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Original Research Article

Antifungal Properties of Ozone Gas in Stored Naturally Contaminated Dry Maize (*Zea mays* L.) Grains

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Abstract: Antifungal properties of ozone (O_3) gas were evaluated in stored naturally contaminated dry maize grains. An experimental design was applied at three concentrations (20, 40, 60 µmol/mol) and different exposure times (30 to 180 min) for Groups I to III, respectively. O_3 gas had evaluated its antifungal efficiency at Day zero and after 30 days of storage, at the lower and upper layers of each silo. Regarding O_3 concentration, it was observed with the increase of its concentration (from 20 to 60 µmol/mol) a decrease of 2.5x10(16.2%) and 0.5x10(3.2%) CFU/g in the silo upper and the lower layers, respectively. On the other hand, regarding O_3 time of exposure, when the ozonation time increased (from 30 to 180 min) on contaminant mycoflora there was a total fungi load decrease of 1.0x10(6.5%) and 0.5x10(3.2%) CFU/g in the upper and lower layers, respectively exposed to O_3 gas. The response surface presented the maximum of 94.5% of spores inhibition. After 30 days of storage no statistical difference was observed between the applied treatments. Therefore, both treatments were effective. It was observed at Control silo (no gas treatment) at day 30th a total fungi load of 22x10 CFU/g. The O_3 treated, at day 30th had in the upper layer 1.3x10 CFU/g and in lower layer 0.4x10 CFU/g, which represents 93.8% (max 97.7%) and 98.1% (max 100%, i.e., NG= no growth) of spores inactivation in the upper layer and lower layer, respectively. In this study, spores, can be efficiently destroyed by the O_3 gas, with 88% inhibition of the spores immediately after application and 30 days after application, 100% of spores do not germinate under the conditions of 60 µmol/mol and 180 min.
Keywords: Contamination; ozonation; maize; quality, fungi.

INTRODUCTION

Maize (*Zea mays* L.) is the second most produced grain in Brazil, its economic importance is given by its different forms of use, ranging from its consumption *in natura* and/or high-tech food industries maize product (for human consumption), as well as the main ingredient for animal feeds.

The purpose of the storage is the preservation of the characteristics and quality of grain over time in order to meet the market demands. In this scenario, the storage conditions are determinant for the final quality of the product, as the mass of stored grain consists of a living system with mutual influences of physical, chemical and biological internal and external sources [1]. According to bibliography [2] if the storage conditions are not suitable for these grains, they will be susceptible to decay and exposed to fungal contamination, which is one of the most worrying factors today.

Fungal growth affects the quality, may cause visible deterioration, loss of germination, color, dry matter and unpleasant odor. It also can promote the development of toxic compounds such as mycotoxins [3-5] suggest that maize is one of the most vulnerable to the development of cereal toxigenic fungi and therefore is a major product contaminated with mycotoxins. They can be found both *in natura* maize and products. Due to extensive use of this cereal in both human and animal nutrition, apart from being one of the storage post-picking steps with fundamental influence on product quality, it is necessary to evaluate storage conditions

which enable food safety by maintaining a higher period [6].

According to bibliography [7] the lack of good agricultural practices and storage quality conditions, which are associated with high temperature and humidity (due to the tropical climate), they favor the fungi activities (spores development), which could lead to losses in the grains nutritional quality and to mycotoxin contamination as some of them are mutagenic, teratogenic and/or carcinogenic [8].

Any detoxification strategies that aim to remove those contaminant (fungi and toxins) in the earlier processing stages or food production, either still in the plant or during storages/processing, without compromising the food nutritional quality are important and necessary [9]. Several decontamination, such as heat ultraviolet (UV) radiation and microwave have been studied. However, regarding heat (sterilization) application, it can cause the development of undesirable compounds, nutrient loss, producing toxic side reactions and changes (in the physical, mechanical and optical properties) as the temperature applied can reach around 140 °C. UV radiation or microwaves, are expensive treatments, lead to food alterations and low consumer acceptance [10,11].

