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OZONE EFFECT ON FUNGI PROLIFERATION AND GENERA SUSCEPTIBILITY OF TREATED STORED DRY PADDY RICE (*Oryza sativa* L.)

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ABSTRACT

This work reports the ozone (O_3) gas effects on stored dry paddy rice (*Oryza sativa* L.) mycoflora (fungi reduction/genera susceptibility/yeast) and humidity microorganism proliferation factors (moisture content [mc]/water activity [a_w]. From the three O_3 concentrations applied (10, 20 and 40 mg/L – groups I, II and III, respectively) on silos stored rice ($n = 10$), it was observed a rather high reduction on the total fungi load especially in group III from an initial count of 3.0×10^5 down to 1.4×10^2 cfu/mL. Regarding the samples naturally contaminated fungi genera isolated, they were *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria* and *Aureobasidium* (a yeast-like fungi), as well as yeast, which were reduced after O_3 treatment. Despite this, some strain gas susceptibility differences were observed as follows (decreasing order): *Acremonium* > *Alternaria* > *Aureobasidium* > *Aspergillus* > *Penicillium*. As expected, mc and a_w reduced after gas treatment, which was proportional to the time of exposure and O_3 gas stream flow rate applied on the rice stored samples. O_3 treatment showed to be an effective green alternative tool to reduce paddy stored rice contamination and keep safety during storage.

PRACTICAL APPLICATIONS

The O_3 gas treatment showed to be an effective green alternative tool to avoid paddy stored rice fungi growth, especially toxigenic species responsible for mycotoxin formation in the paddy rice, and to keep safety during storage. Excess ozone is decomposed rapidly to O_2, thus no food residue is left.

INTRODUCTION

Fungal development and mycotoxin production are results of the interaction between fungi, substrate and environmental conditions, in which mycotoxin-producing fungi and their spores can contaminate crops, including rice (Moreno *et al.* 2009; Duarte *et al.* 2010; Scussel *et al.* 2012; Beber-Rodrigues and Scussel 2013). Because the factors that contribute to fungi proliferation include environmental and ecological conditions that often are beyond human control, world food contamination by fungi represents a significant problem (Hussein and Brasel 2001; Scussel 2005). Thus, studies on developing methods for fungi decontamination in order to reduce its impact on grains and their by-products are necessary. In that sense, modified atmospheres have been applied, including carbon dioxide, nitrogen, oxygen and, more recently, ozone (Scussel *et al.* 2011a; Giordano *et al.* 2012; Savi *et al.* 2014a,b).

One of the important applications of ozone in agriculture is the postharvest treatment of crops (Zorlugenic *et al.* 2008), reducing or eliminating undesirable mycoflora from grains and their by-products (Tiwari *et al.* 2010). Ozone acts through a progressive oxidation of vital cellular components on destroying microorganisms preventing the microbial growth, thus extending the shelf life of several foods (Guzel-Seydim *et al.* 2004; Aguayo *et al.* 2006).

This gas is a powerful sanitizer oxidant recognized since 1997 as a GRAS (generally recognized as safe) substance by the Food and Drug Administration and has been used in a number of applications in the food industry for destruction

or detoxification of chemicals or bacteria (United States Food and Drug Administration [FDA] 1982; Food and Agriculture Organization of the United Nations [FAO] 1996). These applications include the surface decontamination, food storage, and preservation, as well as packaging sterilization (Desvignes *et al.* 2008; Cárdenas *et al.* 2011).

Although commercial ozone application for grain management is not well documented, there are numerous studies that describe the potential benefits of that technology (McDonough *et al.* 2011), especially in wheat, barley, corn and Brazil nuts (Kells *et al.* 2001; Allen *et al.* 2003; Kottapalli *et al.* 2005; Raila *et al.* 2006; Wu *et al.* 2006; McDonough *et al.* 2011; Scussel *et al.* 2011a; Giordano *et al.* 2012).

Ozone efficacy depends on several factors that include its concentration applied, the characteristics of each food, and environmental factors such as temperature and humidity. Each ozone-treated food, i.e., rice, wheat or nuts, may present different behaviors due to their physical structures that get in contact with the gas; therefore, for a better knowledge of its effectiveness detailed studies are necessary (Giordano *et al.* 2012; Savi *et al.* 2014a).

