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Reduction of fungi and mycotoxin decontamination by ozone gas treatment in three stored rice (*Oryza sativa* L.) varieties

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Abstract

The present work brings together different rice varieties (black, brown and white) evaluated for their differences/susceptibilities/resistance to ozone (O₃) gas treatment for safer storage (mycological and toxicological contamination control). The three rice varieties were separated into two Groups –Control (GC) and treated Groups (GT) which had O₃ gas applied (5 L/min, 40 ppm and 60 min for gas flow). Samples were collected during the storage period to check for the O₃ gas effect on fungi reduction (total count and fungi genera identification) and so for the humidity parameters of moisture content (mc) and water activity (aw). It was possible to verify the effectiveness of the O₃ application in the samples when compared to Control. It was observed that even at the shortest time of gas exposure, O₃ application caused changes to fungi (both growth speed & toxin formation). The grains did not change their organoleptic, physical and biochemical characteristics after O₃ application. Recent studies from our Labmico Group indicated that the O₃ application in addition to prevention of the biological contaminants, as reported in the current work, also reduces an insecticide (deltamethrin) residues. As O₃ treated grain has reduced fungi contamination and toxicity of rice grains in all the varieties studied, it can be considered a potential agent to control fungi spoilage and so for toxigenic strains. Considering that there is a growing concern on the use of agrochemicals and their harmful effects on human health and the environment, O₃ application can be a promising to implement decontamination of highly consumed grains worldwide, such as rice.

Keywords: rice, fungi, storage, ozone, mycotoxins.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most consumed cereals in the world, with 90% of its production coming from Asian countries. Outside of Asia, Brazil is the greatest producer of rice. Asian countries' rice consumption varies from 100 to 150 kg/person/year (Sosbai, 2014). There are varieties that express different nutritional characteristics having impacts on the human health. Several consumers around the world prefer rice with translucent appearance, with intact and uniform grains (Castro, et al, 1999). In relation to the grain type, the market indicates a rice consumption migration from type 2 to 1 and parboiled rice in Brazil (Botelho et al, 2010). However, new trends in the food market favor the consumption of other varieties, since the increasingly consumer demand for food-health integration (Aziz et al, 2002).

Due to the demand for quality, it is important to emphasize the post-harvest insect protection management control and what they carry during storage: toxigenic fungi (Hoeltz, 2005; Soares et al, 2018). The presence and action of fungi affect the grains physical structures, compromising their quality & safety with deterioration, loss of nutrients and toxins production (Kreibich et al, 2016). Mycotoxins are secondary metabolites, which are toxic substances able to affect humans and animals, and may be mutagenic, teratogenic or carcinogenic effects (Hoeltz, 2009).

Food contamination can pose risks to consumer health. Grains, such as rice are affected greatly by the presence of fungi and mycotoxins (Hining, 2011). In the field, contamination is influenced by environmental conditions such as air humidity, incomplete drying, product humidity, rainfall at harvest time, insects, soil fungus loads, air and plant health (Fonseca, 2008). The constant movement of insects within an ecosystem contributes to the dispersion of fungal spores, which are carried on the body surface (Bidochka 1997; Saint, 1984; Soares et al, 2018). Water (coming from inside the food or even from the external environment), is the medium that favors microorganisms growth (Pitt, 2009). The main pests present in the grains during storage are beetles and moths. Among the beetles are *Rhyzopertha dominica* F. and *Sitophilus oryzae* L. are some of the most damaging species in rice. Regarding moths, the main ones are *Sitotroga cerealella* and *Ephestia kuehniella* (Lorini, 2010).

Under favorable conditions, fungi develop rapidly during the cultivation, harvesting, transport and storage processes. However, the storage corresponds the main stage in which the grain is susceptible to those types of contamination. (Scussel, 2018). Among the mycotoxins that most affect rice cultivars are aflatoxins, ochratoxin A, zearalenone, citrinin and fumonisins and their main toxigenic fungi are the *Aspergillus*, *Penicillium* and *Fusarium* species, and the same fungus can produce several different toxins (Aziz and Moussa, 2002).

There are several methods of storage to prevent/control the fungi proliferation, among them temperature reduction and also technologies known as green methods, such as the application of ozone (O₃) gas (Kim and Dave, 1999). O₃ is a colorless gas with a pungent odor, unstable and partly water-soluble, and has high oxidizing power. Within 15 min in contact with the air, it ends up oxidizing and turning O₂. It does not generate residues and is a strong disinfectant agent with action on a great variety of pathogenic organisms, including bacteria, viruses and protozoa. (Botelho da Silva, et al, 2011; Savi et al, 2015). In addition, it is internationally recognized as a GRAS - generally recognized as safe (Piacentini, 2015; Christ et. al, 2016, 2017).