Therefore, there has been a growing interest on developing different procedures. Alternatives to control unwanted contaminants (living beings and toxins) in the grain storage, by applying generally recognized as safe (GRAS) methods, such as ozone (O_3) gas.

That gas has been reported being effective to control fungi growth, degrading mycotoxins and pesticide residues in a broad variety of raw and processed food, either at post-harvest or in the industry (without reducing the nutritional value) [12-28].

Therefore, the antifungal properties of O_3 atmosphere application (at different *concentrations* and *exposure times*) in maize grains naturally contaminated were investigated in order to determine its inactivation efficacy - by applying 2^2 factorial design approach.

MATERIAL AND METHODS

Sample

dry, maize grains (50 kg), naturally fungi contaminated (15.5x10 CFU/g) from year 2014/2015 harvest (mc: 11.5%).

Culture media and chemicals

(a) culture media - potato dextrose agar (PDA) and bacteriological peptone, Himedia (Curitiba, Parana, Brazil); *(b) chemicals* - Tween 80, Himedia (Curitiba, Parana, Brazil); chloramphenicol, Vetec (Duque de Caxias, RJ, Brazil); sulfuric acid, potassium iodide and sodium thiosulfate, Synth (Diadema, SP, Brazil) and lactophenol dye, Fluka (Sao Bernardo do Campo, SP, Brazil).

Equipment

Microbiological incubator, Quimis (Diadema, SP, Brazil), drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil); autoclave, Phoenix (Araraquara, SP, Brazil), microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); O_3 gas generator, model OP-35-5L Interozone (Jundiaí, SP, Brazil).

Pilot silos

Made of polyvinyl chloride - PVC (800 x 150 mm for height and width, respectively - total: 6), with two apertures for O_3 gas inlet and to exit (at the bottom and top the silos, respectively) according to Figure 1.

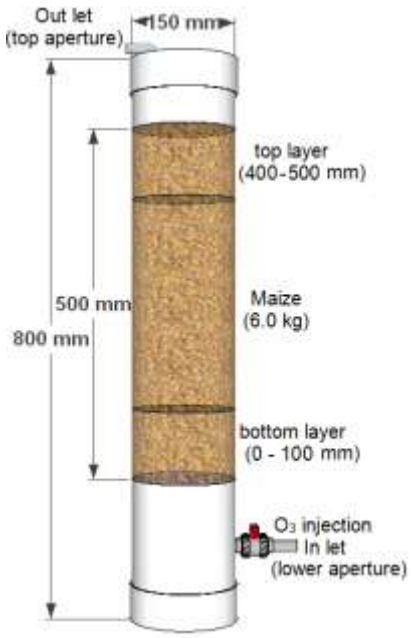

Fig-1: Silo system for maize ozone treatment details and dimensions

Silos maize loading, O_3 application and storage

The procedure was carried out according to bibliography [28] with some modification as follows: *(a) loading* - maize kernels (6.0 kg) were loaded into each silo (Groups: C – not gas treated and I, II, III - O_3 treated); *(b) O_3 application* - the gas was applied at different concentrations (20, 40 and 60 µmol/mol) and different exposure times (30, 105 and 180 min) (Figure 2). Through the silo's lower aperture (gas inlet) by means of a compressed air pump (connected to the ozonizer), room air passed first, for impurity removal (through a filter) then through the calibrated O_3 generator (Corona type) and electrical discharges were produced (between two electrodes) generating O_3. Note: the *O_3 concentrations was measured* in each silo by iodometric titration [gas bubbled through an acidified

potassium iodide solution (pH<2.0 with sulfuric acid), then titrated with sodium thiosulfate (0.005 N) using a starch solution as indicator]; *(c) storage* – as soon as gas concentrations were achieved in each silo, their lids were closed, and maize kernels were kept stored for 30 days; ***(d)*** *sample collection for analysis* - portions (25 g) of maize sample were taken ascetically for total fungi load determination and moisture content – mc (%) analysis. That was carried out prior and just after the gas application (Day zero) and so at the 30th of storage, both at the *upper* (zero to 100 mm) and *lower* (400 to 500 mm) layers of each silo.