Considering that: (1) there is no study carried out on the effect of ozone gas on rice reported to date; (2) commodity is highly consumed in Latin America (mainly by the Brazilian population) extensive to several Eastern countries as staple food; (3) studies have reported stored rice losses due to fungi proliferation (deterioration/fermentation); and (4) ozone gas is reported as GRAS and inexpensive, this study was carried out to evaluate the gas effect (at three different concentrations) on stored dry paddy rice mycoflora destruction and possible water reduction.

MATERIALS AND METHODS

Rice Samples

Paddy rice (12.4 kg) grains were grown under irrigation system, stored free of impurities, dried (moisture content [mc]: 12.03%, water activity [a_w]: 0.67) and had a total fungi count of 3×10^5 cfu/mL. They were obtained from a rice silo (capacity: 25,000 tons) from Juriti Cooperative factory located in Santa Catarina State, Southern Brazil.

Chemicals and Culture Media

Sulfuric acid and sodium thiosulfate (Synth; Diadema, SP, Brazil), potassium iodine (Synth; Diadema, SP, Brazil), lactophenol dye (Sigma; St. Louis, MO, USA) , starch indicator, chloramphenicol (Sigma; St. Louis, MO, USA), peptone, malt extract agar (MEA), potato dextrose agar (PDA; (Himedia; Curitiba, PR, Brazil)).

Equipment

Thermometer and hygrometer (J. Prolab; Sao Jose dos Pinhais, PR, Brazil); vertical silos ($n = 7$), built with vinyl polychloride tubes with dimensions of 25×10 cm for height and diameter, respectively, containing an upper lid and two apertures (top and lower parts of the silos) for sample collection and ozone application, respectively; ozone gas generator model OP-35-5L (Interzone; Jundiaí, SP, Brazil), a_w meter Aqua Lab (Decagon Devices; São Jose dos Campos, SP, Brazil); flow meter 0–15 L/min (Protec; Cotia, SP, Brasil); impurities remover equipment (Ouro Peças; Alvorada, RS, Brazil); autoclave (Phoenix; Araraquara, SP, Brazil); laminar flow cabinet (Veco; Campinas, SP, Brazil); optical microscope (Olympus; Shinjuku, Tokyo, Japan); stereoscopic microscope (Carlzeiss Jena; Cambridge, United Kingdom); incubator (Quimis; Diadema, SP, Brazil); analytical (Shimadzu; Sao Paulo, SP, Brazil) and semi-analytical (Shimadzu; Sao Paulo, SP, Brazil) scales; colonies counter (Phoenix); automatic pipette, 10–100 μL capacity (Digipet; Curitiba, PR, Brazil); oven (Olidef Cz; Riberao Preto, SP, Brazil); inoxidable blender (Metvisa; Brusque, SC, Brazil).

Ozone Paddy Rice Treatment

Silos were primarily cleaned with sodium hypochloride, rinsed with distilled water and dried. Then they were loaded with paddy rice (i.e., grain in the husk) (400 g) for ozone application. Silos were divided into four groups as follows: group I (10 mg/L of ozone); group II (20 mg/L of ozone); group III (40 mg/L of ozone); and group control (no ozone treatment). Each treatment was carried out in 10 replicates ($n = 10$), except for the group control ($n = 1$).

The ozone gas was applied through the silo inlet aperture by means of a compressed air pump and an ozone generator to get the established concentration in each silo (10, 20 and 40 mg/L of ozone at a flow of 1 L/min). The ozone gas stream was kept flowing until the silo volume was fulfilled (1.6 min) and then held for 30 min (Fig. 1). After that period, rice samples were collected immediately for mycological tests and humidity (mc and a_w) analysis including the control group. The ozone concentration was measured by iodine metrical method through the ozone equipment outlet according to APHA (1999), in which the gas was bobbled into a potassium iodide solution and followed by titration with sodium thiosulfate for ozone quantification. Figure 2 shows the flowchart of the sample preparation, ozone treatments and analysis performed on the stored paddy rice studied.

