

Efficacy of ozone in the microbiological disinfection of maize grains

Eficácia do ozônio para a desinfecção microbiológica de grãos de milho

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Abstract

This research aimed to determine the effectiveness of ozone in the microbiological disinfection of maize grains. Two kg samples of maize grains were used with moisture contents of 14.4% (w.b.), and 94% and 97% of natural infection by *Penicillium* spp and *Aspergillus* spp, respectively. The gas was applied at a concentration of 2.14 mg L⁻¹ and flow rate of 5.8 L min⁻¹ for 370 min at 25 °C ± 2 °C in order to determine the ozone concentration and saturation time of the maize grains. The experiment was installed according to a split plot design, with two treatments in the plots (atmospheric air and ozone gas) and exposure times of (0, 10, 20, 30 and 50 h) in the subplots, in a completely randomized design. It was shown that the ozone concentration and saturation time in the grain mass were 0.9874 mg L⁻¹ and 138.56 min, respectively. Ozonation was effective in controlling storage fungi in the grain mass with 50 h of exposure to the gas, reducing the rate of incidence of *Aspergillus* spp (78.5%) and *Penicillium* spp (98.0%), thereby confirming its fungicidal effect under the conditions presented.

Keywords: *Zea mays* L.; Ozonation; Mycotoxigenic fungi; *Aspergillus* spp; *Penicillium* spp; Stored grains.

Resumo

O objetivo deste trabalho foi determinar a eficácia do ozônio para a desinfecção microbiológica de grãos de milho. Utilizaram-se amostras de 2 kg de grãos de milho, com teor de água de 14,4% b.u., contendo 94% e 97% de infecção natural por *Penicillium* spp. e *Aspergillus* spp., respectivamente. Para determinação da concentração e do tempo de saturação dos grãos pelo ozônio, aplicou-se o gás na concentração de 2,14 mg L⁻¹ ao fluxo de 5,8 L min⁻¹, à temperatura de 25 °C ± 2°C. O experimento foi instalado segundo esquema de parcela subdividida, tendo, nas parcelas, dois tratamentos (ar atmosférico e gás ozônio), e, nas subparcelas, os tempos de exposição (0, 10, 20, 30 e 50 h), em delineamento inteiramente casualizado. Observou-se que a concentração e o tempo de saturação do ozônio na massa de grãos foram de 0,9874 mg L⁻¹ e 138,56 min, respectivamente. O processo de ozonização foi eficaz no controle de fungos de armazenamento na massa de grãos, com 50 h de exposição ao gás, reduzindo o índice de ocorrência de *Aspergillus* spp. (78,5%) e *Penicillium* spp. (98,0%), confirmando, portanto, seu efeito fungicida, nas condições apresentadas.

Palavras-chave: *Zea mays* L.; Ozonização; Fungos micotoxigênicos; *Aspergillus* spp.; *Penicillium* spp.; Grãos armazenados.

1 Introduction

The interaction between abiotic factors and living organisms, including microorganisms such as fungi and bacteria, contributes to the effective damage of stored grains,

depending, of course, on the storage and environmental conditions and the plant species or variety. The presence of these microorganisms may lead to the production of mycotoxins



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by some fungal genera and affect the organoleptic traits, nutritional value and further industrial use of the grains and their by-products (FARONI; SILVA, 2008).

Several studies included fungal species of the genera *Aspergillus* and *Penicillium* as the mycotoxigenic agents, considered important in the food industry, mainly with respect to cereal grains and oilseeds (RESTAINO et al., 1995; MCKENZIE et al., 1998; KELLS et al., 2001; MURPHY et al., 2006; KORZUN et al., 2008; KUMAR et al., 2008; McDONOUGH et al., 2011; ALENCAR et al., 2012). Several researchers have reported that these two genera include the species showing significant mycotoxigenic potential, most commonly found in stored maize grains (LAZZARI, 1993; KAWASHIMA; SOARES, 2006).

The constant search for ways of reducing quantitative and qualitative grain losses has become an increasing concern, and has led to efforts to implement safe and effective grain storage conditions. Several issues, such as current food legislation, increased pest resistance to conventional fumigants, as well as increased demand for the use of environmentally friendly products, have encouraged research institutions and even the private sector to pursue technological innovations to meet such needs (TIWARI et al., 2010).