This work evaluated the susceptibility of three rice varieties to fungi and mycotoxins decontamination by O₃ gas.

2. Materials and Methods

2.1 Materials

Samples: Three rice varieties of (a.1) black, (a.2) brown and (a.3) white with initial mc: 14.41, 13.86 and 13.61%, and aw: 0.060, 0.580 and 0.520, respectively).

Culture media and reagents: Potato dextrose agar (PDA) from Himedia (Curitiba, Parana, Brazil) and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil).

Equipment: autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); tweezers, Prolab (São Paulo, SP, Brazil); caliper, Digimatic (Mitutoyo, Tokyo, Japan); drying oven, Olidef-cz (Ribeirão Preto, SP, Brazil); aw meter, Aqua-Lab4TE, Decagon (Sao Jose dos Campos, SP, Brazil), Peagameter, Model Schott-gerate CG 818 (Schott, Mainz, Germany); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); rotary shaker, Marconi (Piracicaba, SP, Brazil) and microbiological incubator, Quimis (Diadema, SP, Brazil); colonies counter, Phoenix (Araraquara, SP, Brazil); sieve system, mesh (2-1mm) Beffer (Caieiras, SP, Brazil); Microscopes - light (LM), CH-BI45-2, Olympus (Shinjuku, Tokyo, Japan); O₃ gas generator, OP-35-5L, Interzone (Jundiaí, SP, Brazil), thermohigrometer, J-prolab (São José dos Pinhais, PR, Brazil), stereo microscope (SM), Opticam (SP, Brazil).

2.2 Methods

Sample collection: rice varieties were collected (1 kg) in October, 2017, by the Vegetal sanitary defense of Santa Catarina Integrated Development Agricultural company.

O₃ application: The storage silos were produced with polyvinyl chloride tubes containing only two openings: one for the O₃ gas inlet and one for the O₃ gas outlet (25 X 10 cm diameter capacity). The three rice varieties were separated into two Groups –Control (GC) and treated Groups (GT). After grains (300 g) of each rice variety were loaded (50 g) into the O₃ chambers, the generated O₃ gas was pumped (through the inlet entrance) into the vessels by a compressor (equipped with a filter to prevent the entry of moisture), at continuous flow rate (5 L min⁻¹) (Savi et al, 2016). The gas concentration applied was of 40 ppm for 60 min (Soares et al, 2018). At the end of the O₃ gas exposure its effect on fungi growths (GT) was evaluated and compared to the GC.

Humidity: MC and aw measurement were prior and after O₃ treatment. Each sample (2 g) was submitted (n=2) to drying in oven (105°C+/-5°C) up to constant weight by the gravimetric method of AOAC (2005). To determine a_w, each sample (2 g) was subjected to analysis (n = 2) using the Aqua Lab equipment, 25°C (AOAC 2005).

Mycology: The GT (O₃) and GC (no O₃ treated) rice grains were incubated (5 grains each) on PDA culture medium at 25°C+/-1 for 7 days. At day 3 and 5 after incubation, the colonies were observed and had their genera identified. After 7 days of incubation, the most representative fungi colony of each plate was evaluated for genus identification, both by analyzing their reverse under ultra violet light and analysis of their hyphae and conidia by SM (x60 and x100) (Ganley, 2006). Identification of fungi: The genera and the species identifications were performed according to Pitt and Hocking (2009). The colony morphology was evaluated by SM analyzes (Scussel et al, 2014).

3. Results

Humidity and fungi: It is known that the mc and a_w are important humidity factors for the development of fungi. Table 1 shows very close values among the varieties evaluated. The variety with the highest mc value had also the highest a_w value. Data also reveals that among the three varieties studied, the difference between nutrient availability and other factors in grain composition stands out in relation to a_w difference, since the grain (White) with lower mc (13.61%) & a_w (0.520) is the one that presents more different colonies and also more toxigenic colonies based on the fluorescence in its reverse and genera identification.

The incubated grains did not have their surfaces sterilized, thus providing favorable conditions for epiphytic fungi. These fungi may have a mutualistic relationship (absorbing nutrients and providing defense to the grain) or commensal, where it only removes nutrients (da Silva, 2006). Germination ceased at the time the colonies began to expand through the Petri dish.