Experiment design

The 2^2 factorial design, reported by bibliography [28], was applied to the current study. Briefly, the ***(a)*** *influence* of the factors - O_3 gas *concentration* (O_3) at 20, 40 and 60 µmol/mol and ozonation *time (t)* at 30, 105 and 180 min on the ***(b)*** *efficiency* of *fungi inactivation* and *humidity* possible variation. They were checked immediately (Day zero) and after 30 days of storage (at the *bottom* layer and *top* layer). Equation 1 below (Eq.1) show the model utilized.

$$\textbf{[Eq.1]} \quad \hat{y} = b_0 + b_i X_i + b_j X_j + b_{ij} X_i X_j$$

b_0: mean/intercept

b_i, b_j, b_{ij}: the model of regression coefficients

X_i (O_3 concentration) and X_j (exposure time): independent factors evaluated in coded values.

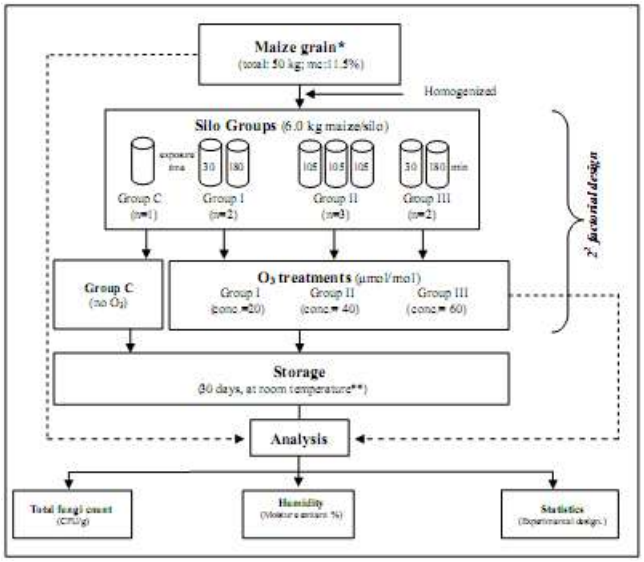

Fig-2: Flowchart of the whole dry maize grains (*naturally fungi contaminated) ozone (O_3) gas treated procedure ([28] – modified) **22 °C (range: 20-24)

Maize analysis

Samples were analyzed for ***(a)*** *total fungi load* - the percentage of fungi growth (a variable that needed to be determined in each sample by microbial load count) was obtained through dilution plating followed by counting colony forming units (CFU/g), to measure the effect of O_3 on fungal population's. After 3 days of incubation, colonies were counted giving CFU/g values. The numbers of CFUs were counted prior and after O_3 treatment, and ***(b)*** *humidity* - mc was determined by the AOAC gravimetric method 31.1.02 [29].

Statistical analysis

Data obtained were analyzed for the main effects and the variables interactions on responses. Thus determining which were the significant factors ($p < 0.1$) and adjusting a model (Eq.1) to correlate variables and their responses. The significant coefficients of the model were evaluated by the "t" test, and the data were subjected to an analysis of variance (ANOVA), to verify the statistical validity and predictive ability of the models obtained for the answers.

RESULTS AND DISCUSSION

From the data obtained on maize kernel O_3 treated to destroy fungi spores, it was possible to observe that the gas was able to highly reduce spores levels (inactivating them). However, that was dependent on the concentration and time of exposure applied, it also showed not affecting the mc. Table 1 and Figures 3-5 show some of the O_3 effect data registered.

Effect of O_3 treatments on fungi counts

The effect of O_3 on fungi maize kernel spores on (Groups I, II and III), evaluated at Day zero and after 30 days of storage (*bottom* layer - zero to 100 mm and *top* layer -400 to 500 mm) together with humidity (mc) studied under the factorial designed (2^2) applied are shown in Table 1 and Figures 3.a,b and 4.a,b and the

results compared to Control Group (maize without O_3 treatment).

