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Ozone as fungicide in rice grains

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Penicillium spp.
Aspergillus spp.
fungistatic activity

ABSTRACT
The fungistatic activity, especially during storage, can lead to rapid deterioration in the nutritional quality of grains, and reduce their use and disposal in industrial process due to contamination with toxins (aflatoxins and others). Among the technologies identified as promising in controlling these microorganisms, there is ozonation. The objective of this study was to determine the concentration and the saturation time of ozone gas in rice grains and set the effective ozonation disinfection time in filamentous fungi and yeast. Rice grains (14.3% w.b.) were inoculated with *Penicillium* spp. and *Aspergillus* spp. and, subsequently, ozonized at the concentration of 10.13 mg L^{-1}, under continuous flow of 1.0 L min^{-1}, in five periods of exposure (12, 24, 36, 48 and 60 h). Ozone gas concentration and saturation time in rice grains were 5.00 mg L^{-1} and 13.97 min, respectively. There was a reduction of 3.8 log cycles (100%) in the count of yeasts and complete inhibition of fungal from the genera *Aspergillus* and *Penicillium* in ozonized grains.

Palavras-chave:
Oryza sativa L.
Penicillium spp.
Aspergillus spp.
atividade fungiostática

Ozônio como agente fungicida em grãos de arroz

RESUMO
A atividade fungiostática pode, principalmente durante o armazenamento, levar à rápida deterioração na qualidade nutricional dos grãos e reduzir seu aproveitamento e inutilização em processo industrial pela contaminação com toxinas (aflatoxinas entre outras). Dentre as tecnologias apontadas como promissoras no controle desses microrganismos se destaca a ozonização. Objetivou-se, com este estudo, determinar a concentração e o tempo de saturação do gás ozônio em grãos de arroz e definir o tempo de ozonização eficaz na desinfecção de fungos e leveduras. Grãos de arroz (14,3% b.u.) foram inoculados com *Penicillium* spp. *e Aspergillus* spp. e posteriormente sua ozonização foi realizada na concentração de 10,13 mg L^{-1}, em fluxo contínuo de 1,0 L min^{-1}, em cinco períodos de exposição (12, 24, 36, 48 e 60 h). A concentração e o tempo de saturação do gás ozônio nos grãos de arroz foram de 5 mg L^{-1} e 13,97 min, respectivamente. Observou-se redução em 3,8 ciclos log (100%) na contagem de leveduras e completa inibição para os fungos dos gêneros *Aspergillus* e *Penicillium* nos grãos ozonizados.

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Introduction

Rice is one of the most important grains in terms of economic value. It is considered as one of the foods with the best nutritional balance, supplying 20% of the energy and 15% of the proteins, per capita, necessary for human. For being an extremely versatile crop, it has greater potential to combat hunger in the world (Brondani et al., 2006).

In Brazil, rice production in the season of 2013/14 was equal to 12,161.7 thousand tons, 2.9% higher in relation to the 2012/13 season. Such production increase was mainly due to the expansion of area caused by the high levels of the prices of the product (CONAB, 2014).

According to FAO (2014), the expected global use of rice in 2013/14 will be 492.1 million tons, 3.0% more than in the previous year. Other uses, including seeds, post-harvest, losses and non-alimentary industrial uses, may reach approximately 67.8 million tons. As a result, the consumption of rice per capita can be above 57.4 kg (FAO, 2014).

Although much progress has been achieved in the prevention of losses in rice post-harvest, these losses reach between 15 and 16% of the production and occur during operations such as drying, storage and milling. The main reasons are the difficult access to and the lack of technical knowledge (FAO, 2004).

Since it is the final product, the damages are irreversible. Thus, the reduction in the nutritive and commercial value of the grains is mainly due to the attack of biological agents such as insects, fungi and mites (Alencar et al., 2012).

Fungi are widely distributed in nature and are common contaminants of food, grains and feed, since they constitute an adequate substrate for the development of microorganisms (Guimarães et al., 2010).

