fm) was below 1%, except for the 2.2% recorded in Mutitu. Initial weevil damage was lowest in Mutomo (1.8%) and highest (9.6%) in Matinyani. Mouldy grains were very low in Central division (1.5%) compared with 3.7% for Matinyani while broken pieces were very close in four divisions (1.1% – 1.2%) compared with 1.7% in Matinyani.

Table 1 Parameters assessed in Mutitu division.

Parameters	**June baseline results**		**August evaluation results**	
	Farmer analysis	**Lab analysis**	**Farmer analysis**	**Lab analysis**
Foreign matter (%)	2.2	2.2	0.8	0.8
Grain moisture content (%)	12.4	12.4	12.4	12.4
Insect damage (I)	3.8	4.5	11.3	11.2
Mouldy grains (M)	6.2	2.9	3.2	3.3
Immature (I)	2.8	---	---	---
Pieces(P)	2.5	1.2	4.4	1.3
Total of IMIP	15.3	8.6	18.9	15.8
Mean	3.8	2.9	6.3	5.3

Table 2 Parameters assessed in Central division.

Parameters	June baseline results		August evaluation results	
	Farmer analysis	Lab analysis	Farmer analysis	Lab analysis
Foreign matter (%)	0.6	0.6	1.0	0.8
Grain moisture content (%)	12.6	12.6	12.4	12.4
Insect damage (I)	2.5	6.2	25.3	20.8
Mouldy grains (M)	0.7	1.5	1.4	1.8
Immature (I)	4.1	---	---	---
Pieces (P)	1.8	1.1	3.9	1.8
Total of IMIP	9.1	8.8	30.6	24.4
Mean	2.3	2.9	10.2	8.1

Table 3 Parameters assessed in Matinyani division.

Parameters	June baseline results		August evaluation results	
	Farmer analysis	Lab analysis	Farmer analysis	Lab analysis
Foreign matter (%)	0.3	0.3	1.9	1.9
Grain moisture content (%)	13.3	13.3	13.4	13.4
Insect damage (I)	0.9	9.6	34.4	30.5
Mouldy grains (M)	2.8	3.7	2.4	3.2
Immature (I)	5.6	---	---	---
Pieces (P)	5.4	1.7	4.8	1.8
Total of IMIP	14.7	15.0	41.6	35.5
Mean	3.7	5.0	13.9	11.8

Table 4 Parameters assessed in Chuluni division

Parameters	June baseline results		August evaluation results	
	Farmer analysis	Lab analysis	Farmer analysis	Lab analysis
Foreign matter (%)	0.4	0.4	1.5	1.5
Grain moisture content (%)	12.4	12.4	12.8	12.8
Insect damage (I)	3.6	2.8	10.1	9.5
Mouldy grains (M)	3.5	2.1	2.7	2.4
Immature (I)	6.4	---	---	---
Pieces (P)	2.2	1.2	2.2	1.7
Total of IMIP	15.7	6.1	15.0	13.6
Mean	3.9	2.0	5.0	4.5

Table 5 Parameters assessed in Mutomo division.

Parameters	June baseline results		August evaluation results	
	Farmer analysis	Lab analysis	Farmer analysis	Lab analysis
Foreign matter (%)	0.4	0.4	0.1	0.1
Grain moisture content (%)	13.0	13.0	12.2	12.2
Insect damage (I)	3.7	1.8	2.7	2.6
Mouldy grains (M)	1.5	2.2	1.8	2.1
Immature (I)	2.5	---	---	---
Pieces (P)	3.2	1.2	2.8	1.1
Total of IMIP	10.9	5.2	7.3	5.8
Mean	2.7	1.7	2.4	1.9

After three months, a rising trend was expected for all the parameters. Grain moisture did not vary much and declined in two out of five divisions. Greatest percent increase in weevil damage was recorded in Matinyani (20.9%) and Central (14.6%) respectively while the same in Mutitu and Chuluni was 6.7%. Mutomo had the lowest at 0.8%. Mouldy grains increased by 0.3-0.4% in Mutitu, Central and Chuluni,

but declined in both Matinyani and Mutomo divisions. The increase in broken pieces was 0.7% and 0.5% for Central and Chuluni compared with 0.1% for Mutitu and Matinyani divisions. In decision making, farmers appeared to use a combined damage factor approach to reject or accept grain for consumption. A weighted average for weevil damage, mouldy grain and broken pieces indicated the order of most affected division to be Matinyani with 11.8–5.0% = 6.8% increase from June to August followed by Central (5.2%), Chuluni (2.5%), Mutitu (2.4%) and Mutomo with 0.2%.

3.3. Effective methods of mixing grain with chemical dusts

The results of farmer evaluation on maize treatment methods in each division are shown in Figures 2-6. In Mutitu, the level of initial weevil damage for 5 treatments including the control varied from 1.9% in the fuffle to 9.3% in farmer-shovel mixing method with 4.5% as the average. Two months later, the same varied from 3% to 29% with 11.3% average. Among treatments, shovel mixing by farmers had the highest increase (6.0%) in weevil damage while the research use of the same had the least change of 0.1%. All methods were better than control where weevil damage increased by 25.5% (Fig. 2).

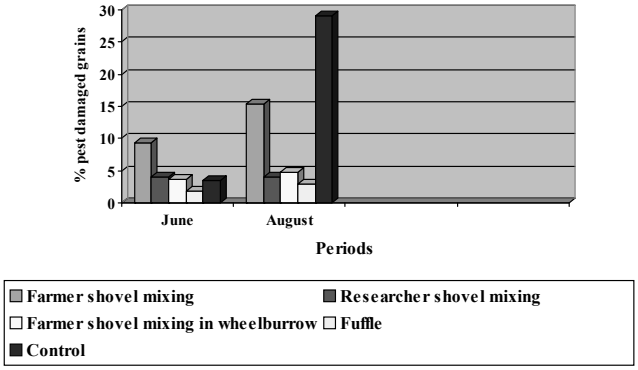

Figure 2 Level of pest damage in different maize treatment methods evaluated in Mutitu division.

The level of initial weevil damage for the five treatments including the control in Central division varied from 3.1% in the fuffle method to 6.5% in farmer-shovel mixing in the wheelbarrow with 4.5% as the average. Two months later, the same varied from 3.8% to 57.1% with 16.8% average. Among treatments, shovel mixing in the wheelbarrow by farmers had the highest increase (7.5%) in weevil damage while the research use of the shovel had 0.8% and 0.7% for the fuffle. All the treatment methods were better than control where weevil damage increased by 54.3% (Fig. 3).

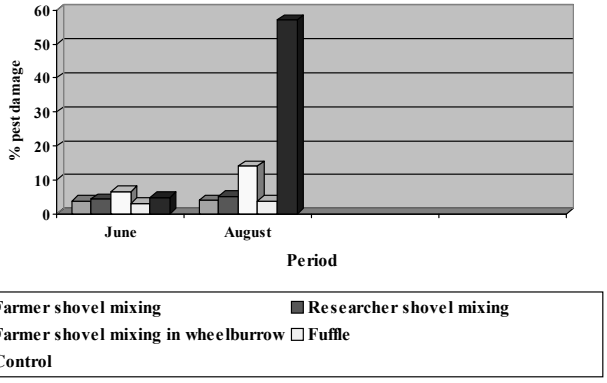

Figure 3 Level of pest damage in different maize treatment methods evaluated in Central division.

In Matinyani, the level of initial weevil damage for the 5 treatments including the control varied from 3.9% in the fuffle method to 9.7% in farmer-shovel mixing in the wheelbarrow with 7.1% as the average. Two months later, the same varied from 4.8% to 89.2% with 26.3% average. Among treatments, shovel mixing in the wheelbarrow by farmers had the highest increase (9.7%) in weevil damage while the research use of the shovel had 2.9% and 0.9% for the fuffle. All the treatment methods were better than control where weevil damage increased by 79.4% (Fig. 4).

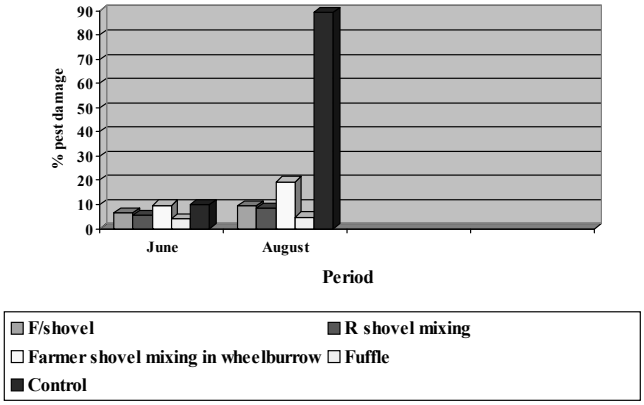

Figure 4 Level of pest damage in different maize treatment methods evaluated in Matinyani division.

The level of initial weevil damage for the five treatments including the control in Chuluni, varied from nil (0%) in the fuffle method to 10.8% in farmer-shovel method 3.0% as the average. Two months later, the same varied from 1.5 to 32.6% with 15.6% average. Among treatments, in-bag stick mixing by farmers had the highest increase (20.0%) in weevil damage while the research use of the shovel had 2.5 and 1.5% for the fuffle. All the treatment methods were better than control where weevil damage increased by 31.6% (Fig. 5).

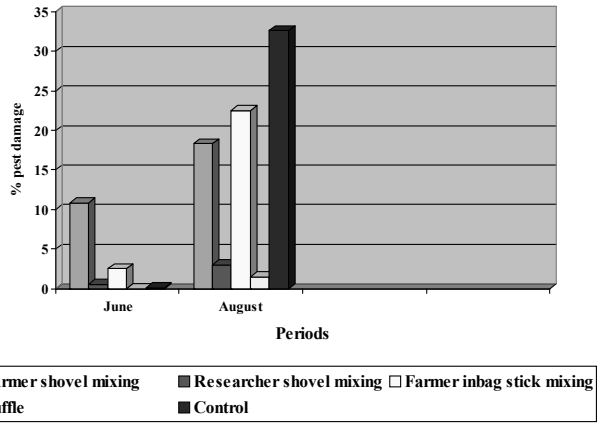

Figure 5 Level of pest damage in different maize treatment methods evaluated in Chuluni division.

The lowest level of initial weevil damage for the five treatments including the control was in Mutomo and varied from (0.3%) in the fuffle method to 4.3% in hand mixing on tarpaulin by farmers with 1.4% as the average. Two months later, the same varied from 1.2 to 5.6% with 3.0% average. Among treatments, hand mixing on tarpaulin by farmers had the highest increase (0.7%) in weevil damage while the research use of the shovel and the fuffle had 0.5% each. All the treatment methods were better than control where weevil damage increased by 5.2% (Fig. 6).

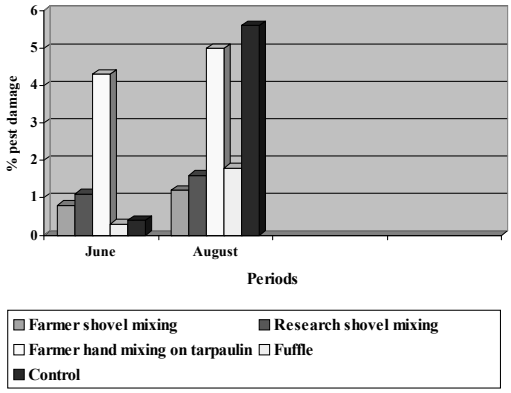

Figure 6 Level of pest damage in different maize treatment methods evaluated in Mutomo division.

4. Discussion

The training was organised to fill the gaps identified during on-farm surveys. The methods used and the approach was ideal for the task. The Farmer Field school setting improved farmer participation as well as learning. Such demonstrations are commonly referred to as "results demonstration" and appear to be most effective as farmers both practised and evaluated the results. They used these results to make informed decisions based on level of damage and increase in insect population found on maize from the evaluated treatments. Also, they were able to compare their methods with the ones demonstrated by the research team.

The demonstrations cannot be regarded as close to experimental trials. Ideally, the grain should have been thoroughly mixed to spread evenly the level of damage and the pest population. This would have cost implications. Despite lack of homogeneity, the results were as expected. Rather than use one factor criterion, the farmers' approach of combined effect was adopted. Farmers reject or accept grain based on extent of weevil damage, mouldiness and size of broken pieces. It was therefore easy to classify divisions according to level of combined damage levels. The delay in taking appropriate measures to protect stored maize was reflected in the level of initial weevil damage. Laboratory experiments have shown that early treatment can maintain grain damage below 5% over a 6-month storage period. However, with initial damage level between 1.4% and 7.1%, it was hard to expect impressive results. Despite the disadvantage of starting with heavy infestation, the fuffle proved to be an effective way to mix grain with chemicals dusts.

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Integrated control of *Ephestia cautella* (Walker) in a confectionary factory

Savoldelli, S.*, Süss, L.
DiPSA (Dipartimento di Protezione dei Sistemi agroalimentare e urbano e Valorizzazione delle Biodiversità), Università degli Studi di Milano, via G. Celoria 2, 20133 Milan, Italy. Email: sara.savoldelli@unimi.it

* Corresponding author
# Presenting author

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Abstract

In a confectionary factory, integrated pest-management techniques were used for 12 months. *Ephestia cautella* is the major pest in this industry. Its presence was monitored using pheromone traps and water traps. The most critical areas were identified and water traps were better at identifying these areas than pheromone traps. Intensive cleaning and structural improvements were carried out when necessary. Water traps have been used as a mass trapping system because they catch both males and females. Catches in water traps showed a decrease in population density after 8-9 months. In a confined area, a mating disruption system was applied to interfere with moth mating. Most females caught with water traps were mated, although in the area where mating disruption was applied, the percentage of unmated females was higher compared with areas where mating disruption was not used.

Keywords: Integrated pest management, Mating disruption, Water trap, Almond moth, Pheromone.

1. Introduction

In a confectionary factory in Italy, which produces a wide variety of chocolate-based products, the almond moth, *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae) is the major pest. It is managed by regularly scheduled fogging with synergized pyrethrins. Fogging affects only exposed insect stages but *E. cautella* growth and development occurs within refuges where the insecticide may not penetrate.

2. Materials and methods

Alternative methods were tested to manage the almond moth because of the difficulties in managing infestations in two well-isolated factory departments (Süss and Savoldelli, 2009). Water traps for mass trapping were placed in both departments. In the department where the moth density was less, a mating disruption system was tested. In the other department, maintenance and cleaning interventions were carried out at different times. No chemical treatments were made during the entire test duration.

Tests were performed in two production areas for 12 months. Area A was about 6500 m^3 while area B was about 8,000 m^3. Both had concrete floors, plastered walls and ceilings. The inside environmental temperature averaged 25°C in winter and 35°C in the summer; the relative humidity (r.h.) was between 28% and 37%. A pheromone trap system was established to monitor *E. cautella*, using 25 Storgard® II traps (Trécé, Inc. Adair, OK, USA) baited with a pheromone lure in area A and 26 traps in area B. Dispensers were changed every two months. In these two areas, a mass trapping system was applied, using water traps (13 in area A and 16 in area B) each consisting of a plastic box (50 cm x 40 cm x 15 cm), filled with about 8 cm of water. The water traps were placed on the floor along the walls. At the end of January, 10 m of "rubber string" dispenser, baited with 50 mg/m of Z9, E12 - tetradeca dienyl acetate (TDA), were placed in area A to study the interference of this pyralid sex pheromone compound with the mating behaviour of *E. cautella*. On the basis of data given by the producer, the release of pheromone was calculated as 200-220 µg/m (airborne concentration: 0.3-0.4 µg/m^3) for 2 months. The "rubber string" was cut up in pieces of about 50 cm, hung horizontally among the machines or near the walls, at a height of about 2 m. The "rubber string" was replaced about every 8 wk. Pheromone trap data were collected weekly, and water trap data about every 10 d. All insects trapped with water traps were taken to the laboratory, identified and sexed.

3. Results and discussion

The results of this study confirm the attractiveness of water traps to both males and females of *E. cautella*. Water traps captured more males for each trap compared to pheromone traps, placed in the same environment (Figs. 1 and 2). There was a large difference in the number of captures for each trap at

the beginning of the tests, when the initial level of moths was higher. Monthly data of water trap captures in area A show a peak of moths about every three months: February, May and August. From August to December, water-trap captures decreased and there were no other peaks. The analysis of the females captured with water traps highlights a larger percentage of unmated females in area A compared to area B.

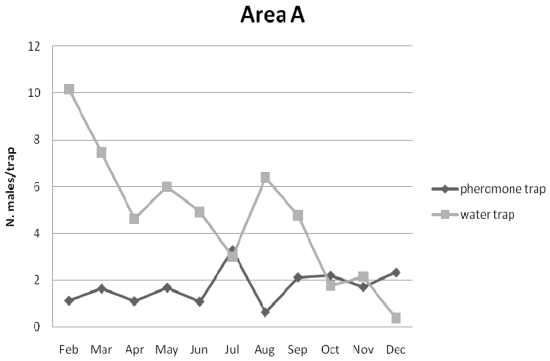

Figure 1 Monthly mean trap catch of *E. cautella* males in pheromone traps and water traps, in area A.

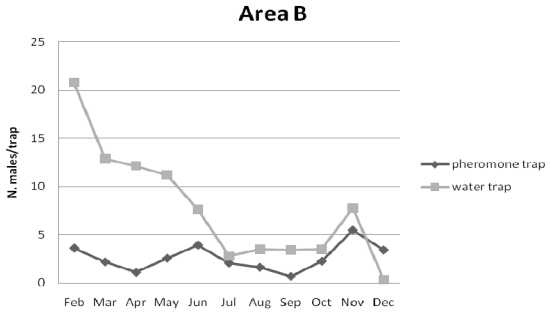

Figure 2 Monthly mean trap catch of *E. cautella* males in pheromone traps and water traps, in area B.

In area B, the initial presence of *E. cautella* was higher. Visual inspections and trap capture data showed an infestation focus in an unused piece of machinery located in the area. After the removal of the machine in May, captures decreased significantly. In August, fittings were cleaned and this intervention eliminated some infestation foci, as confirmed by the low number of water trap captures until October. In November, the milling plant in the area B was stopped for maintenance and cleaning. Captures increased, probably because the plant was opened and partly disassembled. Moths present in cracks and crevices that are usually isolated and difficult to reach may have been attracted to the pheromone and water traps.

4. Conclusion

The control of *E. cautella* can be managed using IPM techniques that combine pheromone traps for monitoring, water traps for mass trapping, pheromones for mating disruption, and, of course, good sanitation is also very important.

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Arthropod monitoring in an automated pasta warehouse

Savoldelli, S.*, Bruschi, E.
DiPSA (Dipartimento di Protezione dei Sistemi agroalimentare e urbano e Valorizzazione delle Biodiversità), Università degli Studi di Milano, via G. Celoria 2, 20133 Milan, Italy. Email: sara.savoldelli@unimi.it

* Corresponding author
# Presenting author

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Abstract

Pest monitoring was carried out in an automated pasta warehouse. It is managed with a logistic process and can contain more than 30,000 pallets. Good pest monitoring is difficult in this environment particularly because of the height (more than 20 m). Several sticky traps were placed on the floor under the shelves to monitor the presence of arthropods. Monitoring was done twice, once in March and once in November, each time for two weeks. Results showed the presence of stored-product pests, but also the occurrence of other arthropods.

Keywords: Pest monitoring, Stored-product pests, Automatic warehouse, Arthropods, Insects.

1. Introduction

In every environment where food is processed and stored, insects, mites and rodents are unwelcome intruders. Italy is an important producer and exporter of pasta and great deal of effort has gone into studying pest problems (Frilli, 1965; Dal Monte, 1985; Süss and Locatelli, 1996; 1997; Trematerra, 2002; 2004, Trematerra and Süss, 2006). Pasta factories, as any other food industry, can be infested by insects that are able to follow manufactured goods (packaged pasta) in stocking warehouses. Economic and commercial consequences of infested pasta can be very negative.

Monitoring traps are normally placed to promptly show the possible presence of pests. Also in automated warehouses, where manufactured foodstuffs are kept, infestation can occur, but monitoring is often impossible to carry out. In fact, pheromone traps for Lepidoptera and Coleoptera can be placed mostly only at the entrances or in some no-transit areas, but certainly not evenly-spaced in the whole warehouse. This is due to the enormous dimensions of the shelf facilities which reach great heights and have transit lanes among shelves. The situation is even more complex in the warehouses where pallets are placed automatically by trailers with a computerized system. In these cases, the presence of insect pests can be detected only when a pallet is collected from the trailer to be sent to the customer.

This study aims at verifying the presence of pests in an automated pasta warehouse, using unbaited sticky traps. Among the captured arthropods, the goal in particular was to highlight which ones are potential pests of pasta and which ones are occasional pests and to show the control measures that can be taken.

2. Materials and methods

The study was conducted in an automated pasta warehouse in Italy. The warehouse is 20 meters high and divided into 4 aisles. It contains more than 30,000 pallets of pasta that can be stored for different lengths of time up to several months. During this time, pasta pallets can be moved automatically to other positions, according to what the computerized system decides, in order to optimize space and storage. The warehouse receives continuously pallets from the pasta factory through an opening in which the conveyor belt passes. The conveyor belt arrives in the warehouse from the left side under the inspection gangway. The outgoing pallets leave the warehouse through a dedicated opening in the center, in the warehouse front. The movement of the pallets is steady and automatic. The warehouse has, in addition, a security exit on the opposite side that is kept closed.

Pest monitoring in the warehouse was carried out through 3 pheromone traps for moths and 3 anobiid traps, that were placed at the plant entrance where there is a narrow gangway among the operating controls of the entire system. The number of traps was certainly not enough to cover the whole warehouse area and inadequate to give information about a possible presence of pests.

To monitor the presence of pests, 54 unbaited sticky traps made of cardboard (13 cm x 10 cm) were placed on the floor of each aisle (216 in total), at a regular distance, in order to capture insects and other possible arthropods present in the area.

The sticky traps were placed in spring (March 2009) and in autumn (November 2009). They monitored the presence of arthropods for 15 days. The placement of the traps and their recovery required the whole computerized system to be shut down in order to enter the aisles between the shelves.

All traps were brought to the laboratory and observed with the stereoscopic microscope. Insects were divided into Orders and Families and classified to species, only if they were pests of pasta.

3. Results

A total of 1668 arthropods were collected in spring and 1591 in autumn. They belong mainly to Insecta, followed by Arachnida. In the autumn, 2 young geckos were also found: they were present in the warehouse as predators of arthropods (Table 1). In spring, among Arachnida, there were many spiders belonging to different species: they were probably preying on many of the insects present in the warehouse.

Table 1 Total amount of animals collected in the pasta warehouse in spring and autumn.

Class	Order	Spring	Autumn
Insecta	Thysanura	0	8
	Collembola	625	101
	Pscoptera	177	280
	Orthoptera	2	2
	Dermaptera	30	113
	Heteroptera	20	117
	Lepidoptera	1	2
	Diptera	348	283
	Hymenoptera	24	67
	Coleoptera	214	464
	Siphonaptera	1	0
Arachnida	Araneae	217	136
	Ixodida	9	4
Malacostraca	Isopoda	0	14
Reptilia	Squamata (Fam. Gekkonidae)	0	2

The collected insects belong to the orders of Thysanura, Collembola, Psocoptera, Orthoptera, Dermaptera, Heteroptera, Lepidoptera, Diptera, Hymenoptera, Coleoptera and Siphonaptera (Table 1). Most insects captured in spring belong to the order of Collembola (43%), followed by Diptera (24%), Coleoptera (15%) and Psocoptera (12%), whereas in autumn the majority of insects were Coleoptera (32%), Diptera (20%) and Psocoptera (19%) (Fig. 1, 2).