Tab. 1 Humidity, fungi genera isolated fluorescence and from different rice (*Oryza sativa*) varieties

Rice Variety	Humidity		Predominant Fungi	
	mc	a _w	Genera	Fluorescence
Black	14.41	0.608	<i>Aspergillus</i>	✓
Brown	13.86	0.580	<i>Mucor</i>	ND
White	13.61	0.520	<i>Penicillium</i>	✓

mc: moisture content; a_w:water activity; ND: not detected; ✓: presence

Fungi and ozone: From the rice samples a.1 (black), a.2 (brown) and a.3 (white) of the image (Fig. 1), that were submitted to O₃ application, it was possible to observe a reduction of fungi colonies formation on the grains (GT) when compared to Control (GC).

Mycoflora: By applying stereo microscopy, the reproductive structures of the fungi isolated from rice husk (tegument) surface and endosperm regions it was possible to visualize and identify different stages of reproductive structures of *Aspergillus* genera and also the species identified (*A. niger*) with their characteristic black color (Fig. 2).

Mycotoxins: In addition to the bromatological changes, the development of fungi can harm animal health and the people/workers handling the husk, due to the production of toxins, especially those related to the toxigenic fungi of the genus *Aspergillus*.

From the white rice strains isolated, where toxigenicity tests were applied, after 7 days incubation of the colonies isolated, it was possible to detect fluorescence production at the reverse of the culture medium under UV light - 365nm. That indicates possible presence of mycotoxins (aflatoxins) through the fluorescence compounds produced (Fig. 3).

Proximate composition: From the three rice samples evaluated, the Black rice had the highest lipid and protein contents (3 and 9.8 %) (Tab.2).

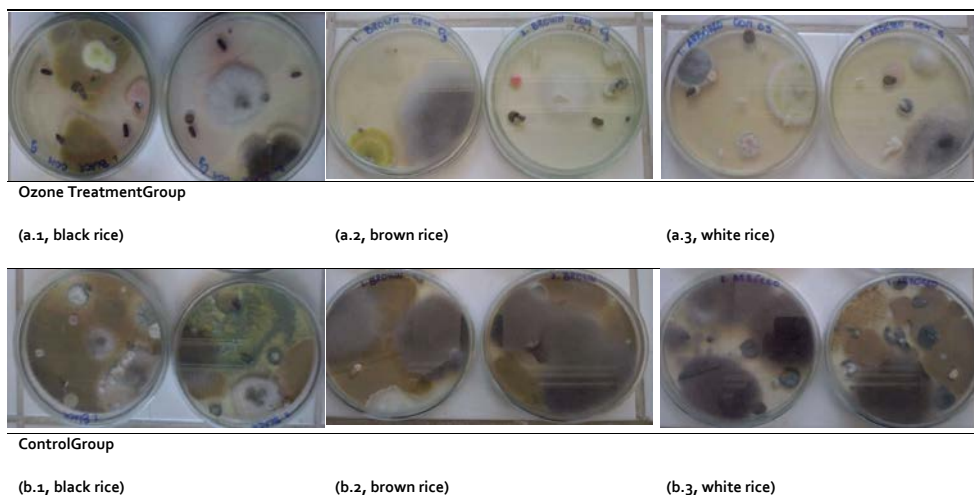


Fig.1 Fungi susceptibility to O₃ gas from three different rice (*Oryza sativa*) varieties: (a) ozone gas treated group and (b) control group.

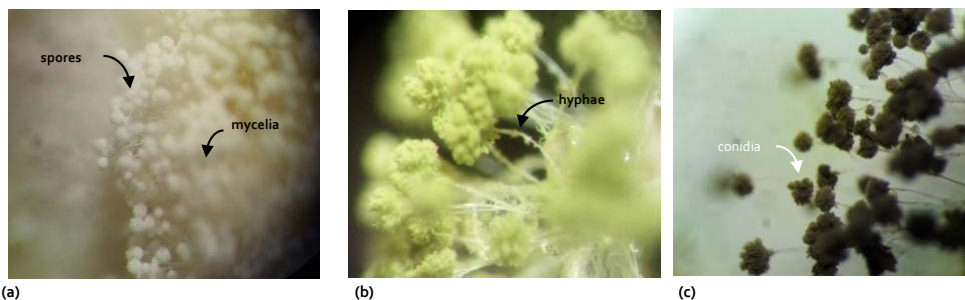


Fig.2 Stereo micrographs of isolated fungi from rice (*Oryza sativa*) grains varieties (a, b) reproductive structures of *Aspergillus* and (c) species identified of *A. niger* [40, 100and 60x, respectively].