Day zero: upper layer (400 – 500 mm)

CFU/g reduction and Percentage of efficiency (Figure 3).

(a) CFU/g reduction: the effect of O_3 concentration and exposure time (Day zero) in the fungi spore inactivation (CFU/g) are shown in Figure 3.a. Data were considered significant for the "t" test. Increasing the concentration of O_3 from 20 to 60 µmol/mol ensure a increase fungi spores destruction of 2.5x10 CFU/g i.e., from 15.5x10 to 4.25x10 CFU/g (low concentration) and 15.5x10 to 1.75x10 CFU/g (high concentration). Indeed when also with increasing the maize kernels ozonation time (from 30 to 180 min) there was a fungi reduction, however from 15.5x10 to 3.5x10 CFU/g (short time) and 15.5x10 to 2.5x10 CFU/g (longer time). The mathematical model (Eq.01) to estimate the total count in CFU/g depending on the O_3 concentration (b_i=-1.25), the exposure time (b_j=-0.5) and mean (b_0=2.64) was found to be predictive test by "F", with a correlation coefficient (r^2) of 0.89 and the surface response (Figure 3.a.2) represents the model.

(b) Percentage of efficiency: the effect of O_3 concentration and exposure time in the efficiency on inactivating fungi spores (%) are shown in Figure 3.b.1 (significant for the "t" test). Increasing from 20 to 60 µmol/mol the gas concentration promotes an increase of 16.1% and the increase the ozonation time (from 30 to 180 min) there was a increasing 6.5%, i.e. 93.5% compared with untreated (Group C). The mathematical model (Eq.1) for estimating the efficiency depending on the concentration of O_3 (b_i=8.0) and the exposure time (b_j=3.2) and mean (b_0=83.0) was found to be predictive test by "F", with a correlation coefficient (r^2) of 0.89. The surface response (Figure 3.b.2) represented the model.

Day zero: lower layer (zero - 100 mm)

CFU/g reduction and Percentage of efficiency (Figure 4).

(a) CFU/g reduction: the fungi spores' inactivation from the initial count (%). In the *bottom* layer (zero-100 mm), viable spores after the application O_3 was 1.35x10 CFU/g, which represents 91.2% reduction of viable spores regarding the initial count. It was not observed statistical difference between the applied treatments. The concentration of O_3 of 60 µmol/mol and time of 180 min (high concentration and longer time) ensured fungi spores destruction of 14.5x10 CFU/g from 15.5x10, i.e. 93.5% of reduction in total counts of fungi spores.

Table 1: Levels of ozone spores decontamination in dry maize kernels (*Zea mays* L.) at different conditions of gas concentration, ozonation time, layer distribution/ … and days of storage

O_3[a] treatment [b]				Total load						Humidity	
				Count [c] (x10 CFU/g)				Reduction[d](%)			
Concetration (µmol/mol)		Time (min)	Layer (mm)	Control		O_3 treatment		Storage (Day)		mc(%)	
				Zero	30th	Zero	30th	Zero	30th	Zero	30th
LOWER Layer	20[e]	30[e]	zero-10	15.5	22.0	2.0	1.0	87.1	95. 5	11.6	11.4
	60[g]	30[g]				1.0	NG	93.6	100.0	11.8	11.2
	20[e]	180[e]				1.0	NG	93.6	100.0	11.5	11.3
	60[g]	180[g]				1.0	NG	93.6	100.0	11.6	11.0
	40[f]	105[f]				2.0	0.5	87.1	97.7	11.3	10.9
	40[f]	105[f]				1.0	1.0	93.6	95. 5	11.3	11.1
	40[f]	105[f]				1.5	0.5	90.3	97.7	11.2	11.0
UPPER Layer	20[e]	30[e]	40-50	15.5	22.0	4.5	1.5	71.0	93.2	11.5	11.3
	60[g]	30[g]				2.5	2.0	83.9	90.9	11.7	11. 5
	20[e]	180[e]				4.0	1.0	74.2	95. 5	11.5	11.2
	60[g]	180[g]				1.0	0.5	93.6	97.7	11.5	11.2
	40[f]	105[f]				2.0	0.5	87.1	97.7	11.5	11.2
	40 [f]	105 [f]				2.0	1.0	87.1	95. 5	11.6	11.3
	40 [f]	105 [f]				2.5	3.0	83.9	86.3	11.4	11.2