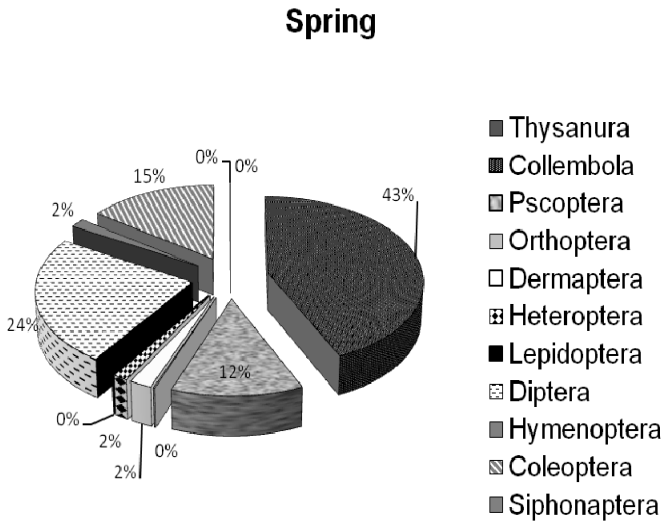


Figure 1 Insects collected in spring.

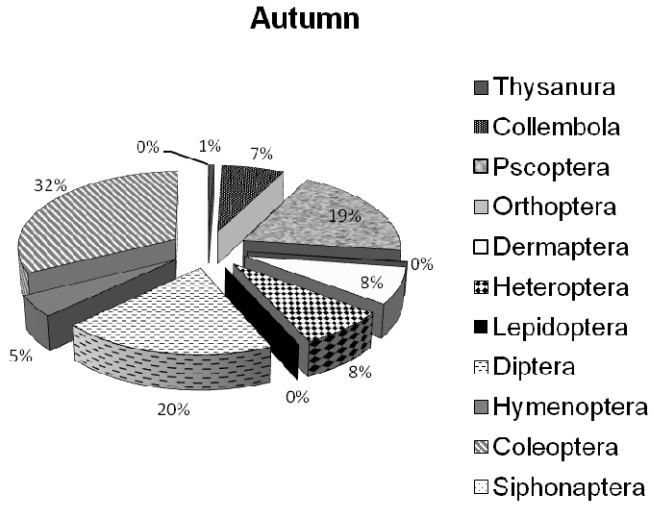


Figure 2 Insects collected in autumn.

The high number of Collembola, with species belonging to the genus *Hypogastrura*, and Psocoptera, especially *Liposcelis bostrychophila* Badonnel, is linked to the high relative humidity of the warehouse floor, caused by some rainwater seepage from the walls. Psocoptera were found throughout the whole warehouse both in spring and in autumn (Tables 2, 3); Collembola were present mostly in spring and, in different positions, more than 10 individuals/trap were captured (Tables 4, 5). It is reported that *L. bostrychophila* is the principal psocid pest species in Europe (Turner, 1998). At high density *L. bostrychophila* taints foodstuffs with waste products and may elicit allergic reactions in sensitized persons.

Furthermore, other species, mostly mycophagous and debris-eating, were found both among Diptera and among Coleoptera. In fact, the majority of Diptera belonged to the Mycetophilidae, albeit in autumn there were also Culicidae (*Culex pipiens* L.) that overwinter as adults in protected areas.

Among Coleoptera we found lathridiid beetles, cryptophagid beetles, *Typhaea stercorea* (L.) and *Ahasverus advena* (Waltl). They are mycophagous and their presence indicates moldy conditions. They do not feed directly on stored foods and their occurrence in commodities may be considered accidental contamination. Both in spring and in autumn, they were found throughout the warehouse, but in small numbers (21 traps in spring and 48 in autumn with less than 5 insects/trap) (Tables 6, 7).

Moreover, there were carabid beetles and staphilinid beetles, predators of insects. Their presence grew significantly in autumn, especially for staphilinid beetles that were found in 102 traps, placed throughout the warehouse (Tables 8, 9). Carabid beetles were found in 45 traps, that were placed more or less evenly throughout the warehouse (Tables 10, 11). The higher presence of these beetles in autumn may have been due to their inclination to find shelter to overwinter.

Heteroptera belonged basically to the families of Pentatomidae and Lygeidae; they were found in high numbers in autumn because they are insects that overwinter, as adults, in protected points and thus found shelter in the warehouse.

The number of species infesting foodstuffs was generally limited. Among the species that attack stored products, there were many *Oryzaephilus surimanensis* (L.) and *O. mercator* (F.), that fed on debris on the floor. They were present both in spring and in autumn. In spring, *Oryzaephilus* spp. was present in all the four aisles but concentrated especially in the first half of the warehouse, towards the entrance, with a density generally less than 5 insects/trap (Table 12). Only 6 traps captured from 5 to 10 individuals, mostly in the first half of the second aisle. In autumn, they were even more spread out as they were present in 79 traps compared to 70 in spring. They were found along the whole length of the warehouse, especially in the first and third aisle (Table 13). Trap number 36 captured more than 10 individuals, the other two traps captured from 5 to 10 individuals and the remaining ones less than 5.

Among pests of pasta, which are mainly *Sitophilus* spp., *Rhyzopertha dominica* (F.), *Lasioderma serricorne* (F.) and *Stegobium paniceum* (L.), there was occasionally only *S. oryzae* (L.). It was present mostly in the spring, in the first half of the first aisle (Table 14). In this area, pallets were heavily infested in the previous year. The infestation was managed by removing infested pasta and treating the floor with pyrethroids. In the autumn, captures of *S. oryzae* were very rare; they were found only in 6 traps, and less than 5 insects/trap (Table 15).

Table 2 Distribution of Psocoptera (*Liposcelis* spp.) in spring.

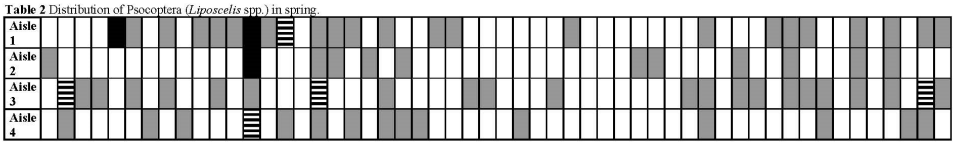


Table 3 Distribution of Psocoptera (*Liposcelis* spp.) in autumn.

Table 4 Distribution of Collembola (*Hypogastrura* spp.) in spring.

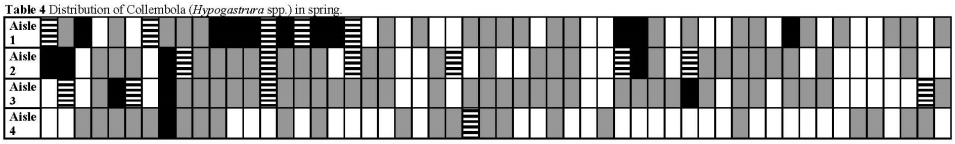


Table 5 Distribution of Collembola (*Hypogastrura* spp.) in autumn.

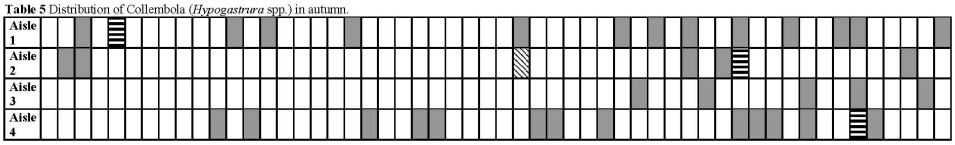


Table 6 Distribution of Lathridiidae, Cryptophagidae, *A. advena*, *T. stercorea* in spring.

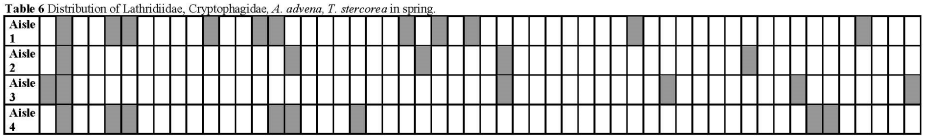


Table 7 Distribution of Lathridiidae, Cryptophagidae, *A. advena*, *T. stercorea* in autumn.

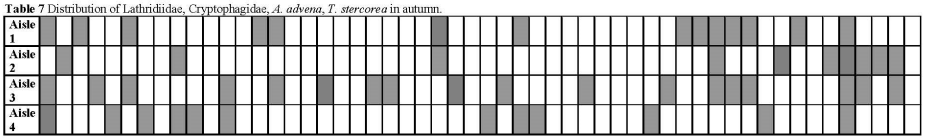


Table 8 Distribution of Staphilinidae in spring.

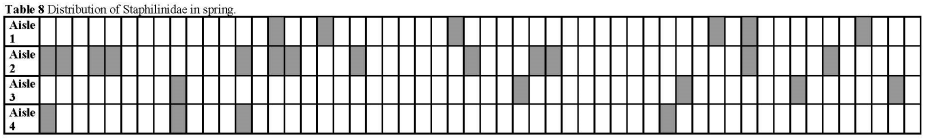


Table 9 Distribution of Staphilinidae in autumn.

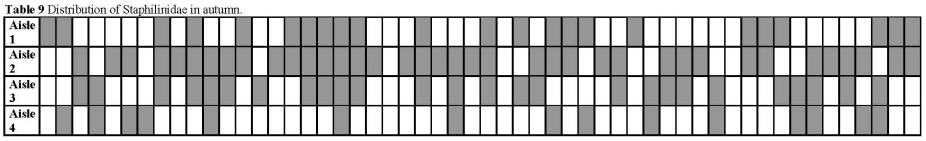


Table 10 Distribution of Carabidae in spring.

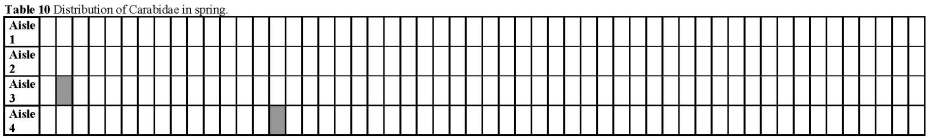


Table 11 Distribution of Carabidae in autumn.

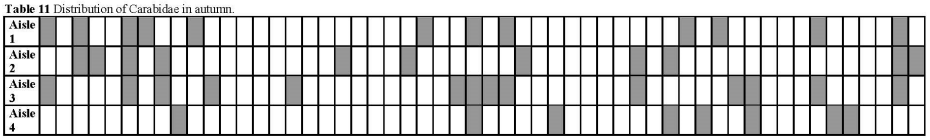


Table 12 Distribution of *Oryzaephilus* spp. in spring.

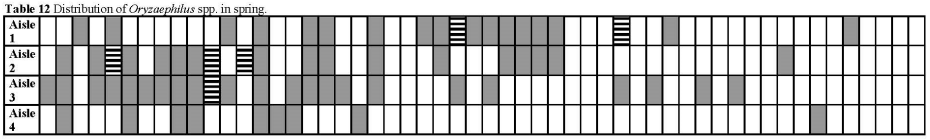


Table 13 Distribution of *Oryzaephilus* spp. in autumn.

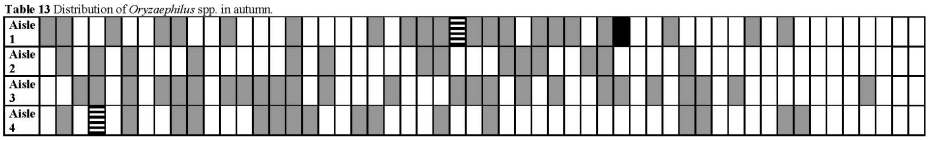


Table 14 Distribution of *Sitophilus oryzae* in spring.

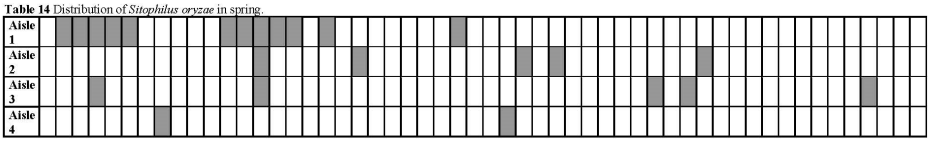
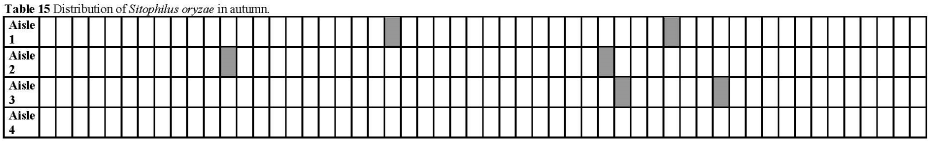


Table 15 Distribution of *Sitophilus oryzae* in autumn.



Distribution of insects collected in each aisle of the pasta warehouse: every square represents the position of a sticky trap.

- no insects
- < 5 insects
- 5-10 insects
- > 10 insects

Furthermore, the finding of a rodent flea in spring gave some cause for concern because it could be linked to the presence or passage of a rodent in the warehouse, although no traces of its activity were noticed. The presence of some hard ticks (*Ixodes* spp.), both in spring and in autumn, can be the sign of the presence of stray dogs outside the warehouse (Table 1). The warehouse is indeed located in an industrial area surrounded by non-tilled fields where some stray dogs were noticed.

The capture of several individuals belonging to different species of pentatomids, lygeids, carabids and staphilinids can thus be explained with the presence of non-tilled areas outside the warehouse. They develop in the countryside and look for nesting areas in autumn to overwinter.

4. Conclusions

This study showed which arthropods were present in the automated warehouse and their distribution. As a result of this study, monitoring is now carried out continuously (every two weeks) by the company. A technician is responsible for placing the traps, collecting them, and examining trap catch. Monitoring is carried out every two weeks. Identification of species is made only for insects infesting pasta (*Sitophilus* spp., *R. dominica*, Anobiidae), so that traps can be replaced more quickly (1-2 days are necessary to replace them). According to the operative protocol established by the Company, if insects infesting pasta are found, all the pallets above the monitoring point and immediately on the right or on the left are to be taken outside the warehouse and examined by trained staff. If a pallet has only one infested package, it is completely removed. The area of the floor where insects are captured is treated with pyrethroids (deltamethrin). Before this management procedure, without this kind of monitoring, there were heavy losses and widespread attacks. Since this new management has been implemented, the number of complaints has significantly decreased. For this reason, although it is an onerous and demanding practice, it continues to be used by this company.

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Early detection of insect infestation in stored grain based on head space analysis of volatile compounds

Laopongsit, W.#, Srzednicki, G.*
Food Science and Technology, School of Chemical Sciences and Engineering, The University of New South Wales, SYDNEY NSW 2052 Australia. Email: g.srzednicki@unsw.edu.au

* Corresponding author
# Presenting author

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Abstract

Insect infestation is a common problem for stored grain. Insects can cause quantitative losses as kernels are consumed by insects. Also, the appearance and organoleptic properties can be altered through physical damage and contamination by faeces, webbing and body parts of insects, respectively. Therefore, several detection techniques have been developed and applied to stored grains. Some methods demonstrate very high sensitivity but are relatively costly, and thus, are not affordable to the industry; whereas cheaper methods lack detection sensitivity. Consequently, volatile isolation techniques could be an alternative approach to monitor insects in stored grain. Odour detection is a useful tool for monitoring grain quality. It has become part of standard method for grading grain in the United States. Although identification of volatile compounds produced by fungi species has been extensively studied in most types of grains, this has been less done for insect infestation. This paper reviews the literature related to detection of insect infestation in stored wheat by using volatile isolation techniques.

Keywords: Grain infestation, Insect detection methods, Volatile isolation techniques, Solid phase microextraction

1. Introduction

Insect infestation is a common problem for stored grain. It can cause quantitative loss to grain as kernels are consumed by insects. Also, the appearance and sensory properties can be altered through physical damage and contamination by faeces, webbing and body part of insects (Bulla et al., 1978). This leads to the loss in economic value of the grain. Therefore, application of insect-detection technologies have raised a lot of interest in the grain industry in order to reduce loss and improve grain and grain-product quality..

There are many insect detection techniques available. Commercial methods include manual sampling, traps and probes which are the most basic tools used on farm. Manual inspection, sieving, floatation and Berlese-funnels are more advanced techniques used in grain-handling facilities (Neethirajan et al., 2007). Adult insects are often easily trapped or detected by these techniques, unlike immature insects, where detection is still limited to some extent. Hence, this requires the application of other techniques to grain which demonstrate higher sensitivity and accuracy. X-ray imaging and nuclear infrared reflectance (NIR) spectroscopy have been extensively studied since they are rapid and non-destructive methods (Milner et al., 1950; Karunakaran et al., 2003; Karunakaran et al., 2004; Rajendran and Steve, 2005; Fornal et al., 2007; Neethirajan et al., 2007; Haff and Toyofuku, 2008). Also, they can detect young insects and hidden insect infestation which cannot be seen by visual inspection. However, the cost of these technologies is relatively high and they require trained labour to operate (Milner et al., 1950; Neethirajan et al., 2007). Due to the high costs of detection technologies some researchers are now investigating odour detection techniques to be applied on infested grains since odour-detection has high sensitivity and accuracy with moderate costs.

Odour is a useful tool in monitoring grain quality. It has become part of standard methods for grading grain in United States (Ram et al., 1999). Along with odour evaluation by the human nose, technologies and techniques have been developed to help extraction for detection of particular odours. They could be classified into two categories: distillation and headspace techniques. The conventional method is distillation where volatiles could be extracted from sample matrices through distillation process. This practice is time-consuming and the sample could be easily decomposed; whereas, the headspace method

often involves shorter time without interrupting sample matrices at all. Therefore, it is suitable for fast screening test and the operations which have high output volume.

Many studies have focused on detection of fungal spoilage and contamination in grain by using volatiles (Borjesson et al., 1989; Magan, 1993; Magan and Evans, 2000; Olsson et al., 2002; Paolesse et al., 2006). However, reference to application to insect infestation is very limited in literature. Therefore, the aim of this paper is to review the potential of using volatiles to detect insect infestation in stored grain on the basis of published studies done so far. Headspace analysis will be focused upon here as it is more applicable to grain industry.

2. Insect pheromones

Insect pheromones are a substance which insects secret to influence the behaviour of other insects (Wilson, 1963). As it is odorous, it allows insects to pass their messages over long distances. Consequently, in the grain industry, this has been applied to stored grain with the aim to prevent insect infestation. Synthesized pheromone or chemical attractant was enclosed in a rubber or plastic case. Then, the insects will be lured and trapped inside the case. Pheromone traps are usually placed far away from stored grain so they can be safely protected from insect pests (Rajendran and Steve, 2005).

In terms of insect infestation detection, pheromones are potentially useful because they are important for insect communication. Hence, if volatile isolation detection techniques are able to detect and identify the chemical compounds that are emitted by insects as pheromones in grain, the results could be used to detect infested grain.

3. Volatile extraction/detection technique based on headspace analysis

3.1. Dynamic headspace extraction (DH)

DHS (or purge and trap) can be operated by flushing a stream of air or inert gas to purge volatiles in the headspace, then an adsorption tube is used to trap all the organic compounds carried by the gas. Many sorbent materials are available today. Tenax resin is probably the most widely used since a wide range of organic volatiles can be adsorbed, particularly aromatic compounds. Besides, it is made of porous polymers which are similar to the materials packed in GC columns. Thus, this allows desorption of the analytes at high temperatures close to those of the GC (Wampler, 1997). Consequently, the volatiles trapped from DHS can be directly introduced into the GC or GC-MS (Wampler, 1997; Rouseff and Cadwallader, 2001).

DHS-GC is a conventional volatile extraction method. Consequently, it has been used to analyze grain odour more widely than other volatile extraction techniques. The initial works of DHS-GC in grain studies involved identification of particular components in grain volatile mixtures and detection of off-odours in grains which were caused by microorganisms (Seitz et al., 1999; Sayaslan et al., 2000; Seitz and Ram, 2000). In terms of insect-damage detection, although it is a conventional method, application of DHS to infested grain is still limited in the literature. This may be because it is more time consuming and more complex than SPME and electronic nose. However, it also been used along with these techniques in some studies (Seitz and Saucer, 1996; Seitz and Ram, 2004).

3.2. Headspace solid phase microextraction (SPME)

SPME is a modern technique that is rapid, inexpensive and good for heat sensitive materials (Richter and Schellenberg, 2007). Headspace analysis of SPME involves insertion of a coated silica fibre above the sample, allowing the adsorption of the volatile compounds for a certain time. Concentrated volatiles can be readily obtained without interference from food matrices and other non-volatile compounds from the headspace (Richter and Schellenberg, 2007). Subsequently, the SPME needle is removed and inserted into the GC. Once the fibre has been placed in the GC inlet, heating causes the volatile compounds, adsorbed by the fibre, to be released into the GC column. Finally, volatiles are separated and characterised by GC or GC-MS (Martos and Pawliszyn, 1998; Reineccius, 2002; Turner, 2006).

Like DH, SPME has been widely applied to detect fungal volatiles in grains more than insect infestation (Jelen and Grabarkiewicz-Szczesna, 2005; Paolesse et al., 2006). However, very limited studies have used SPME to detect insect infestation in grain. Seitz and Ram's (2004) study is probably the only study that generated results that can be used to monitor insect damage in stored wheat (Seitz and Ram, 2004; Fernandes et al., 2010). Some compounds from wheat infested by lesser grain borers were identified by

SPME coupled with GC-MS in their experiment. However, it has not yet been proven to be a reliable method of monitoring insect infestation, because no validation studies have been conducted. There are two more studies with a similar aim, namely to use SPME-GC-MS to identify metabolites and pheromones which are the unique characteristic of particular insect pests (Arnaud et al., 2002). Therefore, it could be concluded that the current position of the SPME application in stored-grain research are still growing; some studies have started using SPME to detect and identify pheromones and their metabolites which may have potential to be used to monitor insect infestation in grains. If this technique is successful, SPME is likely to be applied to grain industry because it is relatively inexpensive and simple to perform.

3.3. Electronic nose

Electronic nose (E-nose) is an excellent approach in odour analysis. It has been widely used in food and flavour industries for over 20 years (Rajendran and Steve, 2005). Persaud and Dodd (1982) were the first to introduce the E-nose with the aim of mimicking the discrimination of the human olfactory system. It is comprised of an array of chemical sensors which are used to detect odour above the sample with different selectivity (Persaud and Dodd, 1982; Martí et al., 2005; Rock et al., 2008).

Studies of E-nose application to grain with the aim of detecting insect infestation are slowly expanding. It was first reported by Stetter et al., (1993), where wheat samples were discriminated as good, sour and insect infested. In this study, 83% of wheat samples were successfully classified by the gas sensor, chemical parameter spectrometry (CPS). In a paper by Hu (2006), E-nose technology was used to detect insect damage in rice. Degrees of insect damage (after 10, 15 and 24 h) could be detected and differentiated. The longer the infestation time, the better E-nose was able to identify infested grain. In the following year, Zhang and Wang (2007) published a paper focusing on similar experiments. They showed that fifteen percent of insect damage could be determined by E-nose. Moreover, volatile profile of different ages of grain was also classified. Future trends in insect damage detection by E-nose seem to improve the detection more and more. With a better pattern analysis software and chemical sensors, E-nose has potential to not only discriminate but also to identify the components of volatile mixtures produced during insect infestation.