Mycological and Humidity Analysis

The mycological tests (total fungi load and fungi genera identification) were performed as follows: *total fungi load*

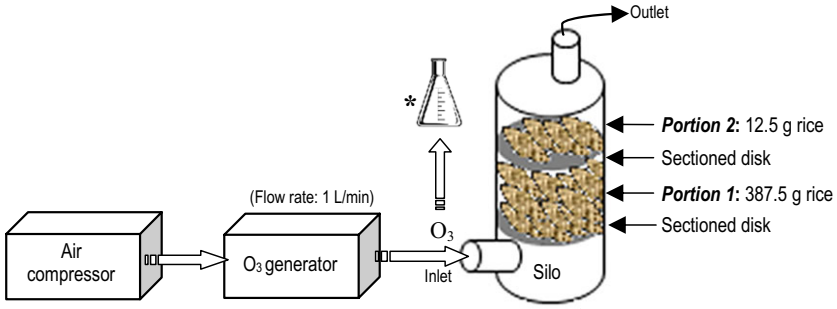


FIG. 1. FLOWCHART OF PADDY RICE O_3 GAS TREATMENT. *IODINE O_3 CONCENTRATION MEASUREMENT

through the method of Samson *et al.* (2004) in duplicate ($n = 2$) by plating serial dilutions on PDA medium with 50 ppm of chloramphenicol; *fungi genera identification* by employing micro-cultivation on MEA according to the Riddell technique described by Weber and Pitt (2000), followed by Samson *et al.*'s (2004) keys of identification; and *rice humidity determinations* in which mc was determined in triplicate ($n = 3$) by drying the sample in an oven (105 ± 5C) according to the gravimetric method of the AOAC (2005), and a_w performed ($n = 3$) using an a_w meter, which electronically obtains data.

Statistical Analysis

Data were organized on absolute relative frequencies, ranges, averages and standard deviations. In order to compare the ozone treatments, the following were used: (1) the *F*-test analysis of variance (ANOVA factor 1) regarding

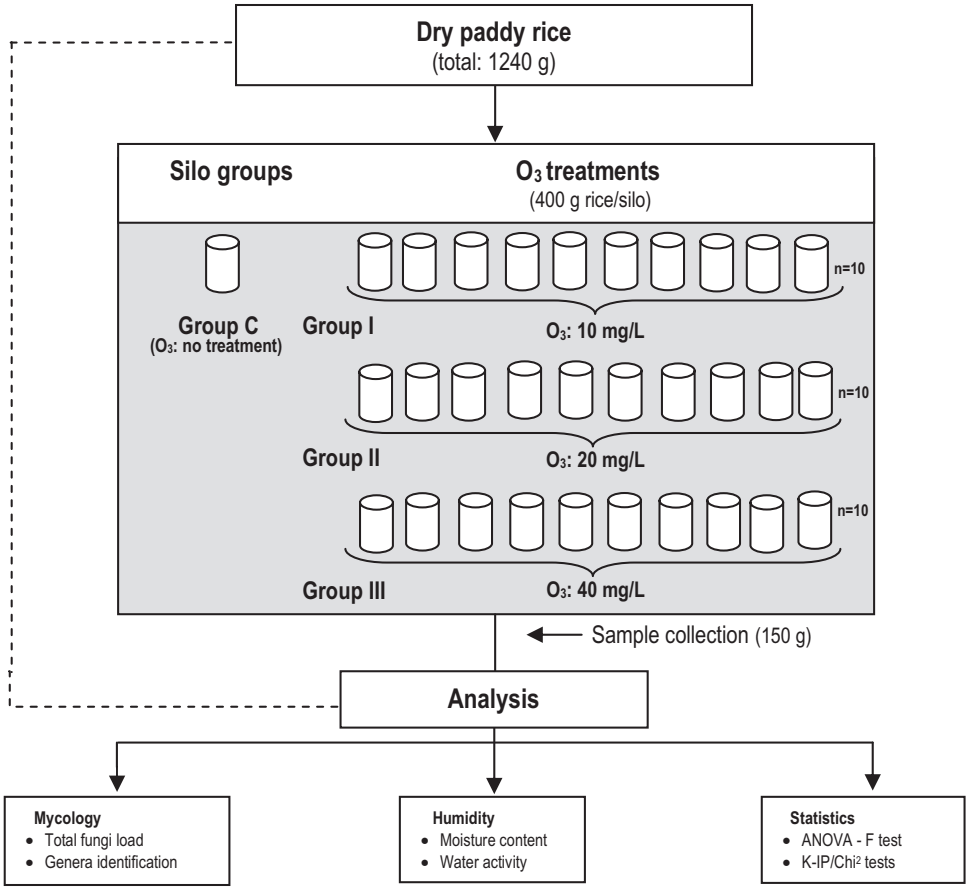


FIG. 2. FLOWCHART OF THE SAMPLE PREPARATION, OZONE TREATMENTS AND ANALYSIS PERFORMED ON THE STORED PADDY RICE STUDIED. O_3, OZONE