Fungal activity, especially during storage, can lead to a rapid deterioration in the nutritional quality of grains and contamination with mycotoxins (Magan & Aldred, 2007). The fungal microbiota that most affects stored rice grains is of two genera: *Penicillium* and *Aspergillus* (Pitt, 2000).

In Brazil, there are no fungicides registered by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) for the post-harvest treatment of rice grains (Brasil, 2014). Thus, when it is necessary to control post-harvest fungi, the processors and storers end up using active principles non-authorized for this purpose or even ignore the presence of pathogens, when they do not pose risks of great economic losses.

A modern and efficient strategy that has been suggested is the use of ozone gas (O_3). Ozone has been widely used in the food industry and in medicine, and is considered as a wide-spectrum antimicrobial agent, because it promotes oxidation and destruction of the cytoplasmic membrane and the cell wall of microorganisms (Cullen et al., 2010). The O_3 gas was classified by the FDA in 2001, in the United States, as a safe sanitizer for the application in food, since its degradation product is oxygen and it does not leave residues in the foods (Gabler et al., 2010).

With respect to the use of ozone for the control of fungi in food, this gas is known to be a strong antimicrobial agent and can act in the inactivation or inhibition of the development of many fungal species in various commodities. It is known that, in agricultural products, the ozone gas inhibits or delays the development of fungi from the genera *Fusarium, Geotrichum, Myrothecium* and *Mucor* (Wu et al., 2006), besides other microorganisms, such as bacteria (Aguayo et al., 2013).

The species that have already been studied and that are sensitive to ozone include the bacteria *Escherichia coli* and *Bacillus cereus* (Akbas & Ozdemir, 2008), *Listeria monocytogenes* (Sheelamary & Muthukumar, 2011) and the fungi *Alternaria* spp., *Aureobasidium* spp., *Cladosporium* spp., *Geotrichum* spp., *Mucor* spp., *Stachybotris chartarium*, *Trichoderma viride*, *Ulocladium* spp. (Hudson & Sharma, 2009), *Botrytis cinerea* (Barboni et al., 2010), *Aspergillus* spp. and *Penicillium* spp. (Brito Júnior, 2013), among others.

Additionally, it should be pointed out that ozone does not either alter the nutritional composition of cereals or form metabolites that are harmful to human and animal health (Young et al., 2006).

Therefore, this study aimed to determine the concentration and the saturation time of the ozone gas in rice grains and define the effective ozonation time for microbiological disinfection.

Material and Methods

The study was conducted in the Sector of Pre-Processing and Storing of Agricultural Products of the Department of Agricultural Engineering and at the Laboratory of Pathology of Seeds and Post-Harvest of the Department of Phytopathology, both at the Federal University of Viçosa (UFV), in Viçosa-MG, Brazil.

The experiment used grains of rice (*Oryza sativa* L.) obtained at the Minas Gerais Agriculture and Livestock Research Company (EPAMIG), with moisture content around 14.3% (w.b.).

Fungi from the genera *Aspergillus* spp. and *Penicillium* spp. were isolated from naturally infected rice grains. After the isolation and identification of these fungi, subculturing was performed in a PDA medium in order to obtain a suspension of conidia, through the deposition of distilled and sterilized water on the surface of the Petri dish containing mycelium and conidia, followed by friction of the colonies using a Drigalski spatula. After removing mycelial and conidial mass, the material was filtered in double layer of gauze; then, the filtrate was subjected to counting in an optical microscope with the aid of a Neubauer chamber to obtain a suspension of conidia with concentration of 1×10^8 conidia mL^{-1}. This solution was sprayed on the grains, which were placed on trays containing germitest paper and stored in B.O.D. chambers at 27 ± 1 °C and relative humidity of 95 ± 5% RH, conditions considered as optimal for fungal growth. The grains were maintained inside the chamber for 48 h, a period sufficient for fungal development and reproduction.

The inoculated rice grains were distributed in cylindrical containers made of PVC (15 x 25 cm) with connections for injection and exhaustion of ozone gas. At 10 cm from the base of the container, a metallic screen was placed for supporting the grains and forming a plenum.