4. Conclusions

This paper has highlighted the relevant literature related to detection of insect infestation in stored grain. Current techniques applied to stored grain are still too low in sensitivity to detect insect infestation and the high sensitivity tools such as x-ray and NIR are too costly. Volatile isolation techniques may demonstrate moderate sensitivity which will compromise with the cost. Therefore, it appears to be an attractive technique to be used as an alternative approach to detect insects in stored grain. Identification of volatile compounds that indicate insect infestation would be the goal for the development of this technique. Headspace techniques appear to be the most suitable technique for volatile extractions because they are non-destructive methods. DH, SPME and electronic nose are the main headspace methods on which grain researchers are currently focusing. Eventual validation of laboratory findings in field trials will provide information on the potential for adoption of these techniques at an industrial scale.

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Summary of commercially available pheromones of common stored-product beetles

Swords, P.*, Van Ryckeghem, A.
Insects Limited, Inc., 16950 Westfield Park Rd., Westfield, In 46074-9374, USA.
Email: P.Swords@InsectsLimited.com; Insecthelp@insectsLimited.com

* Corresponding author
# Presenting author

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Abstract

1. Introduction

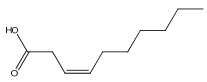
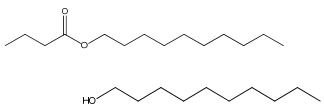
Since the explosion of pheromone identification and synthetic production of the 1960's, pheromone utilization has become a valuable technique for monitoring and control of insect infestations (Cork, 2004) Pheromones are collectively grouped into one family of specific chemical signals designated as semiochemicals. Specific pheromones in this category are synthetically created, placed in traps that are in turn used for population tracking, stages of development and mating disruption of common stored-product pest beetles. These insects have the dexterity to infest processed foods, whole grains such as barley, rye, corn, oat and rice. Trapping and monitoring through pheromone usage can assist in reducing the amount of insecticide used by pest-control managers, only spraying when insects surpass certain levels or when they enter a vulnerable stage.

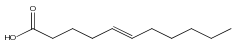
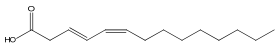
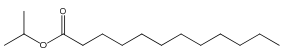
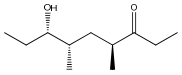
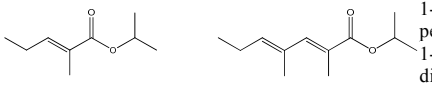
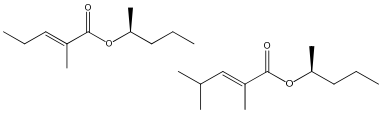
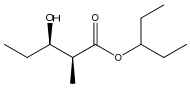
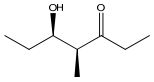
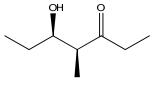
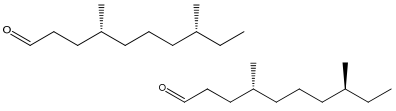
Many stored-product pest beetles produce pheromones that can be classified as long-chain carbon compounds, predominately consisting of acids, aldehydes, acetates and esters. Many of these pheromones are unsaturated and consist of one or more double bonds that can be oriented into an E or Z conformation creating one or more isomers. Pheromones produced by stored-product beetles are generally more complex when compared with pheromones produced by stored-product moths. As a result of this complexity, stored-product beetle pheromones contain a degree of chirality creating additional isomers. The chemistry used for producing these compounds is more involved but can be easily reproduced and contains highly stable intermediates leading to the commercial availability of these particular pheromones.

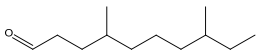
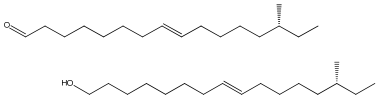
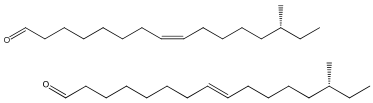
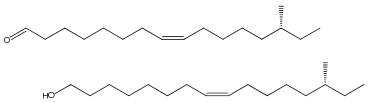
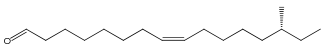
2. Commercially available pheomones for stored-product

The majority of pheromones range anywhere from 7-18 carbons in length producing carbon-chained compounds. Pheromones vary by functional group (i.e., acid, aldehyde, acetate, ester), double bond position and the configuration of the double bond (E or Z). Some of these pheromones are also branched containing methyl groups at various positions on the compound. These methyl groups create chirality in the compound depending upon how they are oriented on the molecule (R or S configuration). These factors are attractant determining for a variety of different beetles. Small changes in double bond placement and orientation of double bond or methyl group substituents may prevent attraction. The same holds true for alterations of functional groups. Through the use of organic syntheses, coupling of smaller organic compounds and the manipulation of long chain carbon compounds, these particular beetle pheromones can be made commercially available.

Table 1 Commercially available pheromones for common stored-product pest beetles.

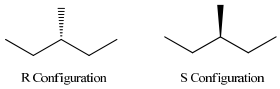
Scientific Name / Common Name	Pheromone Structure	Pheromone Name / Ratio
Anthrenus flavipes LeConte Furniture carpet beetle		(Z)-3-Decenoic Acid
Anthrenus sarnicus Mroczkowski Guernsey carpet beetle		Decy butyrate (1) Decan-1-ol (1)

Scientific Name / Common Name	Pheromone Structure	Pheromone Name / Ratio
Anthrenus verbasci(L.) Varied carpet beetle		(E)-5-Undecenoic acid
Attagenus unicolor (Brahm) Black carpet beetle		(E,Z)-Tetradecadienoic acid
Dermestes maculatus De Geer Hide beetle		11 isopropyl ketones: (Z)-5-dodecenoate, (Z)-7-dodecenoate, (Z)-9-dodecenoate, tetradecanoate, dodecanoate, (Z)-5-tetradecenoate, (Z)-9-tetradecenoate, hexadecanoate, (Z)-9-hexadecenoate, oleate
Lasioderma serricone(F.) Cigarette beetle		(4S,6S,7S)-7-hydroxy-4,6-dimethylnonan-3-one
Prostephanus truncatus (Horn) Larger grain borer		1-Methylethyl (E)-2-methyl-2-pentenoate (2) 1-methylethyl (E,E)-2,4-dimethyl-2,4-heptadienoate (1)
Rhyzopertha dominica (F.) Lesser grain borer		(S)-1-Methylbutyl (E)-2-methyl-2-pentenoate (S)-1-Methylbutyl (E)-2,4-dimethyl-2-pentenoate
Sitophilus granarius (L.) Granary weevil		(2S,3R)-1-Ethylpropyl 2-methyl-3-hydroxypentanoate
Sitophilus oryzae (L.) Rice weevil		(4S,5R)-5-Hydroxy-4-methylheptan-3-one
Sitophilus zeamais Motschulsky Maize weevil		(4S,5R)-5-Hydroxy-4-methylheptan-3-one
Tribolium castaneum (Herbst) Red flour beetle		(4R,8R)-4,8-Dimethyldecanal (8) (4R,8S)-4,8-Dimethyldecanal (2)

Scientific Name / Common Name	Pheromone Structure	Pheromone Name / Ratio
Tribolium confusum Jaquelin du Val Confused flour beetle		4,8-Dimethyldecanal
Trogoderma glabrum Everts Glabrous cabinet beetle		(R)-(E)-14-Methyl-8-hexadecenal (R)-(E)-14-Methyl-8-hexadecen-1-ol
Trogoderma granarium Everts Khapra beetle		(R)-(Z)-14-Methyl-8-hexadecenal (R)-(E)-14-Methyl-8-hexadecenal
Trogoderma inclusum (LeConte) Larger cabinet beetle		(R)-(Z)-14-Methyl-8-hexadecenal (R)-(Z)-14-Methyl-8-hexadecen-1-ol
Trogoderma variable BallionWarehouse beetle		(R)-(Z)-14-Methyl-8-hexadecenal

In some instances, the same pheromone has the ability to attract more than one species of beetle. This holds true for the warehouse, larger cabinet, and khapra beetles. In this example, a 16-carbon aldehyde with a Z conformation of the double bond is used to attract all three. The glabrous cabinet beetle is attracted by essentially the same compound only the double bond having an E configuration. Variations to this pheromone's functional group or E or Z configuration can be blended with the original pheromone to capture one particular beetle exclusively. The ketone, (4S,5R)-5-Hydroxy-4-methylheptan-3-one, attracts both the rice and maize weevil containing a hydroxyl group in the R configuration and a methyl substituent in the S configuration. In a majority of the stored-product beetle pheromones, attractants are strictly isomeric specific. Variations in double bonds, substituents, and functional groups will greatly reduce the effectiveness of insect response to the synthetically created pheromone in most cases.

Most pheromone syntheses start with two smaller compounds to be coupled together to form these larger carbon-chained compounds. Other syntheses can utilize ring opening chemistry which converts a cyclic compound into a straight chain compound with or without methyl or hydroxyl substituents depending upon the structure of the original compound. After an acceptable synthesis is discovered for one particular pheromone, similar pheromones can be created in the same manner only adjusting the length or overall structure of the starting compounds. Functional group, double bond configuration and orientation (E,Z), and the more challenging R or S configurations of methyl or hydroxyl groups need to be considered when choosing starting materials.



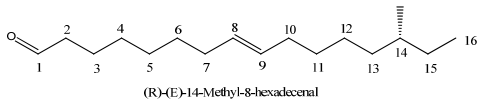


Figure 1 Methyl group R and S configurations and double bond placement of (R)-(E)-14-Methyl-8-hexadecenal.

For the capture of certain pest beetles, two closely related pheromones are used. Most of these multi-compound pheromones are used in very specific ratios, some having only one component making up significantly less of the overall solution. Research has shown that specific ratios of closely related compounds have the ability to increase effectiveness of captures. Some attractants combine more than just a couple of pheromones; the hide beetle attractant contains 11 closely related ketones varying in carbon chain length.

The stored-product beetle pheromones presented are commercially available due to three factors:

1. The starting materials for synthesis are available and inexpensive: It would not make sense to start a multi-step synthesis with costly starting materials. The overall amount of money spent producing the final desired product will skyrocket when the cost of the smaller components used for coupling increase.
2. Ease of organic reactions and reproducibility is acquired: The chemistry preformed results in high yields and high purity. The complexity of the chemistry needed to produce a particular pheromone is low.
3. Syntheses contain highly stable intermediates**:** In multi-step syntheses the product between sequential reactions must be stable and not degrade before moving on to the next reaction.

Other pheromones that don't reach these criteria more than likely are pheromones consisting of more complex compounds and require more complicated chemistry to produce. Any organic synthesis of a stored-product beetle pheromone that meet the criteria above can be used in formulated lures bought from commercial suppliers.

Table 2 Commercial suppliers of formulated lures for stored-product pests

Cooper Mill Ltd., R. R. 3 Madoc, ON, Canada
Fuji Flavor, 358 Midorigaoka, Hamura-Shi, Tokyo, Japan.
Hercon Environmental, Emigsville, PA , USA.
ISCA Technologies, 1230 Spring St., Riverside, CA, USA
Insects Limited Inc., 16950 Westfield Park Road, Westfield, IN, USA.
Russell Fine Chemicals, Unit 68, Third Ave., Deside Industrial Park East, Deeside, Flintshire UK
Suterra Corporate, 20950 NE Talus Place, Bend, OR, USA
Trece Inc., P.O. Box 6278, 1143 Madison Lane, Salinas, CA, USA.

References

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Mayer, M.S., McLaughlin, J.R., 1991. Handbook of insect pheromones and sex attractants. CRC Press, Inc., Boca Raton, FL, USA.

Summary of commercially available pheromones of common stored product moths

Swords, P.*, Van Ryckeghem, A.
Insects Limited, Inc., 16950 Westfield Park Rd., Westfield, In 46074-9374, USA.
Email: P.Swords@InsectsLimited.com; Insecthelp@insectsLimited.com

* Corresponding author
# Presenting author

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1. Introduction

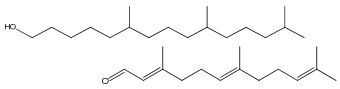
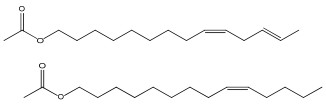
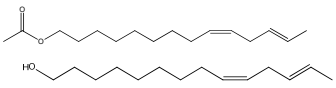
The use of synthetically created pheromones for insect monitoring and control has become a very important technique in the world of pest management. This concept has an origin that reaches back to 1913 when the French naturalist, J. H. Fabre conducted simple experiments with the great peacock moth (Fabre, 1913). In Fabre's study, a female peacock moth had emerged inside a cage. He noticed that male moths were attracted to the empty female's cage as well as the oak leaves the female had rested upon. This simple experiment led to the conclusion that there was a particular scent produced by the female to attract males. This scent is what we now refer to as pheromones.

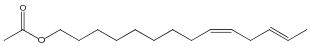
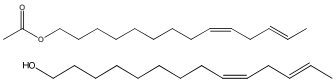
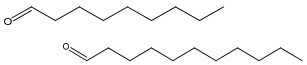
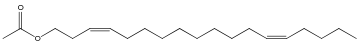
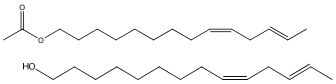
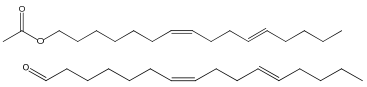
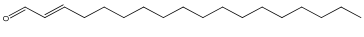
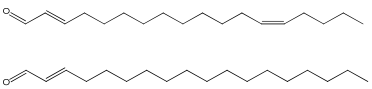
Pheromones are collectively grouped into one family of specific chemical signals designated as semiochemicals. 1961 marks the first year a pheromone was successfully isolated and characterized by the work of Butenandt and Hecker (Butler, 1967). Since then, an eruption of pheromone identification and synthetic creation has taken place. Pheromone use has proven to be an invaluable tool in monitoring and control of pest infestations; therefore, creating a market for synthetically created pest insect pheromones. Many stored product pest moths produce pheromones that can be classified as long-chain carbon, unsaturated alcohols, aldehydes and acetates. The unsaturated compounds consist of one or more double bonds that can be oriented into an E or Z conformation creating one or more isomers. These moth pheromones have become commercially available by designing multi-step organic syntheses that have inexpensive starting materials, easily produced chemical reactions and stable intermediates.

2. Commercially available pheromones for stored product months

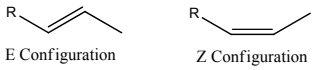
The majority of stored product pheromones range from 14-18 carbon chain compounds. Pheromones vary by functional group (i.e. alcohol, aldehyde, acetate), double bond position and the configuration of the double bond (E or Z). These factors are attractant determining for a variety of different moths, if a double bond is shifted to an adjoining carbon or retains an E configuration instead of a Z configuration, attraction will not occur for a targeted pest moth. The same holds true for alterations of functional groups. Through the use of organic syntheses, coupling of smaller organic compounds and the manipulation of long chain carbon compounds, these particular moth pheromones can be commercially produced.

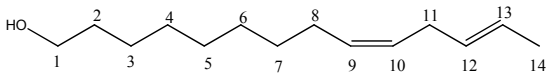
Table 1 Commercially Available Stored Product Moth Pheromones for common stored product pests.

Scientific Name / Common Name	Pheromone Structure	Pheromone Name / Ratio
Corcyra cephalonica (Stainton) Rice moth		6,10,14-Trimethylpentadecan-1-ol (F) E,E-Farnesal (M)
Esphestia (Cadra) cautella (Walker) Almond moth		(Z,E)-9,12-Tetradecadienyl acetate (7) Z)-9-Tetradecenyl acetate (1)
Ephestia elutella (Hübner) Tobacco moth		(Z,E)-9,12-Tetradecadienyl acetate (3) (Z,E)-9,12-Tetradecadien-1-ol (0.3)

Scientific Name / Common Name	Pheromone Structure	Pheromone Name / Ratio
Ephestia figulilella (Gregson) Raisin moth		(Z,E)-9,12-Tetradecadienyl acetate
Ephestia kuehniella (Zeller) Mediterranean flour moth		(Z,E)-9,12-Tetradecadienyl acetate (5.6) (Z,E)-9,12-Tetradecadien-1-ol (1.2)
Galleria mellonella (L.) Greater wax moth		Nonanal (5) Undecanal (2)
Nemapogon granella (L.) European grain moth		(Z,Z)-3,13-Octadecadienyl acetate
Plodia interpunctella (Hübner) Indianmeal moth		(Z,E)-9,12-Tetradecadienyl acetate (1) (Z,E)-9,12-Tetradecadien-1-ol (1)
Sitotroga cerealella (Olivier) Angoumois grain moth		(Z,E)-7,11-Hexadecadienyl acetate (9) (Z,E)-7,11-Hexadecadienal (1)
Tinea pellionella (L.) Case-making clothes moth		(E)-2-Octadecenal
Tineola bisselliella (Hummel) Webbing clothes moth		(E,Z)-2,13-Octadecadienal (2) (E)-2-Octadecenal (1)

There are many similarities that can be observed between these commercially available pest moth pheromones. Looking closely, one can see that most of these pheromones are within two to four carbon chain links of one another. Most pheromone syntheses start with two smaller compounds to be coupled together to form these larger carbon chained compounds. Therefore, given the similarities between length of the pheromones, after an acceptable synthesis is discovered for one, other syntheses can be designed in the same manner by only adjusting the length of the two starting compounds. However, other factors for starting materials need to be considered such as functional group (alcohol, aldehyde or acetate) as well as double bond position and configuration (E or Z).





(Z,E)-9,12-Tetradecadien-1-ol

Figure 1 E and Z double bond configurations and double bond placement of (Z,E)-9,12-Tetradecadien-1-ol.

For the capture of certain particular pest moths, two closely related pheromones are used in specific ratios. This is the case for the almond moth, angoumois grain moth, Indianmeal moth, Mediterranean flour moth, tobacco moth and webbing clothes moth. Most of these multi-compound pheromones are used in very specific ratios, some having only one component making up 10% of the overall solution. Research has shown that specific ratios of closely related compounds have the ability to increase effectiveness of captures. Certain two compound pheromones can be observed to attract more than one moth. (Z,E)-9,12-Tetradecadienyl acetate and (Z,E)-9,12-Tetradecadien-1-ol are used to attract Indianmeal moth, Mediterranean flour and tobacco moth, however varying ratios of these two components has the ability to attract more of one than the others.

The stored-product moth pheromones presented are commercially available due to three factors:

1. The starting materials for synthesis are available and inexpensive: It would not make sense to start a multi-step synthesis with costly starting materials. The overall amount of money spent producing the final desired product will skyrocket when the cost of the smaller components used for coupling increase.
2. Ease of organic reactions and reproducibility is acquired: The chemistry preformed results in high yields and high purity. The complexity of the chemistry needed to produce a particular pheromone is low.
3. Syntheses contain highly stable intermediates: In multi-step syntheses the product between sequential reactions must be stable and not degrade before moving on to the next reaction.

Other pheromones that don't reach these criteria more than likely are pheromones consisting of more complex compounds and require more complicated chemistry to produce. Any organic synthesis of a stored product moth pheromone that meet the criteria above can be used in formulated lures bought from commercial suppliers.

Table 2 Commercial suppliers of formulated lures for stored-product pests

Cooper Mill Ltd., R. R. 3 Madoc, ON, Canada
Fuji Flavor, 358 Midorigaoka, Hamura-Shi, Tokyo, Japan.
Hercon Environmental, Emigsville, PA , USA.
ISCA Technologies, 1230 Spring St., Riverside, CA, USA
Insects Limited Inc., 16950 Westfield Park Road, Westfield, IN, USA.
Russell Fine Chemicals, Unit 68, Third Ave., Deside Industrial Park East, Deeside, Flintshire UK
Suterra Corporate, 20950 NE Talus Place, Bend, OR, USA
Trece Inc., P.O. Box 6278, 1143 Madison Lane, Salinas, CA, USA.

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Protection of stored plant products from rodent pests using chlorophacinone

Vukša, M.*[1], Đedović, S.[1], Jokić, G.[1], Stojnić, B.[2]
[1] Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia.
Email: marina.vuksa@pesting.org.rs
[2] Faculty of Agriculture, University of Belgrade, Serbia

* Corresponding author
# Presenting author

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Abstract

Apart from some preventive measures, advisably taken during construction of storage facilities or at the time of product storage, treatments with chemical rodenticides have been the most widely practiced method of controlling commensal rodents. Their control in storages is normally carried out after animal presence has been observed, and treatments from early autumn onwards, throughout the season, provide the best effect. The paper shows the effects of baits with lower content of the active ingredient chlorophacinone than recommended for protecting stored plant products from rodents. The experiments were set up using the relevant OEPP/EPPO method. Different contents (0.005% and 0.0075%) of the active ingredient chlorophacinone were used in a ready for use (RB) paste bait formulation. Baits were laid in boxes along rodent routes, underneath pallets with sacks and in places where major damage was observed. Baits for house mice were placed at a rate of 10-20 g per 1-3 m, while 30-50 g of baits for brown rats were laid at specific points. Daily bait intake was monitored over a period of 10 d and the portions were replaced with new ones as needed. Placebo baits were laid in identical boxes for 4 d before the experiment began. The abundance of house mice was estimated based on the highest and lowest daily intake of bait divided by the species' daily food requirement.

The data in this experiment show that 0.005% and 0.0075% chorophacinone contents in RB baits changed neither palatability nor bait efficacy in controlling house mouse and brown rat indoors. The average efficacy of chorophacinone was 87-93% against house mouse and 90-100% against brown rat.

Keywords: Chlorophacinone, Rodent, Storage, Efficacy

1. Introduction

Two highy adaptable commensal rodent species, house mouse *Mus musculus* L. and brown rat *Rattus norvegicus* (Berkenhout), have fully adjusted to conditions existing in storage facilities, which provide them with hiding places and readily accessible food resources. Daily food intake of a house mouse is equivalent to 15% of its body weight, which is 1.4 kg annually (Gwinner, 1996). It normally visits food sources near its lair some 20-30 times overnight (Mallis, 1982). Daily food intake of a brown rat is around 28 g, i.e. 100 animals consume a tonne of stored products annually (Buckle and Smith, 1994). Apart from causing damage by feeding, rodents are also able to pollute nine times as much food with their feces, urine, hair and other impurities (Drummond, 2001; Brown et al., 2007).

Protection of stored plant products from commensal rodents was considerably improved in the mid-20th century just after World War Two (Buckle and Smith, 1994; Fall and Jackson, 1998). Nearly three decades later, Davis (1972) and other researchers developed a new approach to rodent control. Integrated pest management (IPM) has thus become undoubtedly the most efficient and most significant strategy of plant protection in terms of ecological and economic concerns (Spragins, 2006), but it has still not taken root fully (Haines, 2000). As a result of technical/technological variability regarding warehouse construction, types of stored plant products, location, environment, climatic and various other factors, it is almost impossible to employ a uniformed approach and strategy of protection from commensal rodents. Their ability to learn, change and adapt to various environmental conditions (Mallis, 1982) adds importance to a development of new and updating of existing methods of protection.

Chlorophacinone (2-[(4-chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione) is one of the most potent of first generation anticoagulant rodenticides which has been used throughout the world as an effective compound for controlling all common commensal and agricultural rodent pests (Santini, 1986; Advani, 1995; Parshad, 1999). Chlorophacinone is moderately palatable to commensal rodents, and its results are

best after intake over several successive days. As in other anticoagulants, its mechanism of activity is based on blocking protrombine formation and prevention of blood coagulation. Due to its ecotoxicological properties, chlorophacinone has been categorized as a group I poison (Tomlin, 2006) and has been used since 1961 for controlling rodent pests (Hadler and Buckle, 1992).