quantitative variables; (2) the *k-independent proportions test* regarding fungi genera proportions observed; and (3) the independent *Chi-square test* to verify differences between fungi and yeast occurrence through the ozone treatments applied. In all the tests used in the survey, results were considered significant if P value <0.05 (Box *et al.* 1978; Siegel and Castellan 1988). Furthermore, a regression equation was developed in order to verify the logarithm behavior of the total fungi load reduction after ozone treatment. For data analysis, the software Statistica version 7 (StatSoft 2004) was used.

RESULTS AND DISCUSSION

From the data obtained, it was possible to observe variations on fungi growth genera susceptibility as well as on the paddy rice ozone-treated humidity parameters.

The statistical test performed showed that the total fungi load and humidity distribution varied with the different ozone concentrations applied ($P < 0.05$), leaving to a reduction through the treatment.

The total fungi load expressed consistent reduction after each treatment as follows: for 10 mg/L a reduction of 90.40% (from 5.5 to 4.4, in log) and an increase with the ozone concentration (20 and 40 mg/L) of 99.95% (from 5.5 to 2.2 and 2.1, in log, respectively). The mc and a_w also presented significant differences among the ozone treatments; however, they had a minor reduction when compared to the total fungi load reduction (mc: from 12.03 to 11.34%; a_w: from 0.67 to 0.61) (Table 1 and Fig. 3).

This was in accordance with Tiwari *et al.* (2010) and McDonough *et al.* (2011), who reported a high reduction (3 logs) on microorganisms in cereal grains. Nevertheless, Antony-Babu and Singleton (2009) verified that the fungal inhibition caused by ozone treatment was prominent even in organisms that have been exposed to quite low levels (0.2 mg/L) of ozone during a short period of time (10 min), being able to degrade both mycelium spores of the fungi studied.

This reduction behavior verified corresponds to a logarithm regression, in which after a specific ozone concentration it has not expected further fungi load reduction. Therefore,

TABLE 1. OZONE-TREATED DRY PADDY RICE DATA REGARDING TOTAL FUNGI LOAD, HUMIDITY FACTORS, FUNGI GENERA ISOLATED AND THEIR DISTRIBUTION

Total fungi load and humidity factors

		Ozone treatment			Data		
Parameters		Group	Concentration (mg/L)	Replicates	Average ± SD (log)	Range (log)	P*
Total fungi load (cfu/mL)		Control	No ozone†	1	$3.0 \times 10^5 \pm$ NA (5.5 ± NA)	NA (NA)	0.00002
		I	10	10	$2.8 \times 10^4 \pm 8.1 \times 10^4$ (4.4 ± 4.9)	1.0×10–2.6×10^5 (1 – 5.4)	
		II	20	10	$1.6 \times 10^2 \pm 5.4 \times 10$ (2.2 ± 1.7)	9.5×10–2.5×10^2 (2 – 2.4)	
		III	40	10	$1.4 \times 10^2 \pm 7.7 \times 10$ (2.1 ± 1.9)	2.5×10–2.9×10^2 (1.4 – 2.5)	
Humidity	mc (%)	Control	No ozone	1	12.03 ± NA	NA	0.00666
		I	10	10	11.34 ± 0.3	11.01–11.85	
		II	20	10	11.68 ± 0.31	11.23–12.02	
		III	40	10	11.81 ± 0.29	11.4–12.24	
	a_w	Control	No ozone	1	0.67 ± NA	NA	0.00025
		I	10	10	0.65 ± 0.01	0.63–0.66	
		II	20	10	0.63 ± 0.02	0.61–0.66	
		III	40	10	0.61 ± 0.02	0.56–0.64	