The use of ozone gas stands out among the various alternatives studied to reduce damage to stored grains caused by fungal microorganisms (RESTAINO et al., 1995; RAILA et al., 2006; WU et al., 2006; ABDEL-WAHHAB et al., 2011; ALENCAR et al., 2012; SAVI et al., 2014; BEBER-RODRIGUES et al., 2015; SANTOS et al., 2016). Ozone is highly reactive and has a great disinfecting potential, which promotes oxidative stress in living cells. It is indicated as an alternative to the use of chemical oxidants (LI et al., 2012).

Several studies have been carried out using ozone gas as a sanitizing agent and microbicide, but little information was provided regarding its potential for reducing microbial populations in stored maize. This research aimed to determine the effectiveness of ozone in the microbiological disinfection of maize grains. In addition, the time of saturation of the grain mass with ozone gas was evaluated. Assessing the reaction kinetics of ozone gas through a porous medium is important to determine the appropriate concentration and time of exposure to avoid failure of the process, since the ozone concentration is reduced due to the decomposition process (PAES et al., 2017).

2 Material and methods

2.1 Ozone generation and measurement

Ozone gas (O_3) was obtained using the O&LM ozone generator developed by Ozone & Life (São José dos Campos, SP, Brazil). In the gas generation process, oxygen (O_2) free from moisture was used as the primary

input, obtained from the concentrator of the ozone generator itself.

The iodometric method was used to determine the concentration of the ozone gas formed by indirect titration, as recommended by the International Ozone Association (IOA). This method, described by Clesceri et al. (1998), consists of bubbling ozone gas into a 50 mL solution of 20 g L⁻¹ potassium iodide (KI). In order to ensure the production of I_2 , it was necessary to acidify the medium by adding 2.5 mL of 0.5 mol L⁻¹ sulfuric acid (H_2SO_4) to the KI solution. It was subsequently titrated with a 0.01 mol L⁻¹ sodium thiosulfate ($Na_2S_2O_3$) solution until the yellowish iodine colour became almost imperceptible. A 1.0 mL aliquot of the 1% (w/v) starch indicator solution was then added and the titration resumed until the blue colour disappeared from the solution.

2.2 Experimental design and description of the installation

The maize grains (naturally contaminated in the field) were packed into cylindrical polyvinyl chloride (PVC) chambers with 19.3 cm of diameter and 23.0 cm of height, each containing 2.0 kg of grains. A metal mesh was fixed at 10.0 cm from the bottom of each chamber to support the grains, forming a plenum for better distribution of the ozone within the chamber. The ozone inlet and outlet connections were installed in the lower and upper taps of the cylinders, respectively. Three chambers were used for treatment with ozone gas and another three for the atmospheric air (Figure 1).

The injection of ozone gas was carried out at a concentration of 2.14 mg L⁻¹ in a continuous flow of 5.8 L min⁻¹ at a temperature of 25 °C ± 2 °C.

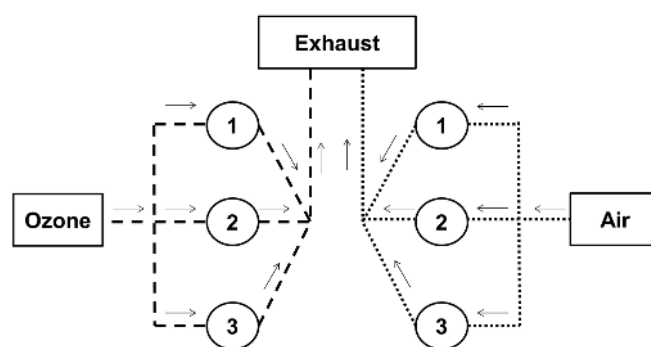


Figure 1. Schematic diagram of the system used for fumigation of the maize grains.

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2.3 Saturation of ozone gas in the maize grain mass

With a view to determining the time of saturation of the grain mass with ozone gas, three 2 kg samples of maize were used with an average moisture content of 14.4% (w.w.b.). The ozone saturation time of the maize grains was defined by determining the residual concentration of the gas at regular time intervals until the ozone concentration remained constant.