As commercial rodenticides are available in various formulations and with different active ingredient contents (Marsh et al., 1977; Advani, 1992), we have compared the efficacies and palatability of RB formulations containing 0.0075% and 0.005% chlorophacinone.

2. Materials and methods

2.1. Sites

The experiments were set up in several storage facilities and their surroundings upon an area totaling 2500 m^2 in which rodents had been observed. The products stored (maize, wheat, barley, sunflower and oats) were either packed in sacks or stored in bulk in feed mixing rooms.

2.2. Experimental design and baiting

The experiments were set up in compliance with the relevant OEPP/EPPO method (EPPO, 1999) and the ready-for-use baits were prepared by adding chlorophacinone to broken cereal grains along with an attractant and fixative adjuvant.

Unpoisoned baits were laid out for 4 d at the beginning and in the end of experiment in order to estimate rodent numbers by census. Poisoned baits were distributed at more or less identical places as placebo baits, underneath pallets, along rodent routes and in places where they had been observed previously. Baits with different chlorophacinone contents were offered simulataneously in separate storage buildings with approximately the same level of infestation over a period of 10 d. During prebaiting and baiting, daily intakes were recorded and new baits were added as required. All baits were offered in identical commercial plastic boxes. Baits for house mice were distributed at a rate of 10-20 g per 1-3 m, while 30-50 g of baits for brown rats were laid out at specific points. Rodent presence was monitored over the following 20 d.

2.3. Data processing

Commensal rodent numbers were evaluated based on the highest and lowest daily intakes of poisonous bait divided by daily requirement, and using the census method (EPPO, 1999). To establish the significance of differences between methods evaluating rodent numbers, Student's T-test was used at a significance level of at least $P<0.05$ (Sokal and Rohlf, 1995). Bait efficacy was calculated using Abbott's formula (Abbott, 1925).

3. Results

Based on census data and visual observation, more than 90 house mice and 40 brown rats were present at the beginning of experiment, and 12 house mice and 4 brown rats at the end (Table 1). Chlorophacinone average efficacy in all baits used was 86.8% for house mouse and 90% for brown rat.

Table 1 Placebo bait intakes (g) in all storage facilities and rodent numbers estimated by census baiting.

Species	Estimation time	Σ placebo bait intakes	Placebo bait intakes/day	Estimated animal numbers
Mus musculus	Beginning	1810	543	91
	End	224	71	12
Rattus norvegicus	Beginning	2468	1124	40
	End	349	113	4

In the experiment involving house mouse, maximum daily intake was recorded on the second day of baiting (Fig. 1). Different active ingredient contents had no effect on daily intakes and palatability of baits. Twenty days after the experiment was completed, house mice were found sporadically.

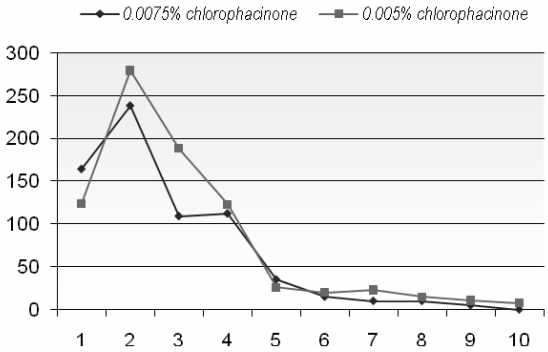

Figure 1 Palatability of the rodent baits in experiment with *Mus musculus.*

In the brown rat experiment, the intake of 0.005% and 0.0075% chlorophacinone baits was highest on the third and fourth day, respectively (Fig. 2). Different contents of the active ingredient had no effect on bait intake and palatability. Brown rats were not found 20 d after the end of experiment.

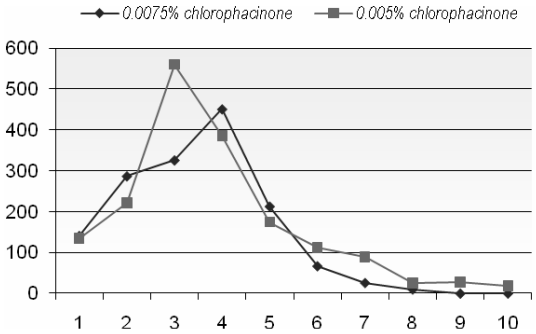

Figure 2 Palatability of rodent baits in experiment with *Rattus norvegicus.*

Data on maximum and minimum daily intakes of poisonous baits and the required daily food portions for commensal rodents indicated a presence of 87 house mice and 40 brown rats at the beginning of the experiment (Fig. 3, Table 2). The estimated efficacy of baits containing 0.005% chlorophacinone against house mouse and brown rat was 87% and 93%, respectively, and it was lower than the efficacy of baits containing 0.0075% chlorophacinone, which achieved 90% and 100% efficacy.

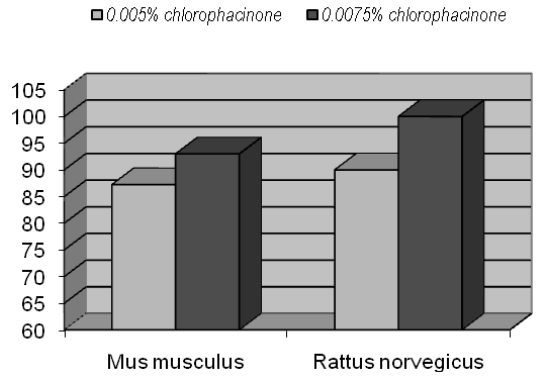

Figure 3 Efficacy levels of the applied rodenticides.

Table 2 Efficacy of products tested for controlling house mouse and brown rat in storage facilities.

Species	Baits	Estimated numbers*		Efficacy (%)
		Beginning	End	
Mus musculus	0.005% chlorophacinone	47	6	87
	0.0075% chlorophacinone	40	3	93
Rattus norvegicus	0.005% chlorophacinone	20	2	90
	0.0075% chlorophacinone	16	0	100

*Animal numbers were estimated based on maximum and minimum daily intakes of poisonous baits

Student's t-test showed significant statistical differences between the estimated numbers of commensal rodents on the chosen site (P=0.0065; df=3).

4. Discussion

Rodent control in storage facilities normally begins after their presence has been observed, and the best effects are ensured by deratization in early autumn and throughout the season (Ružić, 1983). Based on their estimated numbers and visual observations, it is possible to decide whether the level of infestation in any particular site is high enough to conduct an experiment.

The content of active ingredient in baits was not found to affect their palatability. The highest intakes of poisonous baits were recorded during the initial 4 d of the experiment for house mouse, and 5 d for brown rat. Chlorophacinone content in baits had no effect on daily palatablity of baits. Figs. 1 and 2 show a significant reduction in bait intakes during the last several days of the experiment.

The average efficacy of baits containing 0.005% chlorophacinone in controlling commensal rodents in storage facilities was 87% against house mouse and 90% against brown rat, and it was lower than the efficacy of baits containing 0.0075% chlorophacinone, which achieved 93% and 100% efficacy, respectively. However, after inspecting the facilities again 20 d after the end of experiment, house mice were found sporadically and irrespective of bait concentration. No brown rats were found. Marsh et al. (1977) had reported similar effectiveness in laboratory experiments with baits containing 0.005 and 0.01% chlorophacinone. According to Advani (1995), the efficacy of a tracking powder containing 0.2% chlorophacinone was 88% against house mouse.

The experimental data and visual observations, as well as the basic ecotoxicological properties of chlorophacinone, suggest that 0.005% chlorophacinone in RB formulations can achieve satisfactory results in controlling commensal rodent species in storage facilities.

Acknowledgements

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The development of grain storage scientific and technical research in China and relevant theory exploration

Jin, Z.[1], Song, W.[1], Guo, D.[2], Lan, S.[2], Xu, H.[2], Tao, C.[2], Wang, H.[2], Tang, P.[2]
[1] School of food science and engineering, Nanjing University of Finance and Economics, Nanjing 210003, China
[2] Chengdu Grain Storage Research Institute, Chengdu 610031, China

* Corresponding author
# Presenting author

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Abstract

China is a major grain producer as well as a major grain consumer in the world. Grain storage was always highly regarded, and a long history and abundant management experience of grain storage and relevant theory has been accumulated in China. The development of grain storage scientific research in China in the twenty-first century had been summarized in this paper. Grain-storage safety theory and the establishment of Chinese grain storage ecological system is also discussed. New ideas on grain-storage research for the future are also introduced.

Keywords: Grain, Storage, Theory, China

1. Development of strategies of science and technology of grain storage in China

When China enters into WTO, Chinese grain markets are brought into the international marketing system. With the increase of grain production, the task of storing and moving grain is much more critical. With the increase of quality requirements for grain, Chinese experts need to consider the following ideas (Jin, 2007: 2009):

(1) Development of scientific strategies of grain storage in the twenty-first century was to protect and make good use of grain, to increase the living quality of human beings, and to develop techniques of grain storage that reduce losses, pollution and costs, and maintain high quality, high nutrition, and high benefit;
(2) The science and technique of grain storage in China must insist on “human center and sustainable development”;
(3) Consideration of ensuring health of human beings, China should attach importance to green technology in food storage;
(4) Consideration of maintaining high quality of grain storage and the health of human beings, we should think much of building ecological grain storage;
(5) The science and technique of grain storage should realize globalizing grain and green storage.

1.1. The exploration and innovation of a guiding theory in grain storage

Since founding of the new P.R. China, tens of thousands of grain-storage workers using their intelligence, based on learning experiences from China and abroad, achieved a great accomplishment. “Grain Storage Science in China” has become a specialized discipline.

Chinese experts have always attached importance to research related to ecological problems in grain storage. Li Longshu, a master in this area began to research ecology of stored-grain insects in the 1940’s. He has done excellent research in this field and published many articles on the ecology of stored-grain pests, the ecosystem of grain bulks, and stored-product insect ecology. Under his guidance, Li Guangcan, Zhang Qingchun, Fan Jingan, Qin Zonglin and Yan Jian have submitted many research papers on the ecosystem of grain bulks. Zhao Zhimo and Wang Jinjun wrote a book about the ecology of stored-product insects in 1997 (Song et al., 2009). Lu Qianyu (1999) wrote a chapter to discuss ecosystems of stored grain and oil in her book. She described the composition, basic character information, environmental factors and flow of energy in the stored-grain ecosystem.

Jin Zuxun suggested the idea of choosing suitable types of cellars and reasonable storage measures under different ecological condition’ based on the nationwide investigation on potato storage in 1950’s. Since 1990's, he has worked on the concept of Grain Storage Safety Science. With China's continuous

improvement of grain-storage facilities and recently the development of strategies of grain-storage science and technology based on the Safety Science in China and the achievements of international grain-storage ecosystem (Figure 1) (Song et al., 2009). “Security control system of grain storage ecosystem” was implemented based on Grain Storage Safety Science, combined with security measures in squat silos and large warehouses built after 1998 (Figure 2).

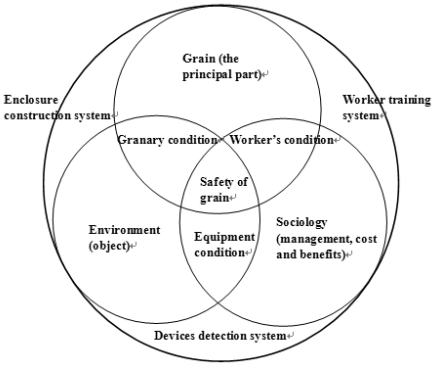

Figure 1 The schematic diagram of agricultural storage ecosystem

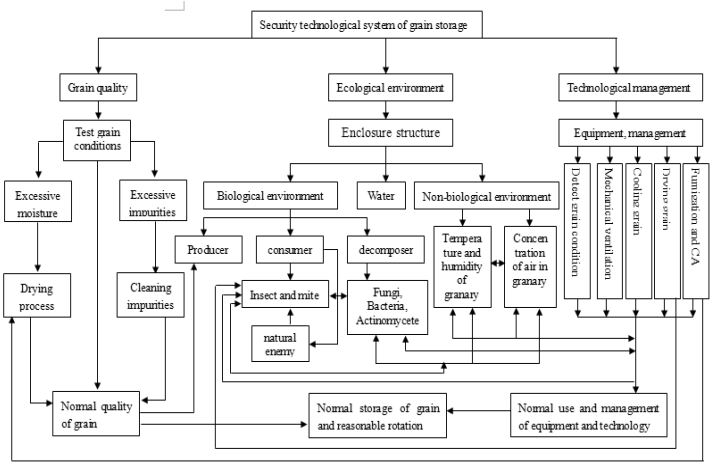

Figure 2 Security control system of grain storage ecosystem

Combined with bin construction and grain storage experience in China and Canada, and considering grain storage ecosystem and engineering ecosystem must be harmonious, Jin Zuxun suggested the idea of setting up “grain storage ecosystem theoretical system with Chinese characteristics“ (Song et al., 2009). Its main contents include:

(1) Definition of different ecological regions of grain storage in China;
(2) Scientific methods to select and designing the type of grain-storage warehouse in different ecological regions of grain storage;
(3) Scientific selection of grai- storage equipment for different ecological regions and different warehouse types;
(4) Development of science-based grain-storage technology and the optimal mode of economic operation in different ecological regions, different warehouse types and different grain species;
(5) Economic evaluation (management, cost-effectiveness and ecology) in different ecological regions, different warehouse type, different grain types and different storage technology;
(6) Security Technology Evaluation System of grain storage.

According to Jin Zuxun's advice, dozens of experts and scholars from research institutes and colleges studied the grain-storage ecosystem in China organized by the State Administration of Grain in the period of the tenth "five year plan", and achieved a number of gratifying results. The results have been the basis for constituting and revising the technical standards of grain storage in China (Song et al., 2009). The following are the main results:

1.1.1. Division of ecological regions of grain storage in China

Based on the climate, growing conditions, accumulated temperature and the relative humidity, China was divided into seven ecological regions. A schematic diagram had been designed as following Figures 3-6 and Table 1.

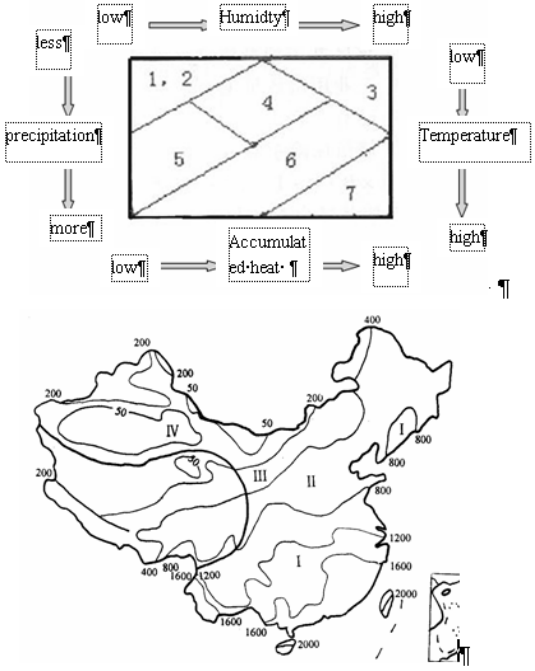

Figure 3 The climatic map of dry or wet based on climate and geographical conditions

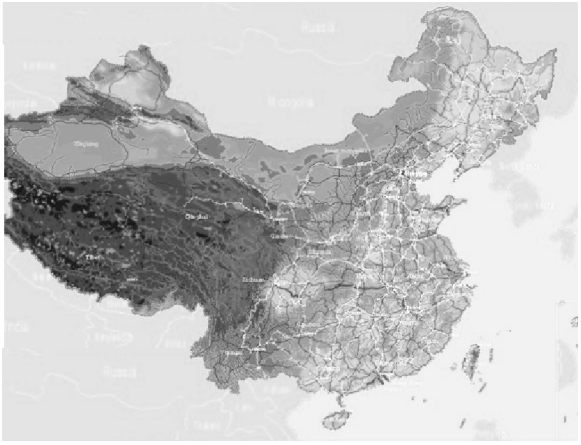

Figure 4 The geographic map based on climate and geographical conditions

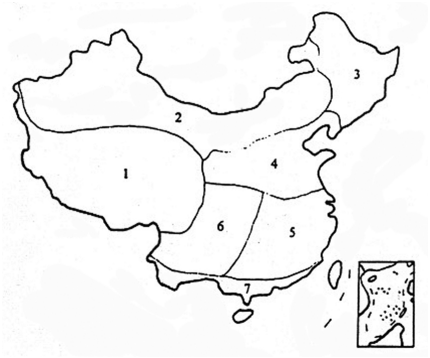

Figure 5 The climate regions based on accumulated temperature more than or equal to 10°C

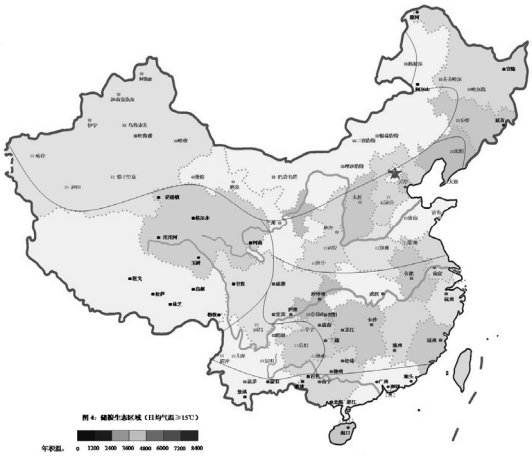

Figure 6 The ecological regions of grain storage based on daily average temperature more than or equal to 15°C

Table 1 The division of ecological regions of grain storage in China

	Region name	Ecological characters	Main technology of grain storage
1	Quite cold and drying region	The effective accumulated temperature that is higher than 15°C is 0~178 D·D. There are 112~194 d that temperature is higher than 15°C throughout the year. Annual precipitation less than or equal to 400 mm. The annual average relative humidity is in the range of 10%~90%. The temperature in January and July is in the range of -8~~20°C and 18~24°C , respectively. The main grain and oil crops are spring wheat, winter wheat and maize. The representational stored-grain pests are *Tribolium castaneum*, *Attagenus brunneus*, *Trogoderma variabile*, *Niptus hololeucus*, *Gibbium psylloides*, *Ptinus japonicus*, *Sitophilus granaries*. It is the most arid region in China. Sunshine and solar radiation is second only to the Qinghai-Tibet Plateau. Very cold and strong wind in winter and spring, is the most appropriate regions to store grain.	1. Drying with wind, airing and natural ventilation; 2. Low-temperature storage in drying season; 3. Waterproofing in rainy season.
2	Low-temperature and drying region	The effective accumulated temperature that higher than 15 °C is 626~2280 D·D. There are 0~70 d the temperature higher than 15°C. Annual precipitation less than 800 mm. The annual average relative humidity is in the range of 28%~90%. The temperature in January and July is in the range of 0~~16°C and 6~18°C , respectively. The main grain and oil crops are spring wheat, winter wheat and highland barley. The representational stored-grain pests are *Attagenus brunneus*, *Trogoderma variabile*, *Niptus hololeucus*, *Gibbium psylloides*. Thin air, solar energy and wind energy resources are extremely rich. Cold throughout the year, dry in dry season. It is one of the most appropriate regions to store grain, however, there is insufficient time to reduce moisture of high-moisture maize.	1. Drying with wind, airing and natural ventilation; 2. Natural low-temperature; 3. Treating high moisture grain with drying with wind, airing and ventilation at the end of spring or the beginning of summer; 4. Airproofing after using protectant before summer; 5. Using grain cooler in special regions of Xinjiang if necessary.
3	low-temperature and high humidity region	The effective accumulated temperature that higher than 15°C is 223~819 D·D. There are 55~122 d with temperature higher than 15°C throughout the year. The annual average relative humidity is in the range of 22%~90%. The temperature in January and July is in the range of -12~~30°C and 19~24.5°C , respectively. The main grain and oil crops are wheat, maize and soybean. The representational stored-grain pests are *Sitophilus zeamais*, *Oryzaephilus surinamensis*, *Tenebroides mauritanicus*, *Tribolium castaneum*. It is the coldest region in China. The climate is cold and wet. There is insufficient time to reduce moisture of high-moisture maize.	1. Mechanical ventilation and tumble dry; 2. Natural ventilation; 3. Drying with wind, airing, ventilation and tumble dry at the end of spring or the beginning of summer; 4. Waterproofing after using protectant before summer.
4	middle temperature and drying region	The effective accumulated temperature higher than 15°C is 828~1690 D·D. There are 143~192 d the temperature higher than 15°C throughout the year. Annual precipitation is in the range of 400~800 mm. The annual average relative humidity is in the range of 13%~97%. The temperature in January and July is in the range of 0~~10°C and higher than 24°C , respectively. The main grain and oil crops are winter wheat, maize and soybean. The representational stored-grain pests are *Sitophilus zeamais*, Gelechiidae, *Plodia interpunctella*, *Oryzaephilus surinamensis*, *Tenebroides mauritanicus*, *Tribolium castaneum*. Cold and dry in winter are favorable conditions to store grain, but high temperatures and wet are unfavorable conditions.	1. Drying after harvest in summer; 2. Airing, ventilation and tumble dry the high-moisture maize; 3. Natural low-temperature; 4 Treat the high-moisture maize with air-cure and ventilation next summer; 5 Waterproofing after using protectant before summer; 6 Pay attention to the situation of stored grain in summer.

	Region name	Ecological characters	Main technology of grain storage
5	middle temperature and high humidity region	The effective accumulated temperature higher than 15°C is 1029~3180 D·D. There are 121~253 d the temperature higher than 15°C throughout the year. Annual precipitation is in the range of 800~1600 mm. The annual average relative humidity is in the range of 34%~98%. The temperature in January and July is in the range of 0~~10°C and near 28°C , respectively. The main grain crops are winter wheat and paddy. The representational stored-grain pests are *Sitophilus zeamais, Rhizopertha dominica*, Gelechiidae, *Cryptolestes pusillus*, *Oryzaephilus surinamensis*, *Tenebroides mauritanicus*, *Tribolium castaneum*. The most rain of China occurs in spring, and the hottest period of the eastern hemisphere is in summer. The high temperature and moisture is unfavorable to grain and oil storage. there is insufficient time to reduce the moisture content of late paddy.	1. Mechanical ventilation and tumble dry after harvest; 2. Ventilation to lower the temperature in spring and winter; 3. Drying high moisture grain next spring; 4. Waterproofing after using protectant before the temperature rise; 5. Fumigation; 6. Pay attention to the situation of stored grain in summer, and take measures timely.
6	middle temperature and low humidity region	The effective accumulated temperature higher than 15°C is 724~1037 D·D. There are 173~224 d the temperature higher than 15°C throughout the year. Annual precipitation is about 1000 mm. The annual average relative humidity is in the range of 30%~98%. The temperature in January and July is in the range of 2~10°C and 18~28°C , respectively. The main grain and oil crops are winter wheat, maize and paddy. The representational stored-grain pests are *Sitophilus zeamais, Rhizopertha dominica*, Gelechiidae, *Cryptolestes pusillus*, *Oryzaephilus surinamensis*, *Tenebroides mauritanicus*, *Tribolium castaneum*. Winters are warm, and summers are hot. More rain and fog in a humid climate; high humidity with little sunshine.	1. Mechanical ventilation and tumble dry after harvest; 2. Fumigation; 3. Ventilation to lower the temperature in spring and winter; 4. Waterproofing after using protectant before the temperature rise; 5. Pay attention to the situation of stored grain in Sichuan Basin, and take measures timely.
7	high temperature and high humidity region	The effective accumulated temperature higher than 15°C is 1566~3476 D·D. There are 289~352 d the temperature higher than 15°C throughout the year. Annual precipitation is in the range of 1400~2000 mm. The annual average relative humidity is in the range of 35%~98%. The temperature in January and July is in the range of 10~26°C and 23~28°C, respectively. The main grain and oil crops are winter wheat, maize and paddy. The representational stored-grain pests are *Sitophilus zeamais, Rhizopertha dominica*, Gelechiidae, *Cryptolestes pusillus*, *Oryzaephilus surinamensis*, *Tenebroides mauritanicus*, *Tribolium castaneum*. Long summers without winter. This is the wettest and hottest region in China. Stored-grain pest problems are serious and it is the most difficult region for grain storage.	1. Ventilation or high temperature drying timely; 2. Fumigation timely if find pests; 3. Ventilation to lower temperature and moisture in dry season, then using protectant; 4. Using special facilities to lower temperature and dehumidification; 5. Using the warehouse with the functions of lower temperature, dehumidification and fumigation.