[a]ozone [b] 2^2 factorial design [c]mean [d]efficiency mc: moisture content NG no growth ozone [e]Group I [f]Group II [g]Group III

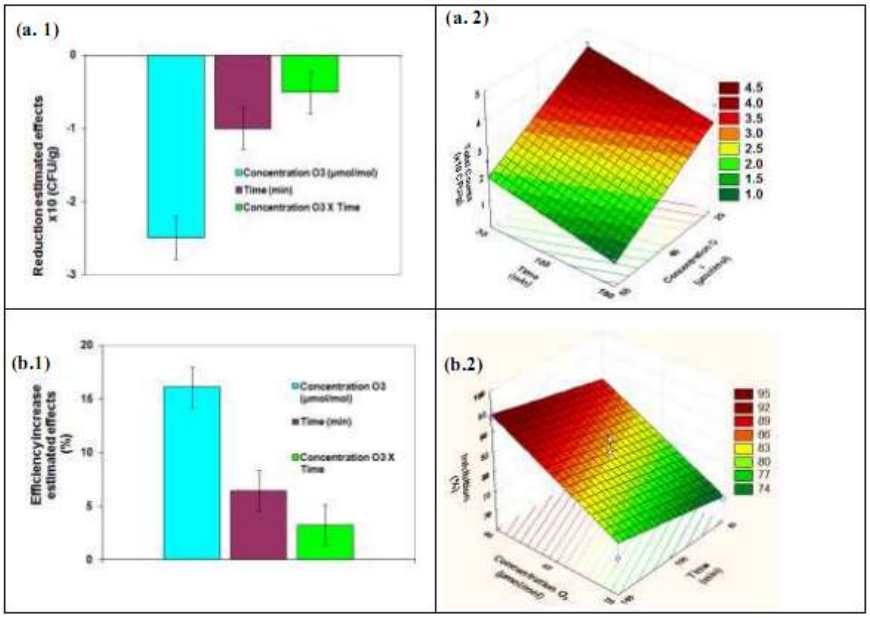

Fig-3: Effect of O_3 concentration (20, 40 and 60 µmol/mol) and time of exposure (30, 105 and 180 min) in maize on (a) total load (*CFU*) inhibition [(a.1) estimated and (a.2) response surface]; (b) total reduction (*%*) [(b.1) effect estimated and (b.2) response surface] – day zero, upper layer (40 – 50 cm)

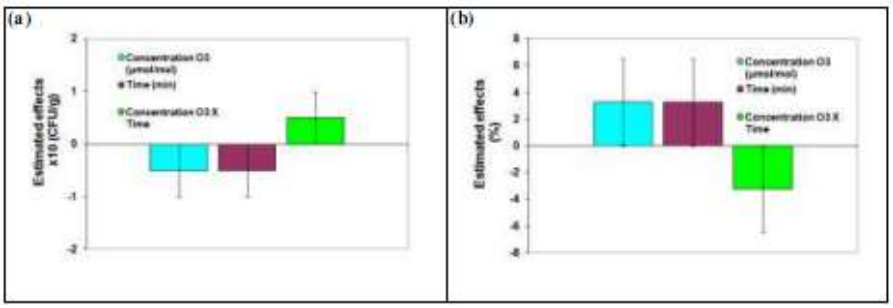

Fig-4: Effect of O_3 concentration (20, 40 and 60 µmol/mol) and time of exposure (30, 105 and 180 min) in maize on (a) effect estimated on total load (*CFU*) inhibition and (b) effect estimated on total reduction (*%*) – day zero, lower layer (zero – 10 cm)

