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Evaluation of tolerance/resistance to phosphine of stored product beetle populations from Europe, by using different diagnostic methods

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DOI 10.5073/jka.2018.463.220

Abstract

We evaluated the susceptibility to phosphine in different populations originated from 14 European countries, by following different diagnostic protocols. In total, more than 200 populations were screened during these tests, classified to 9 beetle species: *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) and *Lasioderma serricorne* (F.) (Coleoprtera: Anobiidae). The different bioassay-related diagnostic protocols that were followed were based on different exposure intervals and phosphine concentrations, ranging between 90 min and 4 d, and between 30 and 3000 ppm, respectively. Our results indicated that one of the populations that had been sampled from Europe was strongly resistant to phosphine. Moreover, the different protocols provide comparable results, which means that a standardized diagnostic can be further designed and adopted. Moreover, molecular assays indicated that the mutations P49S in *R. dominica* and P45S in *T. castaneum* are common among different populations, regardless of the degree of resistance to phosphine. Our results suggest that there are reliable quick tools for the evaluation of resistance to phosphine and that insect sampling in target areas should be conducted on a regular basis.

Keywords: Phosphine resistance, tolerance, fumigation, stored product beetles, protocols

1. Introduction

Phosphine fumigation is the primary fumigation tool to control stored product insects. Nevertheless, although phosphine has been proved effective against most major stored product insect and mite pests, its extensive use meets with several drawbacks (Benhalima et al., 2014). The main disadvantage on the use of phosphine is the development of tolerance/resistance by several stored product insect species. Actually, many species, such as the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) (Opit et al.; 2012), the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) (Konemann et al., 2017), the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Ridley et al., 2012), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Daglish et al., 2014) and the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) (Saglam et al., 2015) have developed a

considerable level of resistance, and there are specific strains of these species that can survive concentrations that are considerably higher than the recommended application rates. Currently, resistance to phosphine is found in several parts of the world, such as Brazil (Lorini et al., 2007), USA (Gautam et al., 2016), Australia (Nayak et al., 2017), India (Kaur et al., 2015) while, for the majority of the species tested, eggs and pupae are considered the most tolerant life stages (Price and Mills, 1987; Rajendran et al., 2001; Ridley et al., 2012). However, there is still inadequate information on the evaluation of the level of tolerance to phosphine in Europe, despite the fact that phosphine is widely used in Europe.

There are different diagnostic tests have been widely used for the evaluation of phosphine resistance. The most widely accepted protocol for the evaluation of phosphine resistance is the Food and Agriculture Organization (FAO) method number 16 (Food and Agriculture Organization, 1975). This method uses a discriminating dose with a concentration based on the $LD_{99.9}$ for different stored product insects. In this concentration, the insects are exposed for 20h and after this interval, the exposed individuals are removed and mortality is recorded usually after a 14-d post-exposure period. Hence, survival after this interval is an indication of resistance in this test. A modification of FAO protocol is the Dose Response Protocol, known also as Dose Bioassay. This protocol is based on a different exposure period (3 days) in a range of concentrations. Another protocol is the one that has been developed by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) for tobacco pests, were the insects are exposed to 200-700 ppm for 4-10 days (CORESTA, 2013). Moreover, a quick test has been developed by Detia Degesch (Steuerwald et al., 2006) which is based on the evaluation of the mobility of the insects after short exposures (usually 15 min or less) to high concentrations (e.g. 3000 ppm).

Apart from the "classic" protocols for the evaluation of phosphine resistance, molecular/genetic methods have been designed, with the use of PCR and molecular markers (Chen et al., 2015; Nguyen et al., 2016). According to literature, there are two major loci, *rph1* and *rph2*, that are responsible for strong resistance. When *rph1* and *rph2* loci are individually homozygous they confer weak resistance, but when they are both homozygous they confer strong resistance (Schlipalius et al., 2002; Jagadeesan et al., 2012; Nyugen et al., 2015; Nguyen et al., 2016). The stored product insects that have been genetically characterized with this method are *R. dominica* (Schlipalius et al., 2008), *T. castaneum* (Chen et al., 2015), *S. oryzae* (Nguyen et al., 2016) and *C. ferrugineus* (Tang et al., 2017).

The present study aims in investigating the tolerance/resistance of different populations of stored product insects in Europe with different evaluation methods. Preliminary investigations were also carried out to detect the genes that are responsible for phosphine resistance in some of these populations. Knowledge of phosphine resistance in different countries in Europe will provide the inferences necessary for improving fumigations and stored product protection measures in general.

2. Materials and Methods

2.1 Populations tested

A total of 500 samples of different commodities (e.g. rice, wheat, barley, chocolate) were collected during 2016-2018 from storage and processing facilities from 14 different European countries. The insects of each sample were identified isolated and transferred to 1L glass jars with commodity to initiate rearing. All rearings have been carried out at the Laboratory of Entomology and Agricultural Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly at 25° C, 65 % r.h., and continuous darkness. In this study, we present the results of 18 different populations of stored product insects, corresponding to nine different species: the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), *S. oryzae*, *S. granarius*, *L. serricorne*, *T. castaneum* , *T. confusum*, *R. dominica* and *C. ferrugineus*. Only adults were used in the tests.