1.1.2. Scientific selection of warehouse type based on the grain storage region

After much research, the design institutes recommended for the first, second, third and fourth grain-storage regions in China that the squat silo and large warehouse should be built; however, in shipping ports or terminal elevators, the squat silo and upright silo should be built. Considering that the most important source of heat to the grain bulk will come from the roof of the warehouse, we must attach great importance to the issue of warehouse roof insulation.

1.1.3. The machines and special equipment needed

The machinery and special equipment in different types of granary ensure the high quality of stored grain (Table 2).

Table 2 The machines and special equipments in different type of granary

Type of granary	machines and special equipments in granary
Large warehouse	Handling equipment, Conveying equipment, Weighing equipment, Cleaning equipment, Palletizing equipment; Detection system, Ventilation system, Fumigation system, Cooling system, Controlled atmosphere system, Security system, Granary management and pest and mold controlling expert system.
Squat silo	Conveying equipment in the top and bottom of granary. Hoisting equipment, Weighing equipment, Cleaning equipment, Dust removal equipment and Control equipment in the tower. Binning equipment, Cleaning equipment, Conveying equipment, Cleaning equipment, Measurement and control system, Ventilation system, Recirculation fumigation system, Cooling system, Security system, Granary management and pest and mold controlling expert system.

1.1.4. The best pattern of economic operation

The best pattern of economic operation in different grain-storage regions, different types of granaries and different grain species is shown in Table 3.

The best pattern of economic operation is to make full use of natural and mechanical ventilation to lower grain temperature, insulation to keep the grain cool during the summer, fumigation if necessary. To prevent condensation, timely ventilation will lower temperature and moisture when weather turns colder throughout the entire country.

Table 3 The reasonable technology of grain storage and the best pattern of economic operation in different grain storage region

Ecological region	The reasonable technology of grain storage and the best pattern of economic operation
1	Cold and dry throughout the year in the region, and wind energy resource is rich, so it is necessary to take full advantage of natural ventilation and low temperature storage. To improve the insulation of the warehouse. Mainly using natural ventilation, supported by mechanical ventilation. The grain temperature is always below 15°C throughout the year. Local fumigation can be carried out, and also can use grain protectants in order to prevent local heating or vermin (natural ventilation + mechanical ventilation + local fumigation + protectant).
2	Taking full advantage of low temperature conditions, low temperature grain storage was carried out in this region. Mainly using natural ventilation especially in winter, at the same time, protectant can be used in whole bin or the surface of grain bulk to control pest. If necessary, the techniques of mechanical ventilation and so on can be used. Grain storage underground is a good method to store grain. The temperature underground about 15 m is 8°C, and it is a nice environment to low temperature grain storage.
3	Three time natural or mechanical ventilation from late September to next January. Grain surface gland, heat sealed to keep cool from June to September. Keeping grain temperature in low-temperature (under 15 °C) or quasi-low-temperature (under 20 °C) throughout the year. If find grain temperature rising in the whole bin or just in local, grain cooler should be used to cooling grain temperature.
4	The best pattern of economic operation in this region: Mechanical ventilation in winter and heat insulation in summer (wheat, paddy and maize); Mechanical ventilation in winter, protectant and heat insulation in summer (wheat, paddy and maize); Mechanical ventilation in winter, heat insulation in summer and fumigation (wheat, paddy and maize); Natural ventilation in winter, heat insulation in summer and fumigation (wheat); Natural ventilation in winter, protectant and heat insulation in summer (wheat); Mechanical ventilation in winter, heat insulation in summer and grain cooler (special requirements on the quality).
5	Natural ventilation, Mechanical ventilation, aeration with grain cooler and detection of grain conditions. Keep grain temperature under 15 °C or 20 °C through Natural ventilation and Mechanical ventilation. If necessary using grain cooler or large-scale air-conditioning to keep grain safety.
6	Mainly mechanical ventilation, if necessary, complemented by grain cooler in the State depots. Natural ventilation with a combination of mechanical ventilation to reduce power consumption in other depot. Automatic-control systems were used in mechanical ventilation.
7	Large warehouse: natural ventilation, fumigation with low dose phosphine, protectant and detection of grain conditions; Squat silo: natural ventilation, mechanical ventilation, aeration with grain cooler, recirculation fumigation, protectant and detection of grain conditions.

1.1.5. Economic evaluation in different grain storage regions and grain storage technology

The economic evaluation of grain-storage techniques can not be separated from management, cost and benefit analysis. The choice warehouse type also can not be separated from economic evaluation. Scientific and economic management is the key to lowering grain-storage costs and increasing economic, social and ecological benefits.

1.1.6. Research on evaluation system for grain storage security technology

Using the theory of grain-storage ecosystems and grain-security science as a guide, with consideration of the condition of the stored grain, climate of the grain-storage region, condition of storage facilities and ecological environment conditions of the grain bulk, we conduct a comprehensive evaluation of the grain-storage security technology using specific scientific guidelines. The evaluation methods proposed by scientists in this project are credible and have been validated in the field in different granaries and different grain-storage regions.

2. A proposal about worldwide ecological grain storage

According to Jin Zuxun's proposal (2007), an information platform about the stored-grain ecosystem should be developed. The main components should include:

(1) Grain ecology (the character and quality of grain, ecological factors): Characterize the effects of different ecological factors on preserving grain quality;
(2) Ecological environment of grain bulks (grain bulk and ecological factors): Characterize the effects of different ecological factors and different physical methods, such as aeration on preserving grain quality;
(3) Grain-storage ecology: research on the effects of storage structures, ecological factors, physical techniques and management measures on preservation of grain quality and rule of energy changing on the process of grain storage;
(4) Long-term research on grain storage ecology.

i. Chemical ecology of grain storage: Research on the relationships between grain and other biological factors or among the biological factors, the structures and functions of natural substances and their effects on safety of stored grain in the stored-grain ecosystems;

ii. Mathematical ecology of grain storage: Research on the relationships between grain and ecological factors or among the ecological factors, and the mathematical expression of the dynamics of relationships of matter and energy in the stored-grain ecosystems;

iii. A branch of engineering ecology: Research on the harmony of natural ecosystems and engineering ecosystems. Engineering ecology is the branch of applied ecology that focuses on the application of ecology in the area of engineering planning, construction and management. Grain-storage project ecology focuses on the harmony of natural ecosystems and engineering ecosystems and makes them harmonious to ensure the preservation of stored-grain quality;

iv. Grain-storage ecology and health: The purpose of stored-grain ecosystem research is not only to reveal the relationships of stored grain and ecological factors, but also to characterize the flow of material and energy, to decrease postharvest losses of grain (include the loss during storage) but also urging ecosystem and engineering systems to harmonize, allowing better use of natural resources and protecting the environment, enhancing the stability of stored grain, maintaining high quality and ensuring health of people by controlling ecological factor and managing engineering ecology.

We have designed many cooperative projects to exchange information. We hope that all the colleagues in the world can cooperate and support each other to contribute to reduce post-harvest of grain losses in the world. China is the developing county with the largest population in the world, and we deeply hope to get your supports and help in the project of stored grain.

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Section: Quarantine and Regulatory

Phaseout of methyl bromide as a fumigant – will PQS uses continue?

Banks, J.*#
Grainsmith Pty Ltd, 10 Beltana Rd, Pialligo ACT 2609, Australia. Email: apples3@bigpond.com

* Corresponding author
# Presenting author

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Abstract

The Montreal Protocol on Substances that Deplete the Ozone Layer (MP) seeks to eliminate emissions to the atmosphere of all ozone-depleting gases. This includes emissions of methyl bromide fumigant. Phaseout of production and consumption of methyl bromide, quarantine and pre-shipment (QPS) uses excepted, is now well advanced. This includes phaseout of non-exempt uses on postharvest durable commodities and structures. Annual non-QPS use on structures and postharvest durable foodstuffs is now less than 300 tonnes a year globally.

There are potential alternatives for almost all remaining non-QPS uses on post-harvest durables. Their actual adoption is constrained by regulatory/registration issues in a number of countries where there is a great need for rapid treatment to meet transport and harvesting schedules. A few recalcitrant and specialised uses remain without recognised alternatives, notably for disinfestation of some high moisture dates immediately postharvest.

QPS uses continue to be exempted from control under the agreed MP control measures for methyl bromide. Production of methyl bromide for QPS uses is now the largest new production on an ozone-depleting substance for an emissive use unregulated under the Montreal Protocol. Regulation of emissions from QPS treatments is under continuing discussion, with a decision whether or not to bring emissions from QPS under some form of MP control possible in late 2010. There are actual or potential alternatives for most of the high volume use of methyl bromide (export grains, export timber and wood packing material (ISPM 15). Both MP and the IPPC already urge adoption of alternatives and other emission reduction measures from QPS use on durables, where feasible.

A further and more detailed discussion of alternatives for QPS treatment of grain, timber (logs) and wood packaging material, following the 2009 Quarantine and Preshipment Task Force Report (TEAP 2009), is to be published prior to the Montreal Protocol Open-Ended Working Group (OEWG) meeting in June 2010.

Keywords: Methyl bromide, Quarantine, Alternatives

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Review of research on the control of pine wood nematode (*Bursaphelenchus xylophilus*) using the fumigant sulfuryl fluoride and current status for inclusion in ISPM No.15

Buckley, S.*#[1], Drinkall, M.J.[2], Thoms, E.M.[3]
[1] Dow AgroSciences S.A.S. 790 Av du Dr Donat, 06254 Mougins, France. Email: sbuckley@dow.com
[2] Dow AgroSciences Ltd, Latchmore Court, Brand street, Hitchins SG5 1NH U.K
[3] Dow AgroSciences LLC, 7257 NW 4th Blvd, #20, Gainesville, Florida 32605 USA

* Corresponding author
# Presenting author

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Abstract

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease, is endemic to North America. The unintentional introduction of the PWN into Asia and now Europe has caused considerable damage to forests in these regions. The PWN was first reported in Portugal in 1999 where it has now become established and it was also reported in Spain in 2008. This situation led the European Union (E.U.) Commission to take exceptional phytosanitary measures for wood trade within the E.U. Preliminary studies on the broad spectrum fumigant sulfuryl fluoride (SF) showed control of the nematode, but complete eradication was not achieved at the dosages tested. Further studies provided evidence that 100% control of PWN could be obtained by fumigating unseasoned wood with SF. A review of these research studies is presented together with a proposed fumigation schedule. This schedule and supporting efficacy data on PWN and quarantine insects has been submitted to the International Plant Protection Convention (IPPC) for inclusion of SF as an approved treatment in ISPM 15 (International Standard for Phytosanitary Measures) which regulates Wood Packaging Material in International Trade. The inclusion of SF in the standard would provide an alternative fumigant option to methyl bromide (MB) which will be banned for all uses in the E.U. in March of 2010.

Keywords: Sulfuryl fluoride, *Bursaphelenchus xylophilus,* ISPM No.15, Quarantine, Fumigation

1. Introduction

The use of wood pallets and packaging materials e.g. crates and boxes, widely used for agricultural commodities and industrial items, has increased significantly as global trade has developed over recent decades. This has resulted in an increased risk of the movement of non-native pest species on wood used in international transport. Some of these present a major threat to forestry and woodlands. This is the case of pine wood nematode (PWN); *Bursaphelenchus xylophilus* (Steiner and Buhrer), Nickle, 1970, which is native to North America and has spread to Asia and Europe with devastating effects on pine species not native to North America. This paper provides an overview on the research conducted on control of PWN with SF and the status of inclusion of sulfuryl fluoride (SF) in ISPM No.15.

1.1. Situation of PWN in E.U. and phytosanitary measures

The PWN is a nematode of about 1 mm length which is the agent responsible for Pine Wilt disease. It is vectored by pine sawyers of the genus *Monochamus* (Coleoptera, *Cerambycidae*). This nematode is native to North America (Canada, U.S.A. and Mexico). It first appeared in Japan at the beginning of 20th century on timber exports then spread to Korea, China and Taiwan. It was first detected in Portugal in 1999 in the Setubal region (Mota et al.,1999) where it appeared to be vectored by *Monochamus galloprovincialis* (Olivier) and other *Monochamus* spp. naturally present in that part of Europe.

In spite of E.U. measures to eradicate this pest since 2000, including the felling of 1 million pine trees, the spread could not be stopped and 70 outbreaks were recorded in 2008 outside the Setubal area, in North and central Portugal. (E.U. Commission, 2009) An isolated outbreak was notified to the E.U. Commission in Spain in November 2008 while several other countries reported presence of live PWN in wood packaging material (WPM) originated from Portugal. This resulted in strengthening emergency measures in an attempt to prevent the PWN spreading to other E.U. countries (E.U. Commission, 2006-2009). Beginning 1 January 2010, all WPM from Portugal to other E.U. Member Countries must be treated in accordance with ISPM 15 which allows two types of treatments: heat or fumigation with methyl bromide (MB).

The treatment choice, however, will be further restricted following an E.U. Commission Decision (2008/753/EC) of non-inclusion of MB in the Annex I of 91/414. This decision means the withdrawal of Plant Protection Authorization for this substance by 18 March 2009 with a transition period of one year. After 18 March 2010, MB treatment of WPM will be illegal in Portugal and throughout the E.U.; the only approved treatment for WPM will be heat i.e. 56°C at the core of the wood for 30 minutes. This requires large investment in fixed installations with powerful ovens to be able to effectively treat WPM. Heat can damage certain packaged goods. Fumigation offers a more flexible alternative e.g. loaded shipping containers with manufactured goods, and requires less investment.

1.2. Sulfuryl fluoride as a potential alternative to methyl bromide

SF has a long history of use being introduced by The Dow Chemical Company as the product Vikane® in 1961 in the USA for the eradication of termites, wood boring beetles, and other structure infesting pests. Since that time, many new registrations and commercial use patterns, e.g. in the food industry, have been established around the world (Thoms et al., 2008).

SF is a broad-spectrum fumigant which is a viable alternative to MB for fumigating a wide variety of structures and post-harvest commodities for pest control and eradication. The physical properties and inorganic nature of SF enable it to achieve deep penetration (diffusion) into matrices and it is more efficient in this respect compared to MB (Scheffrahn et al., 1992).

One area of active research and development of SF is fumigation of unseasoned wood, used in international trade, to control quarantine pests (Woodward and Schmidt, 1995; Mizobuti et al., 1996; Soma et al., 1996; 1997; 2001; Dwinell et al., 2003; 2005; Barak et al., 2006, 2010; Tubajika and Barak, 2006; Flack et al., 2008; Daojian et al., 2010). These data have been developed by leading international quarantine government scientists.

Quarantine specific use patterns already exist in a number of countries which include Finland, Germany, Norway, Sweden and the USA. China has also approved a specific treatment schedule for SF on logs for fumigation prior to export. In the E.U., SF has been granted Annex 1 listing under E.U. Directive 98/8/EC (Biocides) for Product Type 8 - wood preservative (E.U. Commission, 2006) and product Type 18 – insecticide (E.U. Commission, 2009). The submission of modern registration data packages and successful evaluations and registrations at E.U. and country level confirm that the fumigant will be available for use in the foreseeable future.

2. Trials on PWN from Soma et al. (2001) in Japan

2.1. Materials and methods

Red pine, *Pinus densiflora* Siebold et Zuccarini, naturally infested with PWN in Japan were sawn into boards (2 x 15 x 30 cm) and lumber (15 x 15 x 30 cm). Before fumigation, the pretreatment population of PWN was confirmed to exceed 10,000 PWN per 100 g of sample. Five pieces of boards and three pieces of lumber were tied up in a bundle by a plastic band and surrounded by boards and lumber of same size for achieving appropriate fumigation loading. Fumigations were carried out in 100 L fiberglass chambers, at 15°C and with 25% (v/v) load factor. Wood moisture content ranged from 25.8 to 27.3% in the boards and 20.1 to 33.4% in the lumber before fumigation. The mortality was assessed with Baermann funnel method 6-7 days and 20-21 days after fumigation.

2.2. Results and Conclusions

Nematode populations were high (20,400/100 g or greater) in all treatments before fumigation and in untreated controls after fumigation (Table 1). SF showed a clear dose response on the control of PWN with increased PWN mortality as the dosage increased. Nonetheless, the highest SF dosage tested (2932 g-h/m^3) was not sufficient to achieve 100% PWN mortality at 15°C in boards or lumber after fumigation. For the two highest SF dosages tested, PWN survivorship decreased at 20-21 days after fumigation compared to that of 6-7 days after fumigation. This might indicate a delayed mortality response of PWN to SF, which has been documented with insects (Osbrink et al., 1987). The best results were achieved at the highest dosage tested at 20-21 days after fumigation (0.34% survivors).

Table 1 Number of pine wood nematodes (PWN) before and after fumigation (percent survival) with sulfuryl fluoride at 15°C in boards and lumber (data extraction from Soma et al., 2001)

			Number PWN (survival %)				
			Board 2 x 15 x 30 cm		Lumber 15 x 15 x 30 cm		
SF Dose (g/m^3)	Exposure (h)	SF Dosage ($g\text{-}h/m^3$)	Days after fumigation		Days after fumigation		
			0[1]	6-7	0	6-7	20-21
Untreated			39000	33300 (85.4)	38600	39600 (102.6)	53100 (149.6)
30	24	765	-	-	20500	2183 (10.6)	3819 (18.6)
60	24	1539	20400	2704 (13.3)	22700	1906 (8.4)	454 (2.0)
60	48	2932	20400	1277 (6.0)	22700	426 (1.9)	78 (0.34)

[1]0 = prefumigation counts

3. Trials from Dwinell et al. (2003, 2005) in the U.S.A.

3.1. Materials and methods

For the April 2003 field trial, logs used were shortleaf pine, *Pinus echinata* Mill, that had been killed by the southern pine beetle (*Dendroctonus frontalis* Zimmerman) during the prior year were colonised by *Monochamus* spp. and the PWN (Dwinell et al., 2003) . The logs were salvaged ca. eight months after the pines had died, and debarked and sawn into timber *ca*. two weeks prior to each fumigation trial. For all subsequent lab and field fumigation trials, the wood was sawn from loblolly pine, *P. taeda* L.*,* which was also naturally colonised by *Monochamus* spp. and PWN. Sticks (2.5 x 2.5 x 25 cm) were sawn for laboratory chamber fumigations. Boards (2.5 or 5 x 2.5 to 15 cm), cants (12.7 x 12.7 or 10.2 x 10.2 cm), and slabs recovered from logs during the milling process were sawn in *ca*. 97 cm lengths for field fumigations.

Laboratory experiments were conducted by Dow AgroSciences LLC at facilities at the Purdue University, West Lafayette, Indiana, USA using two, 28.3 L fibre glass chambers. The four field fumigation trials were set up between 2003 and 2004 by the USDA at the Whitehall Forest in Athens, Georgia, using 5 m^3 chambers made of timber frame and polyethylene sheeting cover. Wood was assayed for the PWN before and after fumigation, using the Baermann funnel procedure applied on a thin section of wood from the center of each stick (laboratory trials) or removing two wafer-thin sections by augering or sawing boards, cants, and slabs (field fumigations). The wood moisture content (WMC) was determined by drying a second set of samples at 105ºC for 24 h. Slabs, cants, logs and boards were examined periodically for evidence of *Monochamus* spp., activity and emergence after the fumigation.

3.2. Results and Conclusions

Table 2 Survival of pinewood nematode (PWN) in unseasoned pine sticks (2.5 x 2.5 x 25 cm), sawn from naturally-infested logs, following fumigation with sulfuryl fluoride (SF) for 24 h at varying dosages (g-h/m3) in temperature-controlled fumigation chambers, 2003-2004 (Dwinell et al., 2003, 2005).

Chamber ºC[1]	Target (Actual) SF Dose g/m^3	SF Dosage ($g\text{-}h/m^3$)	% Sticks Positive PWN[2]
15	90 (92)	2173	50[3]
15	125 (126)	2996	0[3]
20	30 (31)	694	70[4]
20	60 (61)	1393	10[4]
20	70 (71)	1671	50[4]
20	90 (89)	2099	0[5]
20	110 (109)	2566	0[5]
25	60 (60)	1420	0[4]
30	50 (51)	1204	0[4]
30	60 (60)	1426	0[4]

[1] Two replicated chamber fumigations per temperature and target initial g/m^3. [2] All non-fumigated control sticks were 100% positive for PWN; nematodes extracted using Baermann funnel method. [3] 20 *Pinus echinata* sticks per replicate, including non-fumigated controls. [4] 25 *Pinus taeda* sticks per replicate, including non-fumigated controls. [5] 25 *Pinus echinata* sticks per replicate, including non-fumigated controls

In laboratory fumigations (Table 2), all non-fumigated control sticks were 100% positive for PWN. The minimum dosage of SF tested resulting in no PWN detected in wood sticks was 2996 g-h/m^3 at 15ºC, 2099 g-h/m^3 at 20ºC, 1420 g-h/m^3 at 25ºC, and 1204 g-h/m^3 at 30ºC. The mean water moisture content (WMC) ranged from 37 to 92% on a dry weight basis.

Field fumigations were conducted, respectively, at a mean minimum temperature of 33°C, 32°C, 23°C and 10°C (Table 3). The WMC in fumigation conducted in April 2003 was 34% but was much higher in the other fumigations, between 52 and 87%. At 10°C, live PWN were extracted from timber at all dosages of SF tested (4203-5866 g-h/m^3). There was no presence of nematode on 100% of the boards sampled at a minimum dosage of 1533 g-h/m^3 at 23°C, 1402 g-h/m^3 at 32°C, and 997 g-h/m^3 at 33°C in field trials. There was no evidence of *Monochamus* spp. activity (i.e., fresh shavings) or emergence holes in the fumigated lumber from any field trial.