2.4 Assessment of the fungicidal effect of the ozone gas

Five periods of exposure to ozone (0, 10, 20, 30 and 50 h) were defined in order to assess the effect of the gas on the microbiological disinfection of the maize grains. Three chambers, each containing 2 kg of grain, were used for the treatment with ozone gas, and three for atmospheric air (control). The fungicidal effect of ozone gas was evaluated using maize grains naturally contaminated with *Aspergillus* spp (97%) and *Penicillium* spp (94%). To this end, the filamentous fungi and yeasts in the culture medium were quantified, as well as the rate of incidence of the fungal genera *Aspergillus* and *Penicillium* in the grains.

2.5 Microbiological analysis

The counting technique in Petri dishes containing acidified potato dextrose agar (PDA) culture medium was used to quantify the filamentous fungi and yeasts present in the maize grains. In accordance with the procedures described by Downes and Ito (2001), 25 ± 0.2 g samples of maize grains were properly homogenized in sterile plastic bags containing 225 mL of 0.1% peptone salt solution, using intermittent 60 s movements. Serial dilutions (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) were prepared in sterile salt solution for the above mentioned analyses. The results were expressed in colony forming units per gram of sample (CFU g⁻¹).

The filter paper method (blotter test) was used to detect the *Aspergillus* and *Penicillium*, using a total of 400 grains per sample divided into 8 50-grain sub-samples, placed in Petri dishes containing three sterile filter paper discs moistened with sterile distilled water (BRASIL, 2009). The plates were placed in a BOD incubation chamber for 24 h, with the temperature set at $20 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$, under an intermittent 12 h light/12 h dark cycle. The plates with the grains were then placed in a freezer ($-20 \text{ }^{\circ}\text{C}$) for 24 h. At the end of the freezing period, the plates were placed in the incubation chamber again under the same conditions for a further 5 days, and the samples then examined under a stereomicroscope for the identification and quantification of fungal structures related to the genera *Aspergillus* and *Penicillium*.

2.6 Statistical analysis

The ozone saturation data was subjected to a "Linear Response Plateau" regression analysis as a function of time. The microbiological disinfection experiment

was installed according to a split plot design, with two treatments in the plots (atmospheric air and ozone gas) and the exposure times (0, 10, 20, 30 and 50 h) in the subplots, in a completely randomized design (CRD), with three replicates, except for the rate of incidence of *Aspergillus* and *Penicillium*, where four replicates were used. The results were subjected to the variance and regression analyses. For the qualitative factor, the means were compared using the Tukey test at 5% probability. For the quantitative factor, the models were adopted based on the significance of the regression coefficients, with the application of the t-test at 5% probability to obtain the coefficient of determination and biological phenomenon. The software SAEG version 9.1 (UFV, Viçosa, MG, Brazil) was used to carry out the statistical analyses, and the software SigmaPlot version 11.0 (SPSS Inc., Chicago, IL, USA) for the graphical representation of the data.

3 Results and discussion

3.1 Ozone saturation in maize grain mass

Figure 2 shows the behaviour of the residual concentration of ozone gas according to the exposure time of the maize grain mass during the saturation process, using a concentration of 2.14 mg L^{-1} . Table 1 presents the equation that describes the said behaviour, with its respective coefficient of determination.

For the ozonated maize mass, using a concentration of 2.14 mg L^{-1} and flow rate of 5.8 L min^{-1} , a saturation time of 138.56 min was observed, after which the residual ozone gas concentrations remained constant. The saturation concentration of the gas was 0.9874 mg L^{-1} , which corresponds to about 46.1% of the initial concentration adopted (Table 1).