2.2 Methods for the evaluation of phosphine resistance

Resistance was tested using four different protocols. a) the FAO protocol, based on screening by exposure of the tested insects to 30 ppm for 20 hours, b) the Dose Response protocol, based on the exposure of the tested insects to phosphine to 50, 100, 200, 500, 700 and 1000 ppm for 3 days, c) the CORESTA protocol, based on the exposure of the tested insects to phosphine to 200ppm for 4 days, and d) the Detia Degesch Phosphine Tolerance Test Kit (DDPTTK), based on the exposure of insects at 3000 ppm for 90 minutes. For all protocols, after the termination of the exposure interval, the insects were classified as active, under narcosis or immobilized. Then, the exposed insects were transferred to a clear petri dish for additional 7 days, and then classification was made again. The whole procedure was repeated 9 times (three replicates of three sub-replicates).

2.2.1 FAO Protocol

Twenty (20) adults of the test species were placed in a 1.5 lt glass jar and exposed to phosphine concentrations of 30 ppm for 20 hours. After the termination of the exposure interval, active, under narcosis and immobilized insects were recorded and were transferred to a clean petri dish with food for 7 days. Then, delayed mortality or recovery were recorded.

2.2.2 Dose Response Protocol

The procedure was similar to that for the FAO protocol (including delayed mortality), while the tested adults were exposed for 3 d at 50, 100, 200, 500, 700 and 1000 ppm.

2.2.3 CORESTA

This protocol was based on the CORESTA guidelines, where 20 insects of the test species/population were placed in a 1.5 lt glass jar and exposed to 200 ppm concentration of phosphine for 4 days. After the exposure, active, immobilized and under narcosis insects were recorded, while if there were active insects, then the protocol was repeated with exposure of insects at 700 ppm for 10 days with new individuals (Guide 2 CORESTA).

2.2.4 Detia Degesch Phosphine Tolerance Test Kit (DDPTTK)

Twenty insects were placed in a syringe of 100ml and exposed to a concentration of 3000 ppm of phosphine for 5, 10, 15, 20, 25, 30, 45, 60 and 90 minutes. For strains that active insects were recorded until 90 minutes, the exposure time was extended to 270 minutes (recorded every 30 min). After each exposure interval, active, under narcosis and immobilized insects were recorded, and after the last exposure intervals, insects were transferred to clean petri dishes with food for seven days, to record the delayed mortality or recovery, as above.

2.3 Determination of the mutations that are related to phosphine resistance in *rph2* locus

For the molecular study, two populations of *T. castaneum* and three populations of *R. dominica* were used, based on earlier indications for their susceptibility to phosphine. Specific primers were designed for *T. castaneum* and *R. dominica*. A single band at ~1500-1600 bp was obtained for all the three insect species. PCR products were purified and sent for sequencing in Macrogen sequencing facility (Amsterdam, The Netherlands).

3. Results

Some levels of reduced susceptibility to phosphine, as compared with the laboratory populations, were recorded for many of the populations tested (Table 1). One hundred percent of active individuals were recorded in the population of *T. castaneum* and *C. ferrugineus*. After the exposure of 50ppm for 3 days, for one population of *C. ferrugineus* all (100 %) individuals were recorded as active. Moreover, some populations of *T. castaneum* and *R. dominica* had resistant individuals that could survive at 500 and 200ppm, respectively. Nevertheless, at the highest concentrations (700 -

1000 ppm) there was no survival for any of the populations tested. Moreover, active individuals were recorded after exposure to CORESTA protocol for only one population of *L. serricorne*. Finally, there were certain populations of different species (*S. oryzae* 3Tusc, *S. zeamais* Mach, *L. serricorne* E1, *T. castaneum* BTS and 3SP.18.1, *T. confusum* D1 and *C. ferrugineus* B1) that showed considerable percentages of active individuals after the exposure to DDPTTK.

The P45S allele of *rph2*, which is responsible for strong resistance, was detected from *T. castaneum* populations tested while P49S was detected in the case of R. *dominica*. All populations of *T. castaneum* and *R. dominica* tested are homozygous for the mutant allele, including the lab/susceptible ones, which the exception of *R. dominica* Inj which was found to be heterozygous.

Table 1: Percentage (% ± SE) of active adults of laboratory and field populations of different beetle species after the termination of a 7-d post-exposure period, following the exposure to phosphine under different protocols.