Table 3 Survival of pine wood nematodes (PWN) in pine boards (*Pinus echinata*, April 2003; *P. taeda*, all other fumigations) of varying dimensions sawn from naturally-infested logs following fumigation with SF, Whitehall Forest, USA, (Dwinell et al., 2003, 2005)

		% boards positive for PWN by board dimension (No. of boards fumigated)					
SF Dosage (g-h/m^3)	**Actual Total h Exposure**	**2.5 x 2.5 cm**	**2.5 x 5 cm**	**2.5 x 12.7[1] or 2.5 x 10 cm**	**5 x 12.7[1] or 5 x 10.2 cm**	**12.7 x 12.7[1] or 10.2 x 10.2 cm**	**Slabs**
Fumigation April 2003, mean temperature 35 °C, WMC[2] 34%							
997	6.5	-	-	0 (13)	0 (13)	0 (13)	-
1039	6.5	-	-	0 (13)	0 (13)	0 (13)	-
1192	6.5	-	-	0 (13)	0 (13)	0 (13)	-
1506	6.5	-	-	0 (13)	0 (13)	0 (13)	-
1538	6.5	-	-	0 (13)	0 (13)	0 (13)	-
1751	6.5	-	-	0 (13)	0 (13)	0 (13)	-
Control		-	-	89 (39)	83 (39)	74 (39)	-
Fumigation August 2003, mean temperature 33°C, WMC 52% to 87%							
1151	20	54 (11)	27 (11)	44 (25)	71 (7)	41 (22)	88 (8)
1402	22.5	0 (12)	0 (12)	0 (23)	0 (7)	0 (22)	0 (8)
1916	20	0 (12)	0 (12)	0 (23)	0 (7)	0 (22)	0 (8)
1937	22.5	0 (12)	0 (12)	0 (23)	0 (7)	0 (22)	0 (7)
1942	19.5	0 (12)	0 (11)	0 (23)	0 (7)	0 (21)	0 (7)
2092	21.5	0 (12)	0 (13)	0 (23)	0 (7)	0 (22)	0 (7)
Control		87 (24)	100 (24)	94 (46)	100 (14)	100 (44)	100 (14)
Fumigation February 2004, mean temperature 10°C, WMC 68%							
4203	16.5	-	-	20 (10)	27 (11)	15 (13)	8 (12)
4381	16.5	-	-	30 (10)	64 (11)	62 (13)	33 (12)
5066	16.5	-	-	10 (10)	36 (11)	0 (13)	0 (13)
5515	17	-	-	30 (10)	18 (11)	15 (13)	0 (12)
5866	17	-	-	0 (10)	18 (11)	15 (13)	0 (12)
Control		-	-	80 (20)	73 (22)	100 (26)	85 (22)
Fumigation April 2004: mean temperature 24°C, WMC 72%							
1160	22	-	-	0 (13)	17 (12)	42 (7)	0 (12)
1207	22	-	-	0 (13)	25 (12)	57 (7)	18 (12)
1533	23	-	-	0 (13)	0 (12)	0 (7)	0 (12)
1597	23	-	-	0 (13)	0 (12)	0 (7)	0 (12)
2171	22	-	-	0 (12)	0 (13)	0 (7)	0 (12)
2357	24	-	-	0 (13)	0 (12)	0 (7)	0 (12)
Control		-	-	85 (26)	100 (24)	100 (14)	82 (24)

[1] Only in April 2003 fumigation [2]Wood moisture content

These trials showed evidence that total control of PWN nematode and of its vector *Monochamus* spp. could be achieved with appropriate dosage of SF when temperature was 15°C or higher, even with wood

of high WMC exceeding the saturation point. This research resulted in a fumigation schedule with SF that was submitted to the TPPT by German Federal Ministry of Agriculture for inclusion in the ISPM 15, suggesting the target dosage for PWN applied for all quarantine pests since the other pests required less dosage. The dosages submitted and their associated concentrations in 24 h exposures were as follows: 3000 g-h/m^3 at 15 to 19.9°C, 2100 g-h/m^3 at 20 to 24.9°C, 1500 g-h/m^3 at 25 to 29.9°C, 1400 g-h/m^3 at 30 to 34.9°C, and 1000 g-h/m^3 for 35°C or greater.

4. Trials from Flack et al. (2008) in the USA

4.1. Materials and methods

The TPPT identified four potential issues concerning trials previously submitted (Dwinell et al., 2003; 2005) evaluating SF fumigation for control of the PWN: 1) the absence of number of pests for each treatment and the control; 2) the lack of information on which life stage was present and was the most resistant to SF; 3) requirement to have mortality data using the Baermann funnel after 6 and 21 days to allow for incubation of PWN; 4) requirement to extract nematodes from larger samples than wood slivers used in previous studies. Therefore, additional trials were conducted by Flack et al. in 2008 to provide additional data to validate proposed SF quarantine treatment schedule for 20ºC and 25ºC.

Logs of dead pine (*Pinus* spp.) subsequently colonised with PWN were salvaged, debarked and sawn into sticks one week before fumigation trial. A natural infestation of PWN was augmented with laboratory cultured PWN 24-48 h before fumigation. Fumigations were conducted in 10 l glass chambers at 20 and 25°C for 24 h. Three target concentration x time (CT) dosages (g-h/m^3) were evaluated per temperature. Entire wood sticks were assayed for PWN after 7 and 21 days after fumigation using the Baermann funnel method and the numbers of juvenile and adult nematodes occurring in each extraction sample were determined. The wood moisture content was also measured by drying at 105°C for 24 h.

4.2. Results and conclusions

The mean WMC of wood sticks was 59.1 ± 4.3% (mean ± SD), indicating wood was water-saturated (Table 4). SF dosages ranged from 1947-2287 g-h/m^3 at 20°C and 1342-1586 g-h/m^3 at 25°C which represented dosages less than, approximately equal to, and above the proposed quarantine dosages of 2100 g-h/m^3 at 20°C and 1500 g-h/m^3 at 25°C. Extractions at 7 d indicated high numbers of PWN (3000 at 25°C and 1300 at 20°C) from untreated controls. At 21 d, the number of extracted nematodes from untreated control dropped to 846 at 20°C and 78 at 25°C.

Table 4 Pine wood nematode (PWN) extracted from unseasoned pine 7 and 21 d following 24 h fumigation with sulfuryl fluoride (SF) at 20° and 25°C (data extraction from Flack et al., 2008).

			PWN extracted 7 days post fumigation			**PWN extracted 21 days post fumigation**		
Temp. (°C)	**Chamber #**	**Cumulative SF Dosage (g-h/m^3)**	**No. Juv. PWN**	**No. Adult PWN**	**Total No. PWN**	**No. Juv. PWN**	**No. Adult PWN**	**Total No. PWN**
20	1	1962	2	0	2	8	1	9
	2	2046	46	3	49	30	6	36
	3	2143	334	41	375	220	42	262
	4	1947	2	0	2	0	0	2
	5	2183	0	0	0	0	0	0
	6	2287	0	0	0	0	0	0
	7	0	2850	150	3000	690	156	846
25	1	1342	0	0	0	2	0	2
	2	1487	0	0	0	0	0	0
	3	1586	0	0	0	0	0	0
	4	1334	0	0	0	0	0	0
	5	1495	0	0	0	0	0	0
	6	1568	0	0	0	0	0	0
	7	0	1040	260	1300	54	24	78

At 25°C, no PWN were extracted from sticks in five of the six fumigation treatments. Two PWN juveniles were extracted from one treatment (1342 g-h/m^3) which was less than the proposed quarantine SF dosage of 1500 g-h/m^3. These trials confirm that the proposed quarantine dosages at 25°C are effective.

Results were more variable at 20°C, with some survivors in four out of six fumigations. This was attributed by the authors to the high WMC which may have delayed penetration of the gas. Therefore, the proposed quarantine dosages for 20°C and 15°C were increased by 200 g-h/m^3 to account for variability in WMC and an updated treatment schedule (Table 5) was submitted to the TPPT in 2008.

Table 5 Updated 24 h treatment schedule sulfuryl fluoride (SF) fumigation of unseasoned pine for control of pine wood nematode (PWN).

	Min. Target SF Dosage (g-h/m^3)	SF Dose (g/m^3)	Minimum SF Concentration (g/m^3) at hour:				
Mean ºC			0.5	2	4	12	24
15-19.9	3200	183	188	176	163	131	93
20-24.9	2300	131	136	128	118	95	67
25-29.9	1500	88	94	83	78	62	44
30-34.9	1400	82	87	78	73	58	41
35 or above	1000	60	63	57	53	42	30

5. Current status and perspective of inclusion of sulfuryl fluoride in ISPM 15

Studies by leading nematologists and quarantine experts have shown effective control of PWN by SF. However, further guidance of IPCC on efficacy data requirements for PWN were produced in 2009 (Magnusson and Schröder, 2009) to applicants for proposed treatments in ISPM 15. The requirements included the need for more efficacy data on the dispersal life stage (J3 larvae) and a greater number of individuals for Probit 9 analysis. An additional study on PWN is planned with SF in Portugal in 2010 to meet these requirements. Following inclusion in ISPM 15, the use of SF would contribute to eradication and confinement of PWN alongside other methods of control, leading to enhanced trade within and outside the E.U.

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The use of hand-held computers (PDAs) to audit and validate eradication of a post-border detection of Khapra Beetle, *Trogoderma granarium*, in Western Australia

Emery, R.E.*#[1,2], Chami, M.[1,2], Garel, N.[1,2], Kostas, E.[3], Hardie, D.C.[1,2]
[1] Department of Agriculture and Food Western Australia, Baron-Hay Court, South Perth, Western Australia 6151.
Email: Rob.Emery@agric.wa.gov.au
[2] Cooperative Research Centre for National Plant Biosecurity, LPO Box 5012, Bruce ACT, Australia 2617.
[3] CBH Group, Gayfer House, 30 Delhi Street, West Perth, Western Australia 6005

* Corresponding author
# Presenting author

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Abstract

Most of Australia's agricultural produce is exported. Demonstrating freedom from certain plant and animal pests and diseases is critical to securing and maintaining market access. Surveillance is an important tool in gaining market access and accordingly exporting countries now need to provide accurate, credible evidence to confirm pest freedom status.

In the past nearly all field-collected surveillance information was recorded manually to paper reducing the rate of capture, integrity, conformity as well as security of the data. This paper describes the development of pest surveillance data collection software and hardware using PDAs (Personal Digital Assistants) to provide auditing, validation, chain of evidence and increase the volume of data collected as well as its integrity through relational databases and seamless data transfer to corporate systems. The system's first deployment was during a *T. granarium* eradication.

The khapra beetle (*Trogoderma granarium*) is one of the most serious pests of stored grain and is a regulated quarantine pest in most countries around the world. In April 2007, there was a post-border detection of *T. granarium* larvae and adults in a Western Australian residence. Immediate and uncompromising action was taken to quarantine the home and fumigate it with methyl-bromide at an internationally established rate known to control *T. granarium* (AQIS T9056).

A two-year *T. granarium* trapping program was undertaken which used PDA software to provide evidence of complete eradication via 1273 trap inspections. This achievement was supported by GPS-located traps, digital voice navigation itineraries, digital time and date stamps, field printed barcode labels, site imagery, all in a single hand-held unit.

Keywords*: T. granarium*, Khapra beetle, Eradication, Biosecurity, PDA, Surveillance

1. Introduction

Australia remains committed to World Trade Organisation, Sanitary and Phytosanitary agreements, that require measures taken to protect animal, plant, or human health must be scientifically justified and supported by evidence (WTO 1995). As international concerns about food quality and safety increase, countries' import requirements are becoming more demanding and exporters including Australia must not only declare they are free from plant and animal pests and diseases, but they need to demonstrate it with evidence. It is no longer good enough to provide "absence of evidence"; international markets require "evidence of absence" for quarantine pests.

Trogoderma granarium Everts (Coleoptera: Dermestidae) is a stored product pest of great significance (Szito, 2006). In the 1950's, Australia was inadvertently recorded as a "*T. granarium* country" due to a misidentification of a non-pest, undescribed native beetle This misidentification created trade issues for Australia and took many years to correct (Lindgren et al. 1955). In order to continue to protect its reputation as an exporter of clean grain, Australia maintains a rigorous protocol of pre-loading inspection of export ships supplemented by port and urban based surveillance trapping programs to provide evidence of absence for *T. granarium*.

In April 2007, the Department of Agriculture and Food Western Australia (DAFWA), Pest Surveillance Team uncovered a post-border detection of *T. granarium* in the personal effects of a recently-arrived migrant family in suburban Perth, Western Australia. The residents were disturbed by the presence of

beetles and larvae throughout their belongings and reported this to a pest controller who recognised the khapra beetle from DAFWA extension material. A biosecurity officer was sent out the same day to collect specimens which were identified the next day by the DAFWA taxonomist Mr A. Szito. This identification was confirmed a week later by the CSIRO taxonomist.

Immediate action was taken by Australian government and grain industry to eradicate this post-border detection with a methyl bromide fumigation followed by a two-year trapping program to reinforce complete eradication of the pest (Emery et al. 2008).

This trapping program provided the opportunity for the first use of novel PDA (personal digital assistant) technology developed with the Australian Cooperative Research Centre for National Plant Biosecurity (CRCNPB) at DAFWA, to provide verifiable surveillance data by using 1273 *T. granarium* trap inspections over two years. The PDA pest surveillance tool is supported by GPS-located traps, digital voice navigation itineraries, site imagery along with synchronisation to desktop server databases. It is not just the zero data that is important; we must consider the data behind the zeroes. This metadata can be very expensive and time consuming to collect. Unique user and location identifiers, date and time stamps, GPS coordinates, barcode labels, validation rules and integrity checking are all enhanced with the PDA pest surveillance tool.

2. Materials and methods

2.1. Methyl bromide treatment

The two-year-old two-storey townhouse discovered to have a *T.* granarium infestation was covered with shrink wrapped plastic sheeting in May, 2007 using industrial grade 200 µ low density polyethylene. Shrink wrap plastics have several advantages over older techniques using tarpaulins or canvasses in that they fit more tightly around the structure, reducing leakage due to wear and tear in windy conditions and can be welded together onsite using hand held heat guns and shrink tapes.

The structure was fumigated with 80 g/m³ methyl bromide for 48 hours at 21°C at normal atmospheric pressure with an end point concentration at 48 hours of 20 g/m³ and is regarded as an effective standard by Australian Quarantine and Inspection Service (AQIS T9056). The fumigation was monitored at 24 hours to ensure a minimum concentration of 24 g/m³. An additional 8 g/m³ is added for each 5ºC the temperature is expected to fall below 21ºC to a minimum of 10°C, as this is the minimum temperature during the course of the fumigation that can be used for the calculation of the dose.

Three gas introduction points were installed in the infested house, with the primary one in the upstairs roof-space. Gas was also introduced at two points on the main floor and in the adjoining garage roof-space. Four electric fans for circulation were placed in appropriate locations throughout the house and six gas monitoring points and one temperature probe were installed in areas shown in Table 1 that were distant from in injection point and considered to be the most important for achieving and maintaining the desired gas concentration. Gas concentrations in the house were measured with Drager® tubes and pump and environmental concentrations with an MSA Sirius® Multigas electronic detector.

2.2. Post-treatment surveillance trapping

Insect monitoring was performed by implementing a trapping program over the two years after the fumigation using Trécé Storgard® traps baited with kairamone and ground raw wheat germ. These traps were placed in the treated residence (4 traps in the garage, garage roof cavity, pantry and upstairs roof cavity), 5 in neighbouring residences (1 trap in the kitchen of each house), 12 at the shipping container receival facility (22.0 km SE of the fumigated residence), 12 at a cardboard recycling facility (19.7 km SE of the fumigated residence) and 10 traps were placed at a waste transfer station (5.6 km NE of the fumigated residence).

These traps were checked visually by a biosecurity officer weekly for the first month of the program then monthly during winter when insect activity is low. Over the warmer months of September to April the traps were inspected except for the private residences which were checked monthly and lures replaced quarterly. All insects trapped were recorded and suspect Trogoderma specimens returned to the laboratory for identification by a taxonomist. Non-target specimens not belonging to the Dermestidae were identified in the field.

2.3. PDA trap surveillance tool

In the past nearly all field collected surveillance information was recorded manually to paper reducing the rate of capture, integrity, conformity as well as security of the data. The CRCNPB recognised the need for a more robust field surveillance data collection tool and commissioned a two-year project with DAFWA focussing on development of pest surveillance data collection software and hardware using hand-held computers, PDAs or smartphones. This approach provides chain of evidence control, increases the volume of data collected as well as its integrity through relational databases and seamless data transfer to corporate systems.

Recognising the need to encourage collaborators from different disciplines across the country to add value to each other for years to come, it was decided not to look for a "shrink wrapped solution". This meant ignoring for the time being, less mature platforms and working in the Windows Mobile® PDA. The software development environment chosen was largely wizard driven to encourage collaborators to develop in-house solutions, to share techniques, code and modules. Visual CE® (SYWARE, Inc. Cambridge, MA) was found to provide the functionality this project required.

For field and laboratory mapping the PDA application collected digital latitude and longitudes and ported them to Google Maps for Mobiles® for display in the field. Digital latitude and longitudes (dd.ddddd) were chosen over analogue (dd mm ss) and UTM (eastings and northings) because they are now considered best practice for georeferencing by the Global Biodiversity Information Facility (Chapman et al. 2006) and can be captured directly from the GPS NMEA stream by the surveillance application.

PDA hardware running Microsoft Windows Mobile 5.0, bluetooth, WiFi internet, built-in Sirfstar III GPS and voice navigation were distributed to beta testers from the Australian Department of Agriculture Fisheries and Forestry, Surveillance Reference Group (DAFF 2008) for Urban Surveillance.

Trap run itineraries can be prepared using Google Earth or Google Maps on desktop PCs and uploaded to the PDA. These itineraries can then be used with several popular voice navigation programs to provide in-car voice navigation to sample sites. The ability to maintain itineraries and POIs on desktop computers and send to the PDA is important because it allows the surveillance administrator to keep libraries of the various "trap runs" and upload to PDAs as staff rotate.

On demand printing of specimen barcode labels in the field provides chain of evidence and was achieved with ruggedized, portable, bluetooth thermal printers costing about US$300. These units can print adhesive two inch barcode labels for specimen vial tracking through laboratory information management systems.

The PDA surveillance application, more fully described in Emery, 2009, has a user-friendly interface with sub-forms to "drill-down" from properties through activities, inspections to specimens. The interface incorporates one-click links to Google Maps for Mobiles allowing easy navigation to sites, the ability to view aerial photography of sites and even street views of property frontages.

Several issues needed to be resolved before the application could be deployed. One-click GPS activation from within the application, unique record identifier generation by combining device_ID and an auto-number was required so that data from multiple PDAs could be synchronised without primary key errors. Photographic image recordings of trap locations and specimens can be stored in the database.

3. Results

3.1. Methyl bromide treatment

Two hours after introduction of 100 kg of methyl bromide through the primary line, five of the monitoring points showed the maximum concentration readable by Drager tubes of 80 g/m³ and one point at 68 g/m³ (Table 1). The average recorded could have been well over 80 g/m³ if the gas monitoring equipment was able to read higher. Given the superior state of sealing afforded by the shrink-wrap process, the methyl bromide was able to be introduced through only one point in the upstairs roof space, the gas dispersed throughout the house and adjoining garage rapidly and evenly without the need for the circulation fans to be turned on. After 24 hours the average gas concentration was 39.8 g/m³ (24 g/m³ required by T9056) and at 48 hours 30.8 g/m³ (20 g/m³ required by T9056. The average temperature over 48 hours on the concrete lower floor was 20.7°C. More detail of the fumigation readings can be found in Emery (2008).

Table 1 Fumigation data for *T. granarium* eradication.

		Methyl Bromide Concentration (g/m^3)					
Time	**Temp**	**Line 1**	**Line 2**	**Line 3**	**Line 4**	**Line 5**	**Line 6**
d h:mm	**(°C)**	**Downstairs lounge**	**Kitchen**	**Upstairs bathroom**	**Upstairs roofspace**	**Master bedroom**	**Garage**
0 00:55	20.8	44	40	48	48	40	72
0 01:55	21.1	68	80	72	80	76	80
0 02:55	20.5	64	80	80	80	80	80
0 03:55	20.3	80	80	80	80	80	80
0 04:55	20.4	40	56	60	64	64	80
0 05:55	20.4	64	80	56	72	64	76
0 06:55	20.4	76	80	80	64	48	72
0 07:55	20.5	64	80	76	72	72	76
0 08:55	20.5	72	72	72	72	72	72
0 09:55	20.3	64	72	68	76	72	72
0 10:55	20.5	72	72	72	72	72	72
0 11:55	20.6	76	72	68	72	68	72
0 13:55	20.8	64	72	64	60	64	68
0 15:55	20.4	48	64	64	72	56	64
0 17:55	21.1	48	56	52	64	32	52
0 18:55	21.0				40	32	
0 20:55	21.5					40	
0 21:55	21.4		40				
1 01:55	20.7		38				
1 04:55	20.4		35	36	40	40	48
1 07:55	20.2	36	36	36	40	32	40
1 10:55	19.9	34	32	32	36	36	36
1 13:55	21.1	35	35	30	32	35	35
1 16:55	21.3	35	35	28	35	30	35
1 19:55	21.7	32	35	30	30	28	30
2 00:55							

3.2. Post-treatment surveillance trapping

Trapping and monitoring at the infested site and all suspect residential and industrial premises was undertaken over the two year period from June 2007 to May 2009. No detections of *T. granarium* in any trap were made during this period (Table 2) despite a number of non-target pests being trapped (Table 3).

Table 2 Post-treatment trap inspection data for *T. granarium* eradication.

Property	**Trap Inspections**	***T. granarium***
Residential property 7A Duke	20	0
Residential property 7B Duke	20	0
Residential property 9A Duke	18	0
Residential property 9B (infested premises)	87	0
Residential property 1A	8	0
Residential property 1B	20	0
Industrial transfer station	328	0
Industrial recycling facility	390	0
Industrial container receival	382	0
Totals	1273	0

Table 3 Raw PDA data for non-pest species trapped during post-treatment trap inspections.

Activity Name	Non-target specimens trapped
Infested House	
189BGarage	spider; *Trogoderma* spp native Trogoderma
189BGarageRfspc	cockroach; *Anthrenus* carpet beetle
189BPantry	fly; termite
189BUpstairsRspc	Silvanidae foreign grain beetle
Five neighbouring residences	
Pantries	nil
Refuse recycling centre	
Balcatta1	Curculionidae *Sitophilus oryzae* rice weevil; springtails; *Anthrenus verbasci* European carpet beetle; spider beetle; *Anthrenus* spp; psocids/booklice; *Rhyzopertha dominica* lesser grain borer; Psocidae booklice; Lepismatidae silverfish
Balcatta2	unknown larval skin; Psocidae booklice; *Anthrenus* carpet beetle; Lathridiidae; Trogoderma spp; Portuguese millipede; psocids/booklice; Mezium sp. spider beetle; Tenebrionidae vegetable beetle; spider beetle; *Trogoderma variabile* warehouse beetle; cockroach; Ptinidae *Mezium americanum* spider beetle.
Balcatta3	Psocidae booklice; German cockroach; psocids; Dermestidae
Balcatta4	Psocidae booklice; spider; German cockroach; *Trogoderma* sp.; Rhyparochromidae.
Balcatta5	unknown weevil; Psocidae booklice *Trogoderma* sp.
Balcatta6	Blattidae flower cockroach; Psocidae booklice; silverfish.
Balcatta7	Psocidae booklice; *Typhaea stercorea* hairy fungus beetle; Ant; *Anthrenus* spp. carpet beetle.
Balcatta8	Psocidae booklice; *Trogoderma* sp.; cricket
Balcatta9	Psocidae booklice; Simuliidae fly; Mezium spider beetle; cockroach; *Anthrenus* carpet beetle; spider
Balcatta10	*Anthrenus* carpet beetle; spider beetle; spider; psocid
Cardboard recycling facility	
Visy1	*Trogoderma variabile* warehouse beetle; *Anthrenus* sp. carpet beetle; *Trogoderma* sp.
Visy2	*Trogoderma variabile* warehouse beetle; Psycodidae moth fly; *Anthrenus* carpet beetle; *Trogoderma* sp.
Visy3	*Trogoderma variabile* Warehouse beetle; *Anthrenus* carpet beetle.
Visy4	*Trogoderma variabile* Warehouse beetle; *Typhaea stercorea* hairy fungus beetle; Trogoderma sp.; spider beetle; Hygrobidae water beetle; Dermestidae
Visy5	Psocids
Visy6	*Trogoderma* native Trogoderma; *Trogoderma variabile* Warehouse beetle; *Anthrenus* carpet beetle; Dermestidae
Visy7	*Trogoderma variabile* warehouse beetle; ant; Dermestidae
Visy8	*Trogoderma variabile* warehouse beetle; parasitic wasp; black beetle; hunchback fly; scarab beetle; non-biting midge; Dermestidae *Trogoderma variabile* warehouse beetle; *Anthrenus* sp; Dermestidae
Visy9	Simuliidae fly; *Trogoderma variabile* warehouse beetle
Visy11	*Trogoderma variabile* Warehouse beetle; *Phradonoma bicolor*
Visy12	*Trogoderma variabile* Warehouse beetle; Rutherglen bug; *Anthrenus* carpet beetle; *Trogoderma* native Trogoderma; Staphylinidae
Shipping container receival facility	
Wridgways1	Psycodidae moth fly; moth; spring beetle
Wridgways2	*Anthrenus* sp. carpet beetle
Wridgways3	Dermestidae *Trogoderma variabile*
Wridgways4	*Trogoderma* sp.
Wridgways5	psocids
Wridgways7	*Trogoderma* spp.; *Trogoderma variabile*
Wridgways8	warehouse beetle; drugstore beetle
Wridgways9	psocids; case-making clothes moth.
Wridgways12	Psycodidae hunchback fly; non-biting midge; scale insect; Psycodidae moth fly; non-stinging midge; spider; Rutherglen bug

4. Discussion

Fumigation data shown in Table 1 clearly demonstrate the high standard of gas retention which was well above the CT required and provides a level of assurance of eradication of any *T. granarium* that were in the fumigated residence. However, eradication can only be declared once extensive, validated trapping data are presented.