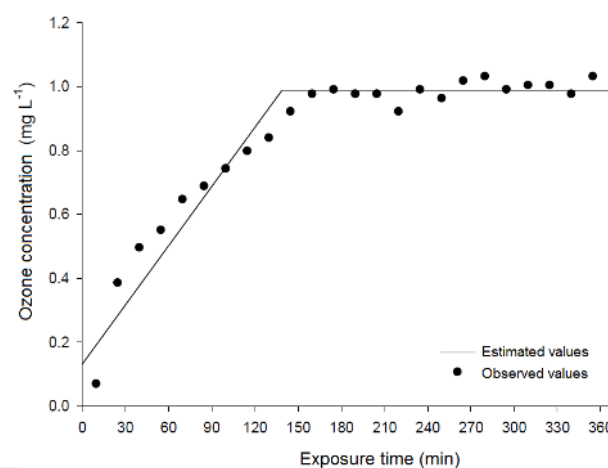


Figure 2. Residual ozone gas concentration (mg L^{-1}) according to the ozonation time (min) during the process of saturation of maize grains, using a concentration of 2.14 mg L^{-1} .

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Table 1. Fitted regression equation of the residual ozone concentration according to the period of ozonation, and the respective coefficient of determination (r^2).

Fitted equation	Interval	r^2
$\hat{Y} = 0.13151 + 0.00617708 * X$	$0.00 \leq X \leq 138.56$	0.89
$\hat{y} = 0.9874$	$138.56 < X \leq 370.00$	

*Significant at 1% probability according to the F test; X = exposure time (h); \hat{y} = residual ozone concentration (mg L^{-1}).

In experiments carried out by Santos et al. (2007), a saturation time of 70 min was obtained for a mass of 1.0 kg of maize grains, under a continuous flow of 4.6 L min^{-1} with a concentration of 0.21 mg L^{-1} . For peanut kernels ozonated with a concentration of 1.59 mg L^{-1} under a continuous flow of 5.0 L min^{-1} , saturation times ranging between 222 and 449 min were obtained by Roberto et al. (2016). For rice grains ozonated with a concentration of 10.13 mg L^{-1} under a continuous flow of 1.0 L min^{-1} , the ozone gas concentration and saturation time were 5.00 mg L^{-1} and 13.97 min, respectively (SANTOS et al., 2016). According to Paes et al. (2017), with increasing ozone concentrations, less time is required to saturate wheat flour. The saturation times were 812, 434, 370 and 342 min for ozone concentrations of 0.54, 1.07, 1.61 and 2.14 mg L^{-1} , respectively. By comparing the results reported by these authors together with those obtained in the present work, it was concluded that, besides the initial application concentration, the flow rate and product (type and quantity) affected the saturation process with ozone gas.

3.2 Microbiological analysis

A significant reduction ($p < 0.05$) was observed in the filamentous fungi and yeast counts in the maize grains exposed to ozone. Reductions equivalent to 0.87, 1.49, 1.58 and 2.0 log cycles were observed as compared to the control maize when the maize grains were submitted to ozonation for periods of 10, 20, 30 and 50 h, respectively. The ozonation process reduced the total fungal counts by 99.0% in comparison with the control maize.

Figure 3 and Table 2 show the reduced fungal counts in the grains exposed to ozone, while the count remained constant in the control maize.

Regarding the filamentous fungal and yeast counts in maize treated with atmospheric air, no effect was observed during the period of treatment. The average and standard deviation values were 3.7585 ± 0.0189 cycles $\log \text{ CFU g}^{-1}$. However, for the maize treated with ozone, the data were fitted to the quadratic model, with the lowest estimated count for filamentous fungi and yeasts being equal to $1.773 \log \text{ CFU g}^{-1}$ after 45 h 42 min of exposure to the gas.

A similar trend was observed in the rate of incidence of *Aspergillus* spp and *Penicillium* spp in the ozonized maize grains. By comparing with the average rates of

Table 2. Fitted regression equations and their respective coefficients of determination (r^2) for the filamentous fungal and yeast counts ($\log \text{ CFU g}^{-1}$) in maize grains exposed to atmospheric air (control) and ozone gas at a concentration of 2.14 mg L^{-1} , according to the exposure period.

Treatment	Fitted equations	r^2
Control	$\hat{Y} = 3.7585$	0.9835
Ozone	$\hat{Y} = 3.71838 - 0.0851728 * PE + 0.000932249 * PE^2$	

*Significant at 1% probability according to the t-test; **Significant at 5% probability according to the t-test.