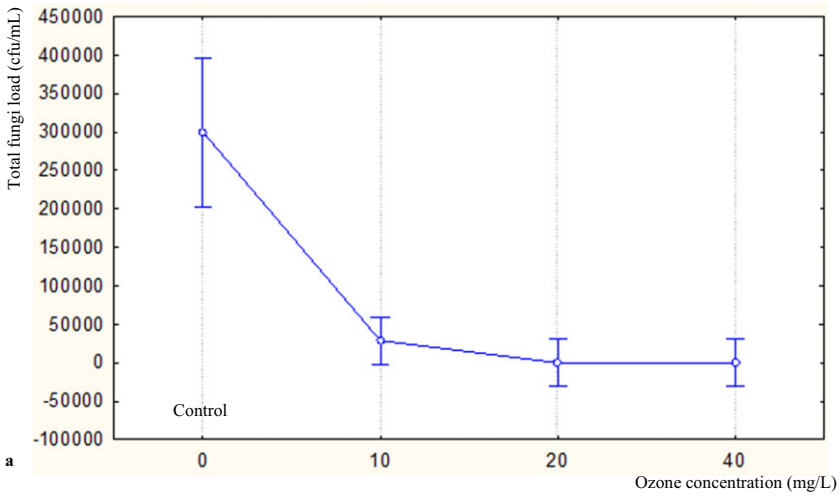
Fungi isolated and distribution

		Ozone-treated positive fungi colony detected rice samples (%)				
Fungi genera		No ozone†	Group I (10 mg/L)	Group II (20 mg/L)	Group III (40 mg/L)	P*
Storage	*Aspergillus*	10 (100)	3 (30)	6 (60)	6 (60)	<0.001
	Penicillium	10 (100)	10 (100)	6 (60)	7 (70)	<0.001
Field	*Alternaria*	10 (100)	1 (10)	1 (10)	NG (NA)	<0.001
	Acremonium	10 (100)	1 (10)	NG (NA)	NG (NA)	<0.001
Yeasts	*Aureobasidium*	10 (100)	1 (10)	1 (10)	1 (10)	<0.001
	Other yeasts	NG (NA)	NG (NA)	3 (30)	2 (20)	<0.001

* $P < 0.05$: significant differences.

† Control group.

NA, not applicable; NG, no growth.



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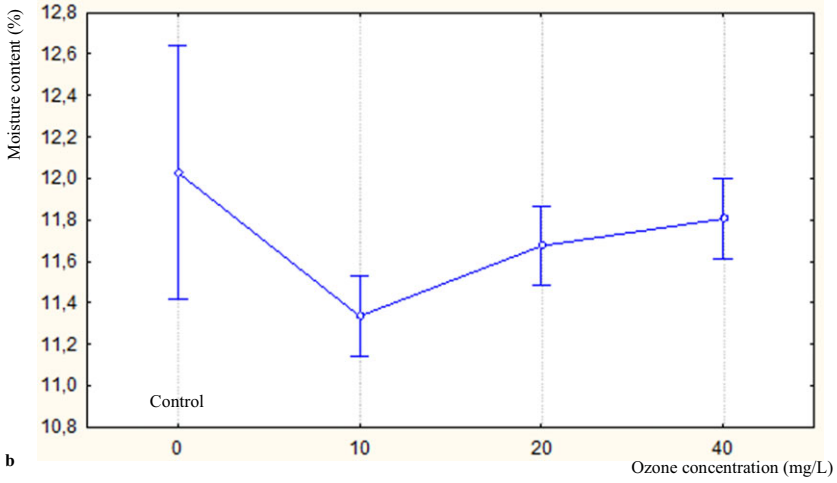