Non-target specimens trapped provide assurance that the traps were serviceable. Over 180 specimens were found in the traps during the two-year survey. They included 43 adult beetles and larvae belonging to the Dermestidae which required determination by a taxonomist. None of the specimens trapped were *T. granarium*. These trap records are summarized in Table 3.

The efficiencies provided by the PDA pest surveillance tool facilitated the collection of high quality data. This first deployment of PDAs for *T. granarium* surveillance in Australia has shown that data can now be collected with utmost integrity. While these data may not be required to support export industries right now, they could be in future if an importing country challenges Australia's stated position that it is free of a quarantine pest. More extensive collection of "evidence of absence" data can be achieved through the efficiencies of the PDA approach. These data have improved credibility and auditing with GPS coordinates, time and date stamps as well as evidentiary chain through sample barcodes and PDA "nag screens" to remind field staff to replace lures all add value to the zeros. Trap data can now be easily correlated with metadata and more traps can be serviced quickly. This is paramount to implementing corrective actions when necessary. The ability to correlate insect trap capture with metadata can also be used with population models to assist in evaluating risk for the regulatory bodies

Information on the habitat/terrain, prevailing weather conditions and training records of the surveillance officer can also be cross-referenced to each inspection made thereby adding metadata veracity to each collected zero.

This project used one dimensional barcodes to identify traps and specimens, however, recently developed two dimensional barcodes could be used on traps to trigger automatic actions such as an emergency response text message that cannot be interfered with by the user.

One area still to be explored is the use of wireless synchronisation. Syware's mEnable wireless synchronisation server has not been installed due to security implications. The location of servers will need to be negotiated as will agreement of industry participants who may have concerns over the security of their data. When this is resolved, field staff will be able to synchronise PDA-collected data from anywhere in the world provided their PDA can be connected to the internet via wifi, GPRS or host desktop PC.

The challenge now is to encourage user uptake of this new technology. Some data collectors see the PDAs as an imposition because they have an established routine, while others are concerned about their privacy being violated by GPS tracking of their movements. While it may take longer to input data on the PDA compared with writing on a clipboard, there is considerably less time spent on data input when returning to the laboratory. These data handling hours are completely removed with the PDA approach as data are seamlessly synchronised with computer systems. User training and support will be the key to gaining acceptance for this type of technology and endorsement from management will be pivotal in this process.

Another concern is that some data collectors continue to store their data in personal spreadsheets and will tout "It's all on the computer". Unfortunately these data have minimal integrity; there are no relational records and no lookup tables with the result that duplication of records through mis-spellings and alternative spellings can be rampant. An erroneous trap location entered in this way could sit among thousands of other records and not be found. The challenge is to encourage field pest surveillance staff to work with PDAs and take advantage of the data integrity that will provide avenues for data to have multiple uses and reduce the level of data displacement to filing cabinets and other non-digital storages.

New applications are under development for other surveillance projects including the Australian bulk handling industry. The grain storage prototype application tracks maintenance tasks, records quarantine pest surveillance and, most importantly, monitors stored grain fumigations to improve fumigations through assistance in extensive monitoring and real time evaluation of fumigation effectiveness.

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A novel approach to limit the development of phosphine resistance in Western Australia

Newman, C.R.*#
Department of Agriculture and Food Western Australia, Bougainvillea Avenue, Forrestfield, Western Australia 6058. Email: chris.newman@agric.wa.gov.au

* Corresponding author
# Presenting author

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Abstract

Escalating development of resistance to phosphine is of concern to grain storage operators world wide. In Western Australia 85% of grain produced is exported with a guarantee through legislation that it is free of all grain insects. Phosphine plays a vital part in shore-based fumigations to achieve this insect-free status but it is also available for unrestricted use by growers for grain stored on farms. For more than 20 years a campaign has been in place to encourage better use of phosphine. Central to the program is the preservation of phosphine for long-term use by exporters and growers. Over this period weak resistance frequency has increased to the current rate of about 48% averaged across all species. Strong resistance has been confirmed in two strains of *Tribolium castaneum*. The three key components of the strategy are:

1. Inspection of central and farm storages for grain insects, and testing insects to discover phosphine resistance.
2. Education of grain-storage managers on farms and commercial premises on effective management of grain stocks and correct use of phosphine.
3. Eradication of highly resistant insect colonies found on farms and commercial premises as well as management of strains with elevated levels of tolerance to phosphine.

Keywords: Farm silo, Fumigation, Phosphine resistance, Extension, Western Australia.

1. Introduction

Australia is one of the few countries in the world where phosphine is available to farmers for the protection of grain in their own storage. The continued effectiveness of phosphine at the farm level in Australia, however, will depend on the ability to slow or arrest the development of resistance to this fumigant in all the major grain-storage insect pests present in the country.

Phosphine has been available to farmers in Australia since the 1950's when the label recommendations included the use of the product in unsealed storages and admixture to a grain stream. In 2008, the label was changed and the two practices removed from the recommended-use table. It is suggested the continued use of phosphine in this manner for many decades in Australia has led to an escalating resistance in stored-grain insects.

In the 1980's, the Western Australian central grain-storage operator, Cooperative Bulk Handling (CBH) abandoned the use of contact insecticides and created sealed storages in which to use phosphine exclusively for protection of export grain.

However, it was of concern that evidence of resistance to phosphine was being found. Champ and Dyte (1976) reported in an FAO survey that 10% of the insect strains tested had an increased tolerance to phosphine. Following this investigation, other reports were presented of phosphine resistance in laboratory strains. The first report of control failure was provided by Tyler et al. (1983) where survival of a number of stored-product insect pest species was discovered in food warehouses in Bangladesh.

This potential for fumigation failure required a sampling regime be put in place in Western Australia to find out if phosphine resistance was present. Grain insects were taken by sampling and screening grain from farm and central storages since 1985 and submitted for bioassay to determine tolerance to phosphine and this provides information on the status of phosphine resistance. The objectives of this paper are to demonstrate how an inspection, education and eradication program assist in mitigating resistance and extending the effectiveness of phosphine as a fumigant in Western Australia.

2. Materials and methods

2.1. Inspection and testing

The inspection and testing proram was facilitated by an existing network of District Officers attached to the Agriculture Protection Board were already visiting farms to advise on and write programs for control of pest plants and animals that were 'Declared' under an Act of Parliament. All major stored-grain insect species were added to the 'Declared' list and all Officers were required to inspect farm-grain storages, sample grain, collect grain insects and offer advice or write programs for the reduction of grain pests. The number of individual strains submitted for phosphine resistance varied annually from 750 to 2500.

District Officers select farms at random and extract approximately 0.5 kg of grain from the base or bagging chute. In some cases, a grain-sampling probe was used to obtain samples from the surface of the grain. The grain is shaken over a 0.9-mm screen and the insects that fall through are collected from a tray beneath. The insects are placed in plastic vials with some of the grain and sent to the stored-grain resistance testing laboratory at the Department of Agriculture and Food Western Australia (DAFWA) for testing. There the insects are separated by species from the grain and approximately 100 of each species are put into a desiccator which is injected with phosphine at 1 mg/L for 30 min. If any insects survive this test it indicates a resistance to phosphine, but not whether the resistance is 'weak' or 'strong'. Another sample of the insects captured are put into culture on a medium relevant to their feeding habits and placed in a controlled temperature room at 28°C and 60% r.h. The progeny produced are subjected to the FAO 'discriminating-dose test' where the insects are exposed to a concentration of the fumigant under controlled conditions that approximates the amount needed to kill 99.9% of the adult insects of a fully susceptible strain (Taylor, 1986). Survivors of this test are classified as having 'strong' resistance.

The classification of 'weak' or 'strong' resistance was developed by Ebert et al. (2003) who demonstrated that the tolerance to phosphine in *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) is controlled by two major genes and the insects can carry either of these genes. One gene is responsible for 'weak' resistance and the other 'weak' resistance gene on its own has little effect, but in combination enhances the effect of the other gene. It is assumed that over a series of fumigations in poorly sealed silos, insects carrying the 'weak' resistance gene survive and as they increase in numbers there is greater chance they will mate with the carriers of the other gene resulting in the progeny expressing the 'strong' resistance characteristics. For the purposes of this work, it is assumed that all stored-product insect pest populations will behave in a similar fashion.

2.2. Eradication of strong resistant strains and education

Identification of a strong resistant strain initiated a process to eradicate the insects at the source. This action by the DAFWA was to demonstrate the process needed to achieve eradication of the strongly resistant insects to the grain store manager to keep the strain in check and to create procedures for future control work.

The procedure is as follows:

- Re-sample insects from the property where strong resistance has been determined and re-test in the laboratory for resistance factor;
- Send sample of insects to a collaborative testing laboratory of the Cooperative Research Centre for National Plant Biosecurity in Brisbane, Queensland for confirmation testing;
- Confirmation of strong resistance initiates a farm visit to assess the scale of the problem and plan a clean up and fumigation with the owner;
- DAFWA personnel visit the farm to commence a hygiene program. This involves sealing silos where possible, fumigating any grain in situ and cleaning around the silo complex to remove food and harbourage for stored grain insects outside the silos;
- A further visit to the farm prior to harvest is required to ensure hygiene procedures have been completed and the silos sprayed internally with a contact insecticide. In addition, contact insecticide is applied underneath and around the silos and in any former derelict grain storage areas that might provide refuge for grain insects. Grain-handling equipment, including harvesters are also treated with contact insecticide. Silos are checked for gas tightness by replacing rubber seals as needed, checking the oil in the pressure relief valve and conducting a pressure test on the silo which should be able to hold an introduced pressure of 250 Pascals decaying to 125 Pascals for five min or longer;

- After harvest and shortly after the farmer has finished loading grain into the silos, DAFWA personnel revisit the property to fumigate the grain. This involves a further pressure test of the silo and loading phosphine, aluminium phosphide (AlP) at the label rate of 1.5 g/m³ into the headspace onto a wide tray that allows the AlP tablets to lay one deep and facilitate release of the phosphine. Grain is usually hot and dry (>25°C, <12% m.c.) and fumigation protocol is 7 d at concentrations greater than 100 ppm when temperature is greater than 25°C and 10 d when temperature is less than 25°C, assuming the silo is appropriately sealed;
- Over the course of the fumigation, monitoring is conducted using a Canary™ brand 'Silo Chek' phosphine monitor. On well-sealed silos, only one point is monitored at the base just above the lower seal plate, which is the most difficult point for gas to penetrate and the point at which the fumigation is most likely to fail. (Newman et al., 2004) On the less well sealed silos, a headspace reading is also taken. The aim is to achieve >200 ppm for 9 d at all monitored points. In less-well-sealed silos this usually requires additional AlP to be applied to achieve protocol;
- To validate the treatments, traps are baited around the silos with whole and crushed grains and grain samples removed from the silos and sieved.

3. Results

Since commencement of the testing program, results indicate there has been a steady escalation in grain insects showing a resistance to phosphine from approximately 10% to over 40% in the 20 years that the program has been in effect (Fig. 1). In Western Australia, the average across all species shows that up to 48% of insects tested have a 'weak' resistance to phosphine with a range of 15–70% between species. The selection of grain farms for sampling is completely random, and the variability each year in the numbers found to have weak resistance is most likely the result of sampling intensity (Emery, personal communication, 2010). In the eastern states of Australia 70 to 100% of insects in the Northern GRDC region (North New South Wales (NSW) and Queensland) and 53–83% in the southern GRDC region (NSW, Victoria and South Australia) exhibit a weak resistance (Collins, personal communication,.2006). In 2007, strong resistance to phosphine had been detected in the Northern region but remained below 10% of the 253 insect samples analysed. (Collins, personal communication, 2007).

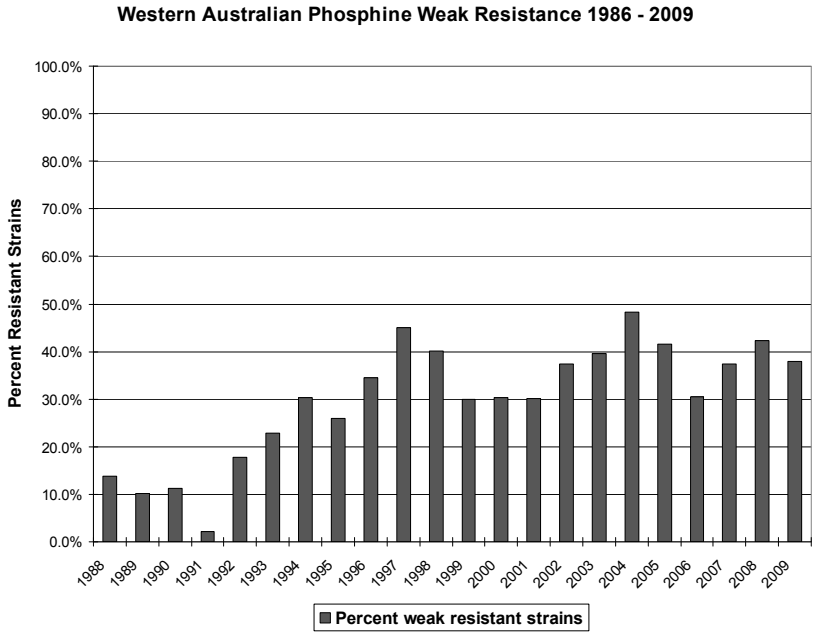

Figure 1 The occurrence of strains with weak resistance in Western Australia, Australia, from 1988 to 2009.

When test results of individual insect species are tabled for Western Australia (Fig. 2), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) showed the highest weak resistance and two cases up to 2009 have been found to have 'strong' resistance (Emery and Chami, personal communication). Few samples of *Crytolestes* species (Coleoptera: Laemophloeidae) collected prior to 1999 demonstrated resistance and as the population becomes more numerous they appear to be exhibiting trends similar to *R. dominica* and *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Fig. 2).

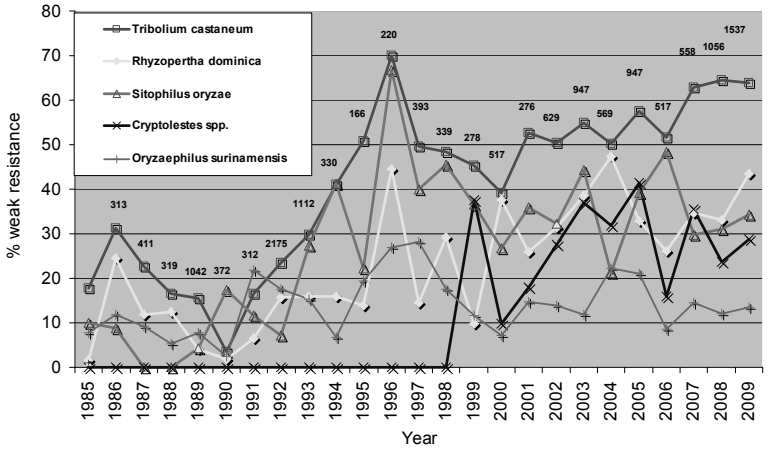

Figure 2 Occurrence of weak resistance to phoshine of stored product insects found in Western Australia from 1985 to 2009.

4. Discussion

Stored-product insect pest resistance to phosphine is important in Western Australia and in order for phosphine to remain an effective pest-control product in the future, action must be continued to slow the development of weak resistance. The emergence of strong resistance is an indication of continued selection of the weak resistant genes in farm silos. In both cases. *T. castaneum* is the dominant species making it more likely that a high proportion of the insect population contains the 'weak' resistance gene and that there will be a cross fertilisation of the two major genes. A failure to effectively eliminate an identified strong-resistant strain during subsequent fumigations will further select the most resistant individuals in the population.

The Western Australia program has been successful in avoiding the levels of resistance observed in eastern Australia and the program will likely need to remain in effect for as long as phosphine or any other single pest-control product is put into use. Slowing the development of phosphine resistance is the key to extending the economic life of the fumigant. In Western Australia, extension of information on improved grain storage and fumigation was put in place soon after stored-grain insects were 'Declared'. Written information was published and distributed to the farmer as insect samples were being collected.

The change from contact insecticide application to aluminium phosphide fumigation by CBH in the early 1980's initiated a new phase in the prevention of phosphine resistance. As more grain is being initially stored on the farm, quality fumigation is essential to managing grain quality and maintaining the effectiveness of phosphine. To elevate the standard of grain storage across the state, manufacturers of farm silos were approached to alter silo designs to enable them to be sealed easily and more effectively and, therefore, retain gas long enough to eliminate all life stages of grain insects in the silo. Silo manufacturers modified their production processes to enable silos to be pressure tested to a standard that would hold phosphine long enough to eliminate all species of insects. (Newman, 1996) However, this change has been somewhat reduced in its effectiveness by poor maintenance of the silos on farm, effectively preventing a higher level of control of the resident insect population than has been observed. (Newman, 1989). The extension message moved to focus on repair and maintenance of farm silos and correct fumigation. Phosphine monitors are uncommon on Western Australia farms so the aim has been to promote 'set and forget' fumigation. From earlier work by Newman et al. (2004) it was effective if

the silo can pass the standard pressure test and the correct dose is applied. The standard pressure test requires that the silo hold an introduced pressure of 250 down to 125 Pascals for five min. Failing this test means it is unlikely phosphine gas will be retained at the required concentration x time factor to eliminate all life stages of all insects in the grain.

The silos are produced from the factory with robust, long-lasting sealing between the wall sheets and all major joints. The vulnerable points are the rubber seals in the inlet and outlet ports and the oil in the pressure relief valve which needs to kept at a level that allows air within the silo to expand and contract, thus preventing vacuum pressures on the roof that could be potentially damaging. Replacement of damaged seals and checking oil levels is a relatively simple and inexpensive task but it is this lack of maintenance that is the most significant factor behind the development of resistance in grain insects.

The author conjectures that the instructions on the first available containers of aluminium phosphide in the 1950's are an important contributor to the continued use of phosphine in unsealed and poorly maintained, sealable silos. The instruction to use in unsealed storages and admixture with a grain stream has fallen into 'folklore' and passed on through generations. In addition, the purchaser of the silos expected that the manufacturer would provide very long lasting seals and that the silo should remain sealed for life.

The extension campaign continues to encourage time be allocated to inspection, repair and maintain the hygiene of grain storages though written material, press articles, radio interviews and most importantly, through on-farm workshops where essential maintenance techniques are demonstrated (Figures 3 and 4).

The funding for extension came from the state government in the early years but since 2000 has been partly funded by the GRDC though several defined programs. These focused on better use of phosphine, aeration for quality control and reduction of grain-insect populations and early harvesting and drying of grain. The current extension program is funded until 2012, providing 75% full time equivalent to promote quality grain-storage principles. Campaigns of this nature collectively raise the awareness of the importance and principles of grain storage as part of the farm business and are now invaluable since Australian growers have the opportunity to store and market their own grain directly off farm.

From 2008, the abolition of the centralised marketing system in Australia has allowed growers opportunity to market their own grain. However, as the length of time that grain is stored on farm increases, there is a need to ensure the product meets buyer specifications. In the case of export from Australia, this specification is that it is free of stored-grain insects. Prior to 2008, the central grain-handling and warehousing system in each state had exclusively received the grain, protected quality and marketed the product domestically and overseas.

Figure 3 Replacing old worn seals of grain silo hatch to improve fumigation efficacy.

Figure 4 Demonstration of airtight silos.

This new aspect to the farm-grain business will focus the importance of high-quality grain storage for farmers who adopt a self-marketing policy. There remains the need to provide information to growers who continue to deliver to the central system but also use phosphine for protecting their own seed and feed in farm storage.

5. Conclusions

Through the efforts of inspection, program development and implementation of eradication programs, fumigation with phosphine is maintaining effectiveness in Western Australia. However, the results of this work would suggest that an insect-management model as explained here, needs to continue if strong resistance is to be limited. The unique approach to the control of stored-grain insects taken by DAFWA, the sealing of the central storage system by CBH, the response by silo manufacturers to seal transportable silos and the ongoing extension campaign have all contributed to the lower level of phosphine resistance encountered in Western Australia. In addition, other mitigating factors may also have played a part in slowing the development of phosphine resistance. For example, the smaller amount of grain held on farms for domestic trading compared to eastern Australia and the majority of grain grown in Western Australia is delivered direct from field to the central system. Of this 85% of grain held under high quality, central storage conditions is exported, allowing better monitoring and control implementation from professionally trained staff.

With the dismantling of the centralised marketing system, it is anticipated there will be more storage installed on farms, larger amounts of grain traded in small parcels and greater use of phosphine. To ensure the grain meets customers specifications, there will need to be more professionalism applied to farm-grain management than in previous years. This includes more effective fumigation measures to avoid resistance selection. Continued extension of quality grain storage principles is a primary method in creating change along with sampling and testing for resistance of stored-grain insects. Both will play a major role enabling the grain-storage manager to deal with escalating phosphine resistance.

The novel component of this strategy is that a large proportion of the funds to conduct this work are derived from grain growers though a levy on delivered grain. The Grains Research and Development Corporation (GRDC) receive this levy and invest the funds in defined research and extension projects maximising the return for the grain growers who contributed the levy. Eradication of strong resistance outbreaks at source cannot continue to be funded by government and industry in the long-term and will most likely revert to advice provided on site by qualified personnel to enable individual growers to manage the problem.

Into the future there will be a need for independent advisers to provide this service and it is unlikely that this will be funded from government sources as the activity is considered a private good. Funding from industry should be sought for this work beyond 2012.