Gas injection was performed at the concentration of 10.13 mg L^{-1} obtained by the adjustment of a voltage regulator of the ozone generator and the oxygen flow at a continuous flow of 1 L min^{-1}, in the exposure periods of 12, 24, 36, 48 and 60 h, with the purpose of evaluating the effect of the gas on the microflora and quality of rice grains.

The test was performed in three replicates for each treatment and each replicate consisted of a 500-g rice sample. The same procedure was adopted for the control treatment, which consisted in the application of atmospheric air (oxygen, nitrogen, carbon dioxide and noble gases) under the same conditions of moisture of the ozone gas.

The relative humidity (RH) of the ozone gas, after generation, was controlled by passing the gas through a saturated solution of Sodium Chloride (NaCl, 75% of RH), following the method proposed by Ozkan et al. (2011).

The ozone gas saturation time in rice grains was determined through the injection of the gas in the fumigation chambers, containing 500 g of grains each. The saturation time was defined by determining the residual gas concentration in regular time intervals and using the iodometric method until the ozone concentration became constant.

After ozonation of the rice grains, specific methods were used for each situation.

For yeast quantification, the method of counting on the plates was used. In the plating, dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were used, with results expressed in colony-forming units (CFU) g^{-1}.

In the detection of the genera *Aspergillus* and *Penicillium*, the Filter Paper Method was used ("Blotter Test") (Brasil, 2009). The blotters were arranged under white fluorescent light bulbs, in chambers with photoperiod of 12 h for 7-8 days at the temperature of 25 ± 2 °C.

Grains were individually analyzed using a magnifying glass to verify the occurrence of fructifications typical of growth of fungi from the genera *Aspergillus* and *Penicillium*. The results were expressed in percentage of occurrence of fungi.

The experiment was set in a completely randomized design with five periods of exposure to ozone (12, 24, 36, 48 and 60 h) and three replicates. The data were subjected to the Linear Response Plateau regression analysis, as a function of the period of exposure and the models were selected based on the significance of the regression coefficients, coefficient of determination (R^2) and on the analysis of the residue, using the program SAEG (UFV, Viçosa, Brasil).

Results and Discussion

The equation that describes the residual ozone concentration as a function of the period of exposure to the gas, during the process of saturation of the mass of rice grains at the concentration of 10.13 mg L^{-1}, according to the Linear Response Plateau regression analysis, with its respective coefficient of determination, is shown in Table 1. The estimation and the mentioned behavior are shown in Figure 1.

A saturation time of 13.97 min was calculated for the ozonized mass of rice grains, from which the values of residual concentration of the ozone gas remained constant. The saturation concentration was 5.00 mg L^{-1}, which corresponds to approximately 49.44% of the adopted initial concentration. Using similar procedures, Brito Júnior (2013) ozonized 2.0 kg of corn at the concentration of 2.14 mg L^{-1}, for the flow of 5.8 L min^{-1}, and obtained a saturation time of 138.56 min.

Table 1. Linear Response Plateau regression equation and its respective coefficient of determination (R^2) for the residual ozone concentration (mg L^{-1}) during the process of saturation of rice grains

Ozone concentration (mg L^{-1})	Adjusted equation	Interval	R^2
10.13	$\hat{y} = 0.3713 + 0.332\ X_i$	$0 \leq X_i < 13.97$	0.94
	$\hat{y} = 5.0075$	$13.97 \leq X_i \leq 50.00$	

X_i - Period of ozonation (min); $\hat{y}$ - Residual ozone concentration (mg L^{-1})