	Sample Code	Concentration of phosphine (ppm)/ exposure time (hours or days or minutes)								
		30ppm 20h	50ppm 3 days	100ppm 3 days	200 ppm 3 days	500ppm 3 days	700ppm 3 days	1000ppm 3 days	200ppm 4 days	3000ppm 90-270min
ae	3T	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	3Tusc	82.2 ± 6.8	18.5 ± 5.1	1.7 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 1.7
	lab	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
arius	3W	2.2 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
ais	Mach*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.8 ± 2.2
corne	E1*	11.7 ± 5.2	2.5 ± 1.4	5.0 ± 0.0	1.25 ± 1.25	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 1.7	32.2 ± 9.6
	lab	1.1 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
neum	BTS	29.4 ± 12.4	29.4 ± 12.4	2.8 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	90.6 ± 2.6
	3SP.18.1	100.0 ± 0.0	43.3 ± 8.4	6.7 ± 4.3	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	75.0 ± 2.9
	molab	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
usum	BPM	1.7 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	D1	0.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 0.8
	Lab	1.1 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
namensi	1W	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
inica	Inj	63.9 ± 9.5	25.7 ± 4.7	10.0 ± 2.9	1.7 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	lab	16.1 ± 8.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
rugineus	B1*	100.0 ± 0.0	100.0 ± 0.0	89.4 ± 2.6	50.0 ± 0.0	15.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	83.3 ± 3.3

* these populations had been sampled from counties outside of Europe

4. Discussion

The results presented here indicate that there are some certain survival patterns in some of the populations tested after exposure to phosphine, but very few indications of possible strong resistance (i.e. populations that had survived after 3 d at 500 ppm). In a similar screening from Morocco, Benhalima et al. (2004) noted that all samples tested were phosphine resistant according to the FAO protocol. In the current work, there were some field populations that were susceptible to phosphine, e.g. *O. surinamensis* 1W and *S. oryzae* 3T. Bell et al. (1977) underlined that FAO protocol is a successful method of identifying resistant strains, while at higher doses of phosphine for 20h, the results from that study showed also resistance to some populations. In general, the FAO protocol could be used with success as a quick diagnostic tool to indicate possible resistance, but Dose Response at higher exposures can be performed to cross-check and quantify resistance. By using a similar approach, Konemman et al. (2017) reported that phosphine resistance in *C. ferrugineus* is common in Oklahoma. Specifically, at the discriminating dose of 56.2 ppm all field populations were resistant to phosphine with frequency that ranged between 6 and 100%. Nayak et al. (2013) also reported extremely high levels of resistance for populations of *C. ferrugineus* from Australia. In our study, one *C. ferrugineus* population was able to survive at 500ppm at the Dose Response test. The

current data set indicate that some of the populations that had been sampled from non-European areas, were much less susceptible to phosphine than the ones that had been collected from Europe.

Our study initially identified the presence of the P45S and P49S mutations that are related with phosphine resistance to *T. castaneum* and *R. dominica*, respectively. Genetic studies of phosphine resistance are focused especially to four major species: *T. castaneum*, *R. dominica, S. oryzae and C. ferrugineus*, and are based on the presence of two loci, *rph1* and *rph2* that are responsible for weak and strong resistance. Most studies were focus on *rph2* locus (Schlipalius et al., 2008; Kaur et al. 2013; Chen et al. 2015). More recently, Schlipalius et al. (2018) identified *rph1* locus for *R. dominica*, *S. oryzae*, *C. ferrugineus* and *T. castaneum*. They found one orthologous gene, a cytochrome b5 fatty acid desaturase (Cyt-b5-r), to be associated with the *rph1* locus in all four species. A more thorough research on these indicators will reveal the genetic basis for the resistance of different populations, in terms of frequency patterns in Europe and elsewhere.

In this work we performed a surveillance on the presence of resistance in populations that had been sampled from Europe and some comparable populations sampled from other areas. Our results showed no evidence of strong resistance in the European populations tested, whereas the common mutations that are related for phosphine resistance were identified for *T. castaneum* and *R. dominica*. Finally, we found that different protocols for the evaluation of resistance to phosphine, although they often provide dissimilar results, are comparable and could be revisited on the basis of designing a novel standardized protocol, which can be adopted further in laboratory trials and "real world" applications.

Acknowledgment

This work was partially supported by the General Secretariat for Research and Technology (GSRT) and the Hellenic Foundation for Research and Innovation (ELIDEK/HFRI) in the context of the action «1st Proclamation of Scholarships from ELIDEK for PhD Candidates» (Scholarship Code: 891).

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Potential for using pheromone trapping and molecular screening in phosphine resistance research

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DOI 10.5073/jka.2018.463.221

Abstract

Phosphine resistance monitoring typically involves bioassays of beetles from population samples collected from grain storage facilities. Insects are classified into susceptible or resistant phenotypes based on mortality or survival at one or more discriminating doses. Although valuable, phenotype testing has several drawbacks. First, phenotype testing needs live insects, and considerable effort is required to collect and maintain them before testing. Second, population samples may contain multiple genotypes expressing different levels of resistance that may not be distinguishable using discriminating dose bioassays. Third, collections are likely to be focussed around grain storages to maximise sampling success. Recent research shows that several key pests are actively dispersing through flight. The availability of commercial pheromone lures and recent advances in molecular screening provide an opportunity to provide information on resistance gene frequencies more broadly across the landscape. This approach is proving to be a valuable adjunct to traditional resistance testing in Australia.

Keywords: pheromones, DNA markers, traps**,** phosphine resistance, allele frequency