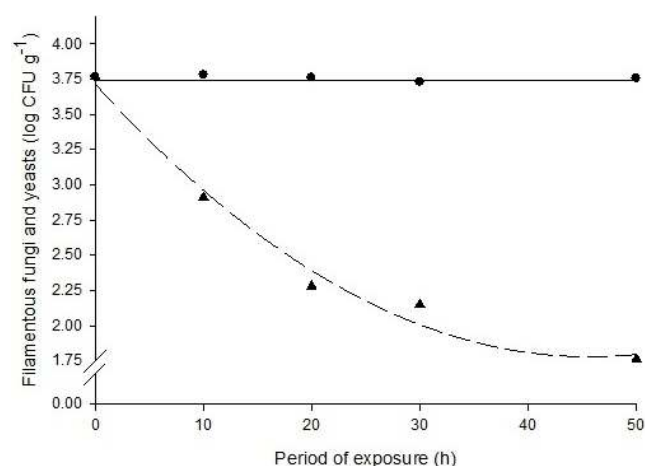


Figure 3. Estimated and observed values for the filamentous fungal and yeast counts ($\log \text{ CFU g}^{-1}$) in maize grains exposed to atmospheric air and ozone gas at a concentration of 2.14 mg L^{-1} for different exposure periods. (• Observed values: control; — Estimated values: control; ▲ Observed values: ozone; --- Estimated values: ozone).

incidence of the fungal species *Aspergillus* spp. in the control maize, reductions of 5.0, 10.5, 55.1 and 78.5% were observed for ozonation times of 10, 20, 30 and 50 h, respectively. Regarding *Penicillium* spp, reductions of 66.8, 84.6, 96.1 and 98.0% were found for the same exposure periods. Thus under such conditions, the results suggest that species of the genus *Aspergillus* are more tolerant to the fungicidal effect of ozone gas than species of the genus *Penicillium*. It is noteworthy that there was a slight decrease in the *Aspergillus* genus count after 10 h of ozonation, while for the *Penicillium* genus, a marked reduction was detected.

Figures 4 and 5 show the changes found in the fungal incidence rates in grains after different exposure periods.

Table 3 shows the fitted regression equations and their respective determination coefficients for the incidence rates of *Aspergillus* spp and *Penicillium* spp in maize grains exposed to atmospheric air and ozone for different exposure periods.

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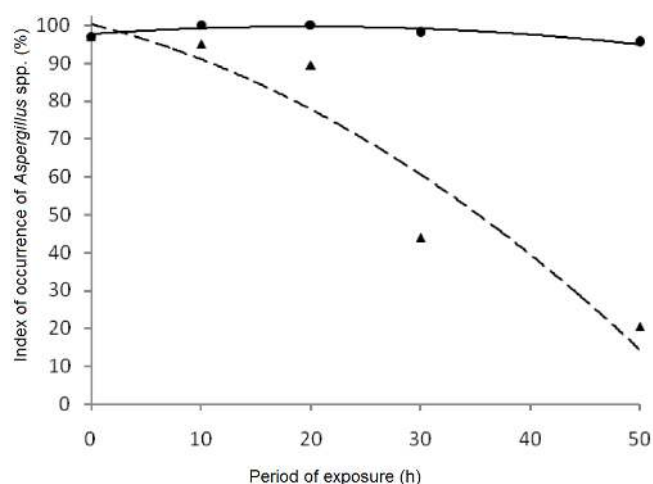


Figure 4. Estimated and observed values for the incidence rate of *Aspergillus* spp in maize grains exposed to atmospheric air and ozone gas at a concentration of 2.14 mg L⁻¹ for different exposure periods. (● Observed values: control; — Estimated values: control; ▲ Observed values: ozone; --- Estimated values: ozone).