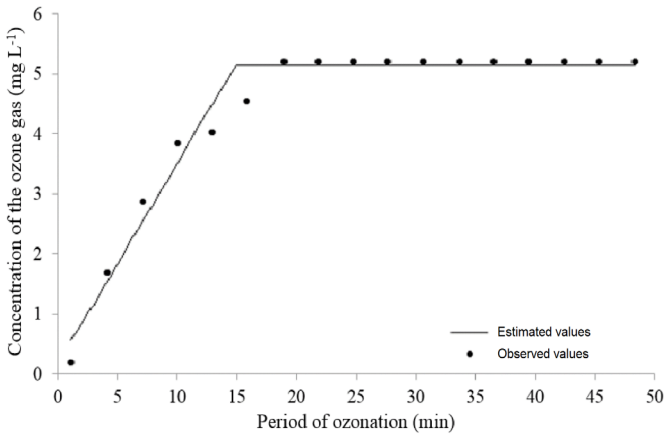

Figure 1. Residual concentration of the ozone gas (mg L^{-1}) as a function of the period of ozonation (min) during the process of saturation of rice grains, at the concentration of 10.13 mg L^{-1}

With the increase in the period of exposure of rice grains to the ozone gas, there was significant reduction ($p < 0.01$) in yeast count (YST). Significant reductions of approximately 3.8 log cycles (100%) were observed, in relation to the control, when the rice grains were subjected to the ozonation process for 60 h (Table 2). Data regression analysis allowed the adjustment of a linear equation to the yeast count of the grains exposed to ozone as a function of the period of exposure (Table 3). As the period of exposure to ozone increased, there was a decrease in

Table 2. Mean values of yeast count (log CFU g^{-1}) in rice grains subjected to atmospheric air and ozone gas

Period of exposure (h)	Yeast count (log CFU g^{-1})	
	Control	Ozone
12	3.83 a	3.29 b
24	3.82 a	2.36 b
36	3.81 a	2.23 b
48	3.82 a	1.64 b
60	3.81 a	0.00 b

Means followed by the same letter in the row do not differ at 0.01 probability level by t-test

Table 3. Linear Response Plateau regression equation adjusted to the yeast count (log CFU g^{-1}) as a function of the period of exposure to the ozone (h)

Variable	Adjusted equation	Interval
FFL	$\hat{y} = 4.2268 - 0.078\ X_i$	$12 \leq X_i < 37.66$
	$\hat{y} = 1.2924$	$37.66 \leq X_i \leq 60.00$

X_i - Period of exposure (h); $\hat{y}$ - Yeast count (log CFU g^{-1})

yeast count and, from 37.66 h on, the count remained constant (Figure 2).

In peanut grains ozonized at the concentrations of 13 and 21 mg L^{-1}, there were reductions of 2 and 3 log cycles, respectively, for the period of exposure of 96 h (Alencar et al., 2012). Likewise, Brito Júnior (2013) obtained reduction of 2 log cycles in yeast count when ozonized corn grains at an ozone concentration of 2.14 mg L^{-1}, for the period of 50 h. However, the concentration of 0.54 mg L^{-1} for a period of 100 h was not efficient at promoting significant reduction in yeast count in wheat grains (Silva, 2011).

Another point that must be taken into consideration is that there is a positive correlation between the relative humidity (RH) of the environment and ozone toxicity, i.e., the higher the RH, the higher is also the toxicity of the ozone gas (Ozkan et al., 2011). These authors observed greater effect of ozone in environment with 95% RH, i.e., low-RH environments need higher ozone concentrations for the same sanitizing effect of high-RH environments. The use of saturated solution of Sodium Chloride (NaCl, 75% of RH) in the present study may have increased the fungicide effect of ozone in rice grains. In previous tests, without the use of saturated solution, such fungicide effect was not observed.

There was significant reduction ($p < 0.01$ and $p < 0.05$, respectively) in the percentage of grains contaminated by *Penicillium* spp. and in the percentage of grains contaminated by *Aspergillus* spp., with the increase in the period of exposure. Data regression analysis allowed the adjustment of a linear equation for the index of occurrence of *Penicillium* spp. and *Aspergillus* spp. of the grains exposed to ozone as a function of the exposure period (Tables 4 and 5).