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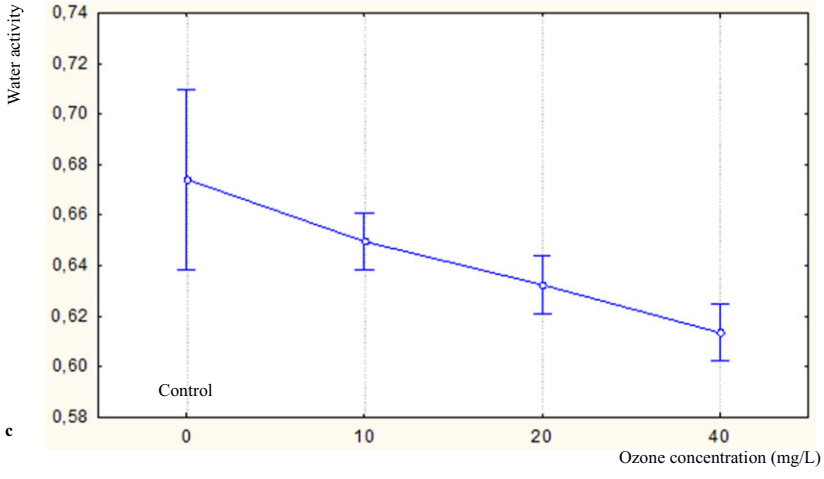

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FIG. 3. GRAPHIC REPRESENTATION OF THE RANGE AND AVERAGE RESULTS OF (a) TOTAL FUNGI LOAD, (b) MOISTURE CONTENT, AND (c) WATER ACTIVITY FOR EACH OZONE TREATMENT PERFORMED ON THE PADDY RICE (0, 10, 20 AND 40 MG/L)

this logarithm regression can be used as a tool to develop cost benefit assessments on the large scale of paddy rice ozone application.

Table 1 shows the distribution and proportion of fungi genera isolated from the control samples analyzed (not treated) in the presence of *Aspergillus*, *Penicillium*, *Acremonium*, *Alternaria* and *Aureobasidium* apart from yeasts and the variation on their total counts throughout the ozone gas treatment applied (groups I–III). Significant differences ($P < 0.05$) were observed between the fungi genera distribution and proportion for each ozone gas treatment by the ANOVA test.

Furthermore, the data suggest an ozone resistance by the genera *Aureobasidium*, *Aspergillus* and *Penicillium* as well as for yeasts, as their proportions were maintained when ozone concentration rose. Despite that, the time between the gas exposure and sample collection (which was immediately after application in the current study) should be longer to allow better ozone effect on spores (resistant structures). The occurrence of fungi and yeasts simultaneously and only yeasts for each ozone treatment was analyzed and Chi-square test indicated significant differences ($P < 0.05$) between these two events, confirming that yeasts are more resistant to ozone than fungi in the conditions applied in the present study.

Cárdenas *et al.* (2011) reported that a diversity of results has been registered in the scientific literature, depending on the ozone different concentrations applied as well as on their types of application (gas or liquid) as well as the food matrix (grain, fruits, feed). Despite this, the results obtained were in accordance to the White *et al.* (2010) findings conducted an enumeration study of ozone-treated maize and found ozone-deactivated fungi genera in the following order: *Rhizopus* > *Fusarium* > *Aspergillus* > *Mucor* > *Penicillium*. Furthermore, the same authors suggested that ozone may be more effective on certain fungal genera at different concentrations, which were observed in the current research too.

It is important to emphasize that *Fusarium* genera were not isolated from the current stored paddy rice samples, because they were already stored, the sample mc was low (12.03%), and storage conditions were not adequate/optimal for that fungi genera development. The optimal mc for field fungi including *Fusarium* is known to range from 20 to 23% (Scussel *et al.* 2011b). White *et al.* (2010) reported that *Fusarium* growth in maize at mc was as low as 16%. Nevertheless, the current paddy rice samples did not reach those mc levels. In addition, the rice stored conditions of temperature, light and aeration could also hamper *Fusarium* growth.

As far as mc and a_w data and the conditions of ozone treatments applied are concerned, although there were some differences registered, that reduction was not quite high, as the time of gas exposure and flow rate of the ozone gas stream applied were not long enough to allow a large percentage of humidity release from rice inner tissues. Other studies report treatments of foods with longer exposure to ozone, from 60 to 189 min, allowing more time for gas effect on the fungi and humidity reduction (Tiwari *et al.* 2010; Scussel *et al.* 2011a; Giordano *et al.* 2012).

The ozone gas treatment was effective in inactivation fungi growth, especially at 40 mg/L concentration, keeping safety during storage. Considering the results, ozone gas treatment could be an effective green method in the grains storage to avoid fungi growth, mainly toxigenic species responsible for mycotoxin formation in the paddy rice.

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