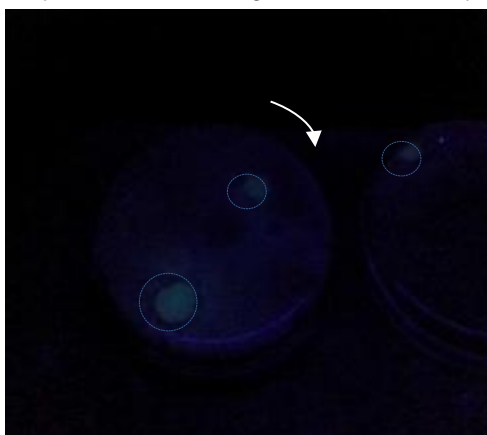





Fig.3 Fungi colonies fluorescence formation seen under ultraviolet light (λ : 365nm) of white rice (*Oryza sativa*) not ozone treated after 7 days of incubation (GC: no gas treatment).

Tab.2 Proximate composition of rice (*Oryza sativa*), varieties -black, brown and white.

Nutritional values*	Proximate composition (per 100 g)	Rice characteristics
BLACK		
Fat	3	
Carbohydrates	67.9	
Fiber	4.2	
Protein	9.8	
Salt	0.002	
BROWN		
Fat	1.9	
Carbohydrates	77.5	
Fiber	4.8	
Protein	7.3	
Salt	ND	
WHITE		
Fat	1.3	
Carbohydrates	77	
Fiber	1.9	
Protein	6.9	
Salt	0.002	

*average ND: not detected

4. Discussion

Rice composition versus fungi: Regarding main composition rice presents the highest percentage of starch, followed by proteins and small amounts of lipids and fibers. In addition, the nutrients are not uniformly distributed in the different fractions of the grain. The (a) outer layers have higher concentrations of proteins, lipids, fibers and vitamins, while (b) the center is starch (Walter, 2008), so non-polishing causes the whole rice grain to have its starch mass protected by a layer of nutrients. Fungi have affinity for media with high concentration of starch and so the grains (Lorini, 2018). This explains the fact that black rice has been the least affected by fungi, its amount of vitamins and other components are higher than that of other varieties, likewise, white rice is polished and therefore facilitates access to fungi, which feed on this starch. After a systematic investigation of rice straw (Chen et al, 2015) verified that the mixed culture of *Trichoderma viride* and *A. niger* had a greater capacity of biodegradation when compared with the pure strains of *T. viride* and *A. niger*.

Ozone gas fungi development control: According to Botelho da Silva (2011), the application of O₃ is efficient however does not rule out the need for good storage conditions. It is noteworthy that due to the complexity of the processing and storage of these grains, O₃ does not dispense need to store under the proper humidity and temperature conditions. In addition, it was realized that even at short O₃ exposure time reduced fungi (only fewer colonies) during the incubation time.

Beber et al, 2015 reported a high reduction in total fungi, especially *Aspergillus* and *Penicillium* fungi genera. O₃ treatment applying concentrations of (10 - 40 mg/L) in silos was shown to be an effective green strategy to reduce the contamination of rice stored in the husk, maintaining safety during storage. Apparently the grains did not have their organoleptic characteristics affected by the application of O₃. Recent studies in our laboratory indicate that the application of O₃, in addition to preventing the presence of biological contaminants as shown in this work, also reduces an insecticide (deltamethrin) residues. (Savi et al, 2015).

5. Conclusions

As O₃ reduced the toxicity and contamination of rice grains in all varieties studied, a potential agent in the treatment of fungi is shown. There is a growing concern about the use of pesticides and their harmful effects on human health and the environment. Its application brings benefits in general and much lower degrees of contamination than the pesticides themselves. Combining its application and safety / control of the amount of gas, O₃ is promising the highly-consumed grain production chain around the world, such as rice.

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Safe Storage Guidelines for Soybeans at Different Temperatures and Moisture Contents

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Abstract

Poor storage capacity of soybean makes it prone to fungal spoilage and heating during storage, resulting in lower quality. Early prediction of the fungal spoilage in stored soybeans is very difficult because fungi are often too small to be seen with the naked eye. Here a new method for fungus to early detection is adopted: it is called counting fungal spores. Soybeans with moisture contents of 11.4, 12.1, 13.0, 13.9, 14.3 and 14.7%, were held at 6 temperatures 10, 15, 20, 25, 30 and 35°C for 180d. Samples were taken at regular intervals and the fungal spores counted. The safe storage conditions (temperature, moisture content, duration) were estimated by means of a curve fitted using the power function fitting. It can predict of soybean spoilage by fungus before there is visible damage.

Keywords: soybean, storage, fungal spoilage, early prediction, spores