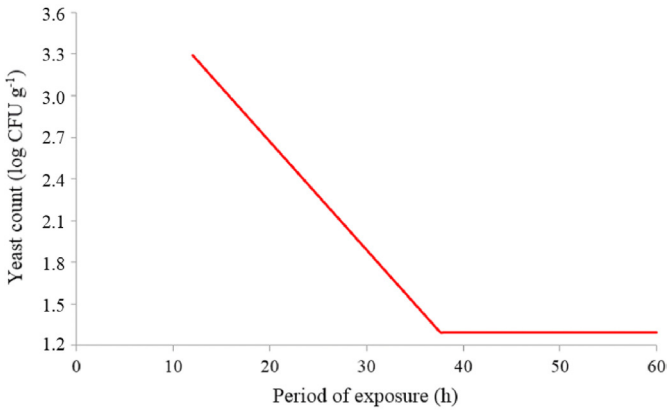

Figure 2. Estimation of yeast count (log CFU g^{-1}) in rice grains exposed to ozone as a function of the period of exposure (h)

Table 4. Mean values of the index of occurrence *Penicillium* spp. and *Aspergillus* spp. (%) in rice grains subjected to atmospheric air and ozone gas

Period of exposure (h)	*Penicillium* spp.* (%)		*Aspergillus* spp.** (%)	
	Control	Ozone	Control	Ozone
12	93.42 a	63.58 b	93.67 a	90.92 b
24	92.33 a	26.42 b	92.08 a	74.42 b
36	90.92 a	7.17 b	92.92 a	45.50 b
48	89.92 a	0.00 b	92.17 a	17.92 b
60	89.42 a	0.00 b	92.42 a	0.00 b

Means followed by the same letter in the row, for each variable, do not differ at *0.01 and **0.05 probability levels by t-test

Table 5. Linear Response Plateau regression equation adjusted to the index of occurrence of *Penicillium* spp. and *Aspergillus* spp. (%) as a function of the period of exposure to ozone (h)

Variable	Adjusted equation	Interval	R^2
Penicillium	$\hat{y} = 100.75 - 3.097\ X_i$	$12 \leq X_i < 31.76$	
	$\hat{y} = 2.3889$	$31.76 \leq X_i \leq 60.00$	
Aspergillus	$\hat{y} = 119.1667 - 2.051\ X_i$	$12 \leq X_i < 58.09$	0.9854
	$\hat{y} = 0.00$	$58.09 \leq X_i \leq 60.00$	

X_i - Period of exposure (h); $\hat{y}$ - Index of occurrence of *Penicillium* spp. and *Aspergillus* spp. (%)

With the increase in the period of exposure to ozone, there was a decrease in the percentage of grains with the presence of colonies of *Penicillium* spp. and *Aspergillus* spp. From 31.76 and 58.09 h on, respectively, the index of occurrence of these genera remained constant (Figures 3 and 4).

Comparing the mean indices of occurrence of *Penicillium* spp. and *Aspergillus* spp. (Figures 3 and 4), there was a reduction in the growth of both in ozone-treated grains. The results suggest that *Aspergillus* spp. was more tolerant to the fungicide effect of ozone in comparison to *Penicillium* spp.

It should be highlighted that the ozone gas, besides acting as an inhibitor of growth and development of *Aspergillus* spp. and *Penicilium* spp., also extended its fungicide effect to the other fungi present on the surface of the grains, such as *Rhizopus* spp. (not object of this study). This occurred due to its large antimicrobial spectrum, which makes it also efficient in the control of bacteria and protozoa (Akbas & Ozdemir, 2008; Wu et al., 2006).

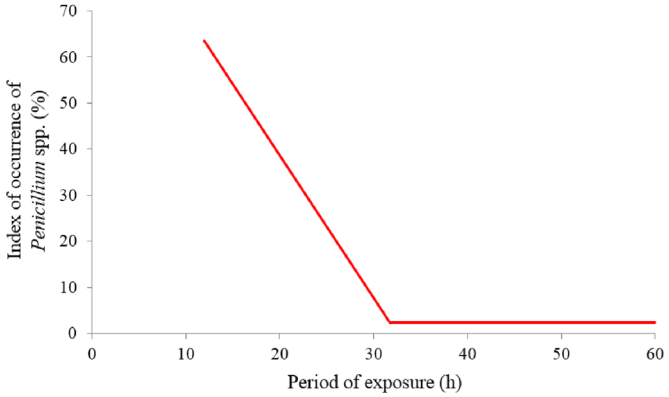

Figure 3. Estimation of the index of occurrence of *Penicillium* spp. (%) in rice grains exposed to ozone as a function of the period of exposure (h)