Day 30th: lower and upper layers (latent effect)

(a) CFU/g reduction and percentage of efficiency: the latent effect of O_3 application in maize is shown by the total fungi count after storage 30 days (Figure 5). It was not observed statistical difference between the applied treatments. Both treatments were effective. It is observed (in Control Group), in the day 30th 22x10 CFU/g. Treated with O_3, at day 30th was observed in *upperpper* layer 1.3x10 CFU/g and in *lower* layer was observed 0.4x10 CFU/g, represents 93.8% (max 97.7%) and 98.1% (max 100%) of inactivation of spores in the *top* and *bottom* layers, respectively.

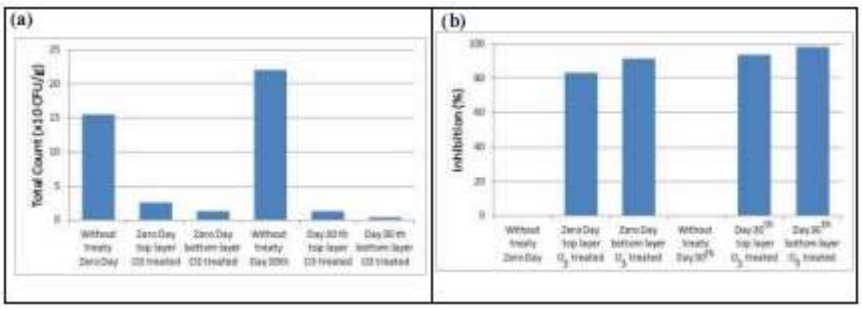

Fig-5: Effect 30th Day (a) Total count (CFU / g) (b) inhibition efficiency of fungi spores. Effect latent (day 30th) of O_3 treatment (20, 40 and 60 µmol/mol) and time of exposure (30, 105 and 180 min) in maize on (a) total count (CFU/g), (b) inhibition efficiency (%) of fungi spores

Studies by several authors have demonstrated the economic viability of O_3 application to fumigate stored grain, supporting its use as a viable alternative for both environmental and economic perspectives [15]. Reductions as high as 10^3 CFU/g of microorganisms associated with stored grains were achieved with O_3 treatment, as well as significant reductions in the levels of mycotoxin [30]. Moreover, investigations of grain treated with O_3 indicate that it has no impact on the intrinsic quality of the grain and the nuts [13,21,25,26,27,31]. Authors have been evaluating the starch oxidation, lipid peroxidation, proteins degradation, morphological tissue changes and germination and no effect, or just slight alterations, which does not interferes to its quality both, processing and nutritional. Bibliography [25] showed that O_3 gas did not change the physical-chemical characteristics at effective concentrations and exposure times compared to the contaminants (fungi and mycotoxins) in wheat grains. Bibliography [28] showed an around 90% *A. flavus* spores inhibition immediately after the maximum gas concentration and time of exposure (60 µmol/mol and 180 min) reached cocoa beans, followed by total inhibition (NG: no growth) as the time of storage increased.

Effect of O_3 gas treatment on moisture content

The mc of the maize after treatment with O_3 reached a mean of 11.5% and there were no statistical differences in mc between treatments ($p<0.1$). After 30 days of storage, maize had a mean mc of 11.2%, statistically similar for all treatments ($p<0.1$). This slight reduction in mc (0.3%), just after the O_3 treatment and 30^{th} day of storage, was probably due to the temperature and the relative humidity during that period, which established new mc equilibrium (external environment x inner silos stored grains). The fact that there was no statistical differences among the mcs at different O_3 treatments, corroborates that there was no maize mc influence on the treatments responses.

CONCLUSIONS

Fungi spores were efficiently destroyed by the O_3 gas under the conditions of 60 µmol/mol and 180 min (93.5% of spores did not germinate). The O_3 effect with the concentration increase was more pronounced than the exposure time increase for the destruction of spores fungi in Maize. It was observed evidence of latent O_3 effect on fungi spores, where observed after 30 days was 100% inhibition (NG: no growth).

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