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Evaluation of ozone treatment in vacuum for in-shell Brazil nuts shipment and aflatoxins

Scussel, V.M.*#[1], Giordano, B.N.E.[1], Simao, V.[1], Manfio, D.[1], Galvao, S.[1], Scussel, J.N.[2]
[1] Food Science & Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, P.O.Box 476, Florianopolis, SC, CEP 88034-001, Brazil. Email: vildescussel_2000@yahoo.co.uk,
[2] Mechnical Engeneering Dept. Technological Center, Federal University of Santa Catarina, PO.Box 476, Florianopolis, SC, Brazil.

* Corresponding author
# Presenting author

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A study utilizing ozone (O_3) and vacuum packaging to find out their effect on in-shell Brazil nuts fungi and aflatoxin (AFL) degradation was carried out together with lipid stability and sensory evaluation after 60 days of storage at 26ºC. In-shell Brazil nuts were O_3 treated at 31.5 mg/L (5h.), vacuum packaged in low oxygen permeability polyethylene bags, heat sealed and stored (Group I). Groups of in-shell nut packs were kept for Control: with (Group II) and without (Group III) vacuum. The nuts initial fungi load was 4.83 log cfu/g, moisture content of 9.37% and 11.58 µg/kg of AFLs. Any fungi load change (on MEA media), *Aspergllus flavus* and *parasiticus* (on AFPA media) growth/inhibition, AFL presence (analyzed either in-shell and after shelling by LC/FD), lipid oxidation (TBA test) and nut acceptance/rejection by sensory evaluation (attributes: nut shell and edible part appearance, strange odor, residual taste, rancidity and firmness) were registered. Right after the O_3 treatment no fungi and yeast count (cfu), neither the toxigenic species of *Aspergillus* (*A. parasiticus* and/or *A. flavus*) growth were detected in the nuts and the same persisted throughout the whole storage period. As expected, different behavior was observed in the Control Groups. In Group II, the nuts kept similar fungi count as the beginning of the experiment; however, slightly lower, probably due to lack of oxygen by the vacuum environment. With the exposure to O_3, AFLs were not detected up to the LOQ of the method (0.50; 0.17; 0.50; 0.17 µg/kg) since Day One and up to the end of the storage, different to the untreated nut packs (Control Groups). The sensory evaluation showed that nuts O_3 treated and vacuum packaged were still palatable and were accepted by the panelist groups with scores ranging from 4 (like) to 5 (like very much), with no significant changes ($p<0.05$) between nut sensory attributes per panelist. From the data obtained, O_3 gas did not affect the lipids of the treated in-shell Brazil nuts vacuum packaged. The malonaldehyde values were constant throughout the whole storage period. The data obtained here on O_3 + vacuum + packaging showed that it can be an alternative procedure, easy to apply, for transporting in-shell Brazil nuts through long distances such as: in the forest (raw) by boat in the long and curved Amazon river, or during export by ship trips can last 3 to more weeks.

Profitable chemical-free cowpea storage technology for smallholder farmers in Africa: opportunities and challenges

Baributsa, D.[1]#*, Lowenberg-DeBoer, J.[1], Murdock, L.[2], Moussa, B.[1]
[1] International Programs in Agriculture, 615 W. State Street, Purdue University, West Lafayette, IN 47907-2053, Email: dbaribut@purdue.edu
[2] Department of Entomology, 901 W State St. Purdue University, W. Lafayette, IN 47907

*Corresponding author
#Presenting author

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Abstract

Cowpea is the most economically and nutritionally important indigenous African grain legume, grown by millions of resource-poor farmers. It is a key cash crop in areas too dry to grow cotton or other export crops. Most of the over 3 million t of cowpea grain produced annually in West and Central Africa is grown on small farms. Storage is often identified as the key challenge for small scale cowpea growers. Many farmers sell cowpea grain at low harvest time prices rather than risk losses by bruchids during storage. Some traditional methods are effective for small quantities (e.g., 10 kg), but are difficult to scale up. Some effective storage chemicals are available, but they are regularly misused by farmers and merchants. The Purdue Improved Cowpea Storage (PICS) Project is addressing these problems through promotion of hermetic storage in triple layer sacks which have an outer layer of woven polypropylene and two liners of 80 µ high-density polyethylene. Village demonstrations with more than 45, 000 PICS sacks have shown the technology to be effective. Good quality affordable sacks have been produced by manufacturers in Nigeria, Burkina Faso and Mali. Over the past three years more than one million sacks have been produced and sold. Despite the success with the outreach activities and the farmer adoption, the challenge remains to develop sustainable sack distribution networks. Issues identified include reluctance of wholesalers to order sacks due to risk associated with a new product, inability of wholesalers to develop effective distribution networks due to difficulties with enforcing contracts, and limited access to capital. The PICS project is exploring new ways to address some of these issues, including using non-traditional distribution systems for PICS sacks such as agro-dealers networks, and adapting distribution systems that have worked for cell phones and other products.

Keywords: Cowpea, Bruchids, Hermetic storage, Supply chain, West and Central Africa

1. Introduction

Cowpea is the most important economically and nutritionally indigenous African legume crops, especially in West and Central Africa (WCA). Cowpea is rich in protein and constitutes a staple food for people in rural and urban areas. It is used for family consumption as well as sold in the local market for much needed cash. In the 1990s in WCA, cowpea production, mostly by small-scale farmers, was estimated at 2.6 million tons on about 7.8 million hectares, 69% of the world production (Langyintuo et al., 2003). Both area planted to cowpea and production has expanded in the last decade, with production now averaging over 3 million t annually. Cowpea is also an important cash crop in the region with potential for entering commerce. It is estimated that around 80% of the cowpea trade in the world is in WCA. In the late 1990s, official cowpea trade accounted for over 300,000 t of cowpea per year within the Nigerian Cowpea Grainshed (Langyintuo et al., 2003; Langyintuo et al., 2005; Langyintuo and Lowenberg-DeBoer, 2006). However, marketing and trade is severely hampered by storage insects, especially the cowpea weevil (Murdock et al., 1997).

Moussa (2006) and Boys (2005) indicate that a conservative estimate of cowpea storage loss is 25%. Cowpea bruchids are seed beetles that develop and reproduce rapidly in stores of cowpeas. A female cowpea bruchid, just emerged from her seed and newly mated, can produce 60-120 eggs (Fox, 1993), hatch into larvae, most of which survive to adulthood and begin reproducing. In warm climates, the time required for a full generation is short, as little as three and one-half to four weeks. As a result, a freshly threshed store of cowpeas with only a small initial bruchid infestation can be rendered inedible and worthless in the market within two or three months.

Farmers use a variety of commercial and traditional methods to control bruchids, many of which have restricted value because of cost, labor and potential toxicity. For instance, insecticides can be used to control cowpea weevils, but poor farmers often do not have access to these insecticides and when they do, they often misuse them resulting in health and environmental problems. Ash is also used for cowpea storage, but only for small quantities due to labor requirement and because many people consider ash as "dirty" and refuse to eat food stored in ash. Other cowpea storage methods include metal drums, widely available and used in northern Senegal and south Benin. It was estimated that about 60% of cowpea production in the main cowpea area in Senegalese was stored in metal drums (Boys et al., 2007). In recent years, there has been a decline in metal drum use due primarily to their cost and the inflexibility of drum storage to production quantities. In addition, many farmers were using insecticides to store cowpea in metal drums because many were rusting (hence, no longer air tight). Moussa (2006) reported that farmers used insecticides when storing cowpea in single or double layer plastic bags. Adoption studies conducted at the village level in WCA revealed that growers were very interested in hermetic storage for cowpea, but lack appropriate containers and information about proper storage (Moussa, 2006; Boys, 2005).

2. Purdue improved cowpea storage (PICS) technology

To further improve cowpea storage and address some of the issues mentioned above, Purdue University with its partners initiated the Purdue Improved Cowpea Storage

(PICS - http://www.ag.purdue.edu/ipia/pics)

project which was funded by the Bill and Melinda Gates Foundation. The goal of the project is to have 50% of farm-stored cowpea in hermetic storage without insecticides in West and Central Africa by 2012. The objectives of the project are to (i) determine the best design for a one-piece commercially available triple-layer plastic cowpea storage bag; (ii) disseminate information on non-chemical cowpea storage methods to extension services, non-governmental organizations (NGOs) and farmers; (iii) demonstrate the most effective cowpea storage methods in each village in the major cowpea production areas of WCA; and (iv) develop and foster local businesses that provide triple-layer plastic storage bags.

The benefits from improved storage include increased cowpea trade in the region, hence, enhanced incomes of small-holder farmers and the food supply to consumers in the region. This project is implemented by Purdue University in collaboration with many partners including the International Center for Tropical Agriculture (IITA), World Vision, the National Institute for Agricultural and Environmental Research (INERA) of Burkina Faso, the National Agricultural Research Institute of Niger (INRA) and other national agricultural research systems, NGOs, farmer associations and private sector partners.

The triple layer technology was originally developed to enable Cameroonian farmers with few resources to store their cowpea grain safe from losses to seed beetles; as experience with the technology grew, it began to spread across into West Africa. The foundation for the current PICS technology was triple bagging of cowpeas, developed under the USAID-supported Bean/Cowpea CRSP in the late 1980's (Murdock et al., 2003). The triple layer bagging technology subsequently began to be adopted in various countries in WCA.

The PICS technology owes its effectiveness to the airtight storage enabled by the PICS bags. Threshed cowpea grain is put into 50-or 100-kg capacity high density polyethylene (HDPE) bags with walls 80 µ thick, taking care to fill the bag completely, without air pockets, except for a neck of 20-30-cm length. This first bag is tied securely shut at the neck and then surrounded by a second bag of the same material and thickness. The middle layer bag, completely surrounding the first, is tied shut at the mouth in the same way as the first. These two sealed bags are then placed inside a third plastic bag, which is woven nylon or polypropylene, for strength. This container thus formed can be handled without bursting the inner bags, and is readily accepted by grain handlers since it is the same type of plastic bag they are accustomed to storing cowpea grain in. A step-by-step how-to-use PICS technology can be found at http://www.ag.purdue.edu/ipia/pics/Documents/PICS_English_Nigeria.pdf.

PICS bags work – as do other hermetic storage contains such as sealed steel drums (Seck et al., 1996) – because insects respire aerobically and thus utilize the oxygen in the airtight container while also raising CO_2 levels. Once the oxygen level in the container falls sufficiently low, insects cease feeding and

become inactive (Margam, 2009). Inactivity itself causes growth and development to cease and in turn reproduction stops. This results in the arrest of population growth. During the oxygen deficit-caused inactivity insects begin dying. the early-instar larvae and pupae appear to be particularly vulnerable.

3. PICS technology capacity building and outreach approach

The PICS project started with pilot programs during the first year in both Burkina Faso and Niger to evaluate the alternative extension methods. The outreach program focused on village activities by trained field technicians. Training was implemented at various levels. First, a workshop for trainers of field technicians was held in each country. The objective of the workshop was to educate trainers about how to facilitate and conduct train-the-trainer sessions for field technicians. Thereafter, there were train-the-trainer sessions for field technicians who would implement village activities.

The core extension program focuses on PICS technology awareness building through village level activities involving multiple visits by trained field technicians. Village activities include sensitization, demonstration, follow-up and open-the-bag ceremonies. Experience in Cameroon in the 1990s by the CRSP and its NGO partners suggested that village level demonstrations were the single most effective method for facilitating adoption and was extremely cost effective (Moussa, 2006). Village activities included a three-step process:

- The technician begins with a sensitization visit to the village to explain the technology, obtain a commitment for the village to participate, and set up a date/time for the village demonstration.
- A subsequent activity is the first demonstration, carried out soon after cowpea harvest. Most farmers in WCA are illiterate, so demonstration is the most effective method of technology transfer or extension education. In each village, the project provided five to 10 triple-layer sacks for conducting demonstrations. Farmers volunteer their cowpea and the sacks are filled during the demonstrations. The filled sacks are then kept in a community storage facility until the open-the-bag events that occur four to six months later. During village demonstrations, the technicians show step-by-step how to fill and tie the bag and then supervise the filling of the remaining bag by farmers. PICS demonstrations include training on how to distinguish an acceptable bag for storing cowpea and how to test bags for air tightness. In the months following the demonstration, the technician would visit the village and hold informal meetings and inquire about the bags of cowpeas.
- The third contact, between four and six months after harvest, is the open-the-bag ceremony. This event is key component of the successful technology transfer because most farmers are illiterate and the concept (depletion of oxygen) that lies behind hermetic storage is abstract to most of them.

The project has made a strong effort to use the media, radio mostly, for communicating the PICS message to rural communities. Most rural people in the region do not have access to television. Radio is the most widely used medium of mass communication in WCA. PICS radio messages are broadcast on radio during village activities (sensitization, demonstration, follow-up and open-the-bag events). In addition, a commercial message is broadcast during the harvest season and storage period, focusing on the availability of PICS sacks in various locations of the country. Comparisons of adoption in villages with and without PICS radio messages indicate that radio has a significant effect in reinforcing the use of the PICS sacks (Moussa et al., 2009).

Posters have also been printed and distributed to PICS sacks vendors, extensions agents and NGOs implementing the PICS activities, and to community radio. Recognizing the expanded use of cellular phones in rural Africa, the project has developed a video sketch on the use of the PICS technology in the Hausa language and in French. The cellular phone videos are being transferred via bluetooth and represent a way to overcome issues related to explaining a concept that is simple but difficult for some farmers to follow without seeing a demonstration.

4. Supply chain development for PICS sacks

To sustain the availability of triple-layer sacks to end-users, the project is developing a supply chain throughout the cowpea growing areas of WCA. To develop this, the PICS project has been focusing on developing processes and systems through the whole supply chain that ensure product quality and assure availability by fostering working relations among the supply chain members, namely: manufacturers,

distributors/wholesalers, semi-wholesalers, sack vendors/merchants and retailers or rooming vendors (Figure 1).

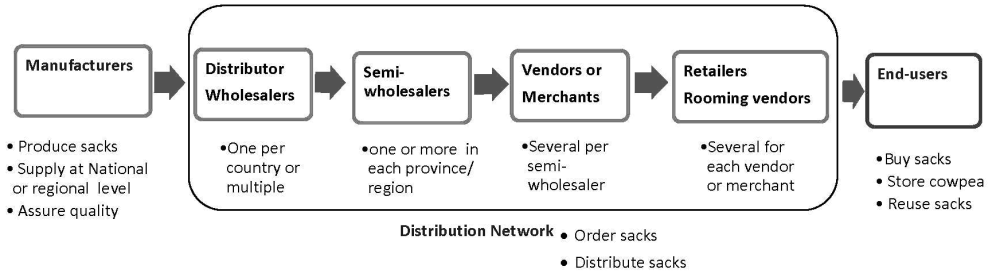

Figure 1 PICS sack Supply Chain- distribution of PICS sacks from the manufacturer to the end-users

Active engagement with the supply chain is achieved through training, relationship building and monitoring. In some cases, this included financial help in making the first order of PICS sacks. The process for the development of the supply chain involved several steps:

- First, the PICS project assessed the availability of plastic manufacturers in each country. If a local manufacturer is not found or local manufacturers cannot meet quality or cost criteria, then the project initiates discussions with manufacturers in neighboring countries or elsewhere in the WCA region. It is best to work with local manufacturers if they are price competitive and can also guarantee bag quality (can meet sacks specifications- e.g., high density polyethylene inner liners 80 µ thick). Given that PICS is a new product, minimizing the cost of production and transportation help keep the price down compared to alternative technologies, which provides an incentive for farmers to use it. However, PICS has relied on outside-the-country manufacturers in some cases.
- Second, the project identifies a distributor who has the means to finance the PICS sack order and also has a network to distribute them. In the past few years, the PICS has experimented with various alternatives for developing the distribution network for selling PICS sacks. During the pilot program, the project used NGOs and extension services as venues for selling PICS sacks to farmers. But this approach did not function well because there were few incentives for selling more bags. Most NGOs and extension services were more concerned about inventory control (e.g., preventing pilfering) than in sales. Recently the project started partnering with traditional sack merchants and emerging networks of agro-dealers, many of whom do not have well-developed distribution networks.
- Third, PICS supported the development of the supply chain by creating demand through media and village awareness activities (sensitization, demonstration, etc.). PICS activities have been carried out in over 23,000 villages in Nigeria, Niger, Burkina Faso, Benin and Togo in the last three years. In addition, the project assists distributors to expand their network, through business consultants, by recruiting and adding new members into their networks. Market and point of sale demonstrations are also conducted to increase awareness of the PICS technology. The project originally targeted only farmers for information and training, but realized that building a sustainable supply chain for the PICS sacks required reaching out to all potential users, including grain merchants, public and private organizations.

While PICS agreements with manufacturers and distributors vary from country to country, the basic strategy is (i) to demonstrate the potential for demand for PICS sacks to both the manufacturer and the distributor; (ii) facilitate expansion of distribution network by working with distributors to identify major cowpea-growing areas not yet covered by their network and to find potential retailers in those areas; and (iii) transfer the risk to both the manufacturer and the distributors by requiring that all orders in the year after full-scale implementation of PICS (usually the second year) be made without financial assistance of the project.

5. Opportunities and challenges in implementing project activities:

The PICS technology has been quickly adopted by small-scale farmers and other organizations because of its effectiveness and ease of use. In addition, the cost of the sack has been relatively low when compared to other alternatives (metal drums, single or double layer plastic bags in conjunction with pesticides or traditional methods that are labor intensive and often less effective). The price of a PICS sack ranges from US $2 to US $4 depending on the country and location. In general, farmers have not complained about the price of the bags, but about availability. They would like bags to be on sale in their local market or local shops. The most vigorous criticism about bag prices come from grain merchants who would need to buy many bags (i.e., hundreds or thousands) if they were to adopt hermetic storage.

Another advantage of the PICS technology is that it can be produced in most countries or in the WCA region. Geographic proximity remains a critical factor that influences the price of the technology at the farm level, given that cost of transport is very high in many countries due to poor infrastructure connecting major cities with rural communities where the sacks are used.

In addition, the PICS technology provides business opportunities to local manufacturers, business entrepreneurs and rural communities for producing and selling PICS sacks, and storing cowpea grain at harvest and selling it later when the prices are high. Farmers and traders have an economic motive for storage because cowpea prices often double or triple from harvest time lows to seasonal highs in the months before the next harvest. The investment in the PICS sack is small compared to the return for storing cowpea in PICS sacks for some months. For example, at harvest in Kano Nigeria, if a farmer sells cowpea he/she earns 5000 Naira (100 kg at 50 Naira per kg). If a farmer decides to sell it later six months later, then he/she may earn 9700 Naira (100 kg at 100 Naira per kg minus 300 Naira for a PICS sack).

Because the PICS sack is a new product to many farmers and sack vendors, there is a reluctance to purchase them in the first year. However, once farmers have witnessed the effectiveness of the technology during the open-the-bags events, many more are willing to purchase and use the sacks. Since the beginning of the project in June 2007, almost 1.25 million PICS sacks have been manufactured (Table 1). Of those, about 148,000 sacks have been used in demonstrations. Over one million sacks have been sold overall. The largest single buyer was Nigerian Office of Food Products (OPVN), with 800,000 bags. Small holder farmers and traders have purchased about 240,000 sacks. While challenges remain, the PICS sack supply chain is rapidly transitioning from a project activity to a private business. Some distributors and wholesalers are adapting models that have worked for other products such as the route-to-markets used by Celtel Company to improve inventory management and cash recovery. During the first and subsequent years of the project, it has been a challenge to estimate the demand for sacks. It is hard to predict the markets given that (i) the PICS sacks is a new product; (ii) there is no reliable data or service that could provide this type of information; and (iii) the potential for reuse of the PICS sacks.

Table 1 Estimate of PICS bag inventory by country and year, number of sacks ordered and remaining for each cowpea harvest season since the beginning of the project in 2007.

		Harvest season						
		2007/2008		**2008/2009**		**2009/2010**		
		PICS	**Others**	**PICS**	**Others**	**PICS**	**Others**	**Totals**
Niger	Ordered	20000	0	100000	400000	0	410000	930000
	Remaining*	15566	0	28,347	0	0	5000	1780
Burkina Faso	Ordered	20000	0	95000	0	0	23000	138000
	Remaining	18396	0	48000	0	0	14000	14000
Nigeria	Ordered			5000	4800	100000	20000	129800
	Remaining			2900	1900	2900	20000	22900
Mali	Ordered					25000	0	25000
	Remaining					11483	0	11483
Benin	Ordered					12500	0	12500
	Remaining					4826	0	4826
Togo	Ordered					12500	0	12500
	Remaining					6000	0	6000
NB:	* remaining inventories are sold the following cowpea harvest season							
	= Rough estimates					= Pre-project in these countries		

Given that most business is based on trust and relationship, growing the distribution network has been difficult. This is because each wholesaler will only work with a limited number of retailers whom they know. Each of the retailers can only maintain relationships with a certain number in the next layer (e.g., vendors who go to traditional markets). This leads to several issues associated with the development and expansion of the PICS sack distribution network, among them: (a) wholesalers are reluctant to provide inventory credit and insist on cash-and-carry to new vendors identified by the project; and (b) reliable vendors are not available in some areas, etc. The lack of access to credit has also made manufacturers require a downpayment at the time of the order so as to manage the risk, even from distributors who are already part of their customer base buying other types of sacks.

Pricing and affordability of the PICS bags is important: the price is too high, farmers and other users will not buy them. In the first year of PICS price was set to cover average manufacturing, transport, handling and retail costs. The manufacturer-to-retail margin was set at 10% to 15% based on interviews with traditional sack merchants. A uniform recommended price was established and enforced by publicizing the recommended price on radio and by regular contact with vendors to monitor prices. The project quickly learned that not allowing vendors to earn adequate margin would compromise the effort to develop a distribution system. Setting the price was leading distributors to keep most of the margin, hence, creating less incentive for vendors in the lower layer of the distribution system to sell the sacks.

Given the lack of quality-control enforcement mechanisms in most WCA countries, it is almost inevitable that when a demand develops for PICS bags, lower quality bags will be marketed. There have already been reports of fake sacks marketed in Niger and Nigeria. To assure quality, Purdue University has trademarked the PICS logo. This will provide a legal tool to pursue those who make low quality bags and try to use the PICS logo.

Some farmers have successfully used PICS sacks to store a variety of crop products such as bambara groundnuts, sorrel grains, maize, sorghum, and millet. Diversifying the use of PICS sacks to other crops may in fact increase the demand for PICS sacks and will provide opportunities to both the PICS sacks vendors and end-users who will be able to preserve their other crops.

6. Conclusions

In countries where PICS hermetic storage technology has been demonstrated, farmers have quickly adopted the technology. The technology is simple to use, affordable, pesticide free and scalable. PICS bags can accommodate whatever quantity the grower has. The PICS bags provide storage opportunity to farmers and consumers interested in organic and bio products. While the project has made significant progress in disseminating the PICS technology, it may take longer than originally planned to develop the sack distribution networks. Like any other new product launched on the market, it takes time to make consumers aware of the product and to get it fully embraced by members of the supply chain. Based on the first two years of the project we have learned the following lessons: i) diversifying/regionalizing distributors will help address issues related to potential size (development and management) of the distribution network; ii) during the first year, requiring the wholesalers/distributors to put a 20% down payment at the time of order helps identify those who are committed and serious; iii) it is better that PICS sack distributors and retailers run out of sacks in the first year, rather than holding sack inventories. Unsold bags create cashflow issues with manufacturers and the PICS project; iv). during the second year, allow vendors to set sack prices and provide them with information on sack demands from the first year; and vi) conduct business with literate merchants – they are more likely to take written or verbal agreements seriously.

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Julius Kühn-Institut

Bundesforschungsinstitut für Kulturpflanzen