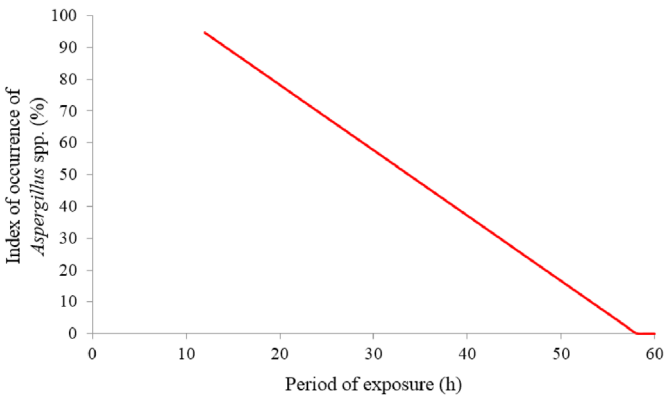

Figure 4. Estimation of the index of occurrence of *Aspergillus* spp. (%) in rice grains exposed to ozone as a function of the period of exposure (h)

Through the observation of the individual rice grains using a magnifying lens, it is possible to notice the effect of the ozonation process. In the ozonized rice grains, not all the fungal spores stopped germinating, but they produced colonies with significantly smaller sizes, sparse and with low vigor (Figure 5).

Vijayanandraj et al. (2006) observed alterations in the morphology of *Aspergillus níger*; the conidia treated with ozone at the concentration of 4.8 mg L^{-1} for 5, 10 and 15 min produced non-sporulating colonies.

Alencar et al. (2012), ozonizing peanut, reported that the percentage of peanut grains infected by *A. flavus* and *A. parasiticus* reduced significantly when they were ozonized at the concentrations of 13 and 21 mg L^{-1}, for a period of 96 h.

The inhibition of the development of microorganisms by ozone is a very complex process, since it acts on many constituents of the cell wall and membrane. The microorganisms are inactivated due to the lysis of the cell envelope, leading to the outflow of the cytoplasmic content (Cullen et al., 2010).

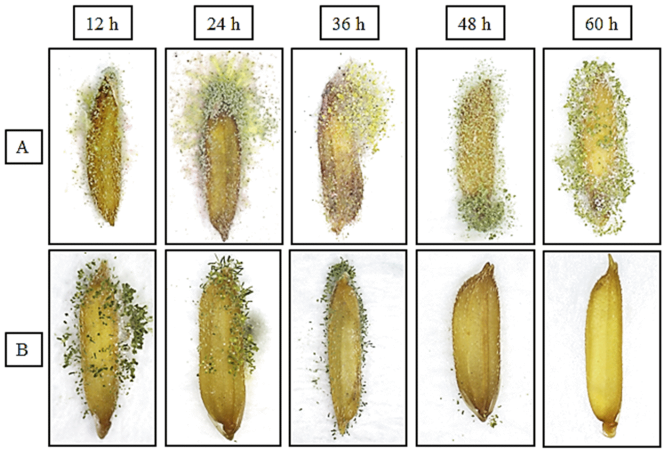

Figure 5. Rice grains observed with a stereoscopic microscope subjected to atmospheric air (A) and ozone gas (B) in different periods of exposure

Conclusions

1. Ozone gas concentration and saturation time in rice grains were 5.00 mg L^{-1} and 13.97 min, respectively.

2. The ozonation of rice grains at the concentration of 10.13 mg L^{-1} and 60 h of exposure to ozone reduced in 3.8 log cycles (100%) the yeast count and in 100% the index of occurrence of *Aspergillus* spp. and *Penicillium* spp.

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