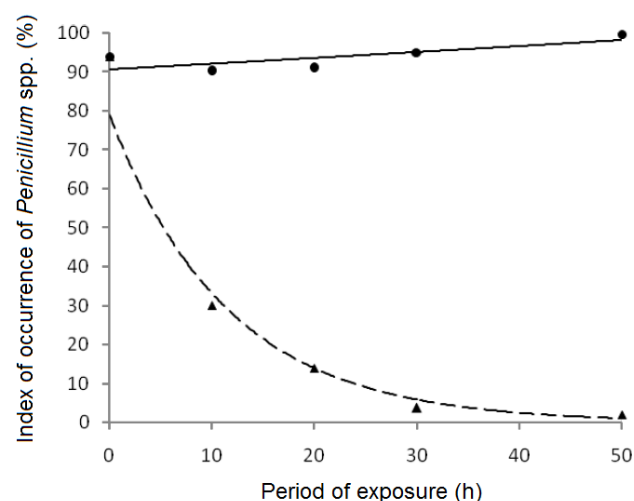


Figure 5. Estimated and observed values for the incidence rate of *Penicillium* spp in maize grains exposed to atmospheric air and ozone gas at a concentration of 2.14 mg L⁻¹ for different exposure periods. (● Observed values: control; — Estimated values: control; ▲ Observed values: ozone; --- Estimated values: ozone).

With regard to the incidence rates of *Aspergillus* spp and *Penicillium* spp in maize grains exposed to ozone gas according to the exposure period, the data were set according to the square root model, where the lowest estimated values were approximately 15.51 and 1.25% after 50 h of exposure to ozone for the two fungal species, respectively.

It is worth noting that in ozonated maize grains, not all the fungal spores failed to germinate. However, those that somehow survived the fungicidal effect of the oxidizing

Table 3. Fitted regression equations and their respective coefficients of determination (r^2) for the incidence rates (%) of fungi of the genera *Aspergillus* and *Penicillium* in maize grains exposed to atmospheric air (control) and ozone gas at a concentration of 2.14 mg L⁻¹ according to the exposure period.

Variable	Treatment	Fitted equations	r^2
<i>Aspergillus</i>	Control	$\hat{Y} = 97.0073 + 1.95096^* - 0.308293^*PE$	0.9732
	Ozone	$\hat{Y} = 97.69 + 7.96521 - 2.75645^{**}PE$	0.9256
<i>Penicillium</i>	Control	$\hat{Y} = 93.9598 - 2.83555^* + 0.522091^*PE$	0.9752
	Ozone	$\hat{Y} = 94.2234 - 26.5496^* + 1.89523^*PE$	0.9990

*Significant at 1% probability according to the t-test; **Significant at 6% probability according to the t-test.

gas produced very small, sparse colonies with vegetative structures presenting an anatomical aspect devoid of force, according to the assessment carried out using a stereoscopic microscope. The traits mentioned showed a general prevalence, but were much more pronounced in the *Penicillium* spp colonies. By studying the effects of ozone on *Aspergillus niger*, the causal agent of black rot in onions, Vijayanandraj et al. (2006) perceived changes in the morphology of the fungus. The authors found that for the conidia treated with ozone at a concentration of 4.8 mg L⁻¹ for 5, 10 and 15 min, their subsequent cultivation in potato dextrose agar culture (PDA), produced non-sporulating colonies, possibly constituted of sterile mycelium, as a result of the ozonation process.

The use of a stereoscopic microscope allowed for a clearer observation of the fungicidal effect of the ozonation process in comparison with the condition of the control maize, almost totally infested by filamentous fungi, including *Aspergillus* spp and *Penicillium* spp (Figure 6).

It is important to emphasize that, besides inhibiting the proliferation of *Aspergillus* spp and *Penicillium* spp, ozone gas extended its fungicidal effect to other fungi present on the surface of the grains, as shown in Figure 6.

Santos et al. (2016) assessed the fungicidal effect of ozone gas in the control of *Penicillium* spp. and *Aspergillus* spp. in rice grains. Rice grains (14.3% moisture content) were ozonized at a concentration of 10.13 mg L⁻¹ under a continuous flow of 1.0 L min⁻¹ for five exposure periods (12, 24, 36, 48 and 60 h). There was a reduction of 3.8 log cycles (100%) in the yeast counts and complete fungal inhibition in the ozonized grains. Similarly, Alencar et al. (2012) demonstrated the fungicidal effect of ozone gas on peanut grains, which caused approximate reductions of 3 log cycles after 96 h of ozonation at a concentration of 21 mg L⁻¹. According to Trombete et al. (2016), by exposing 2 kg wheat grain samples (11.3% moisture content) to 60 mg L⁻¹ of O₃ for 300 min, it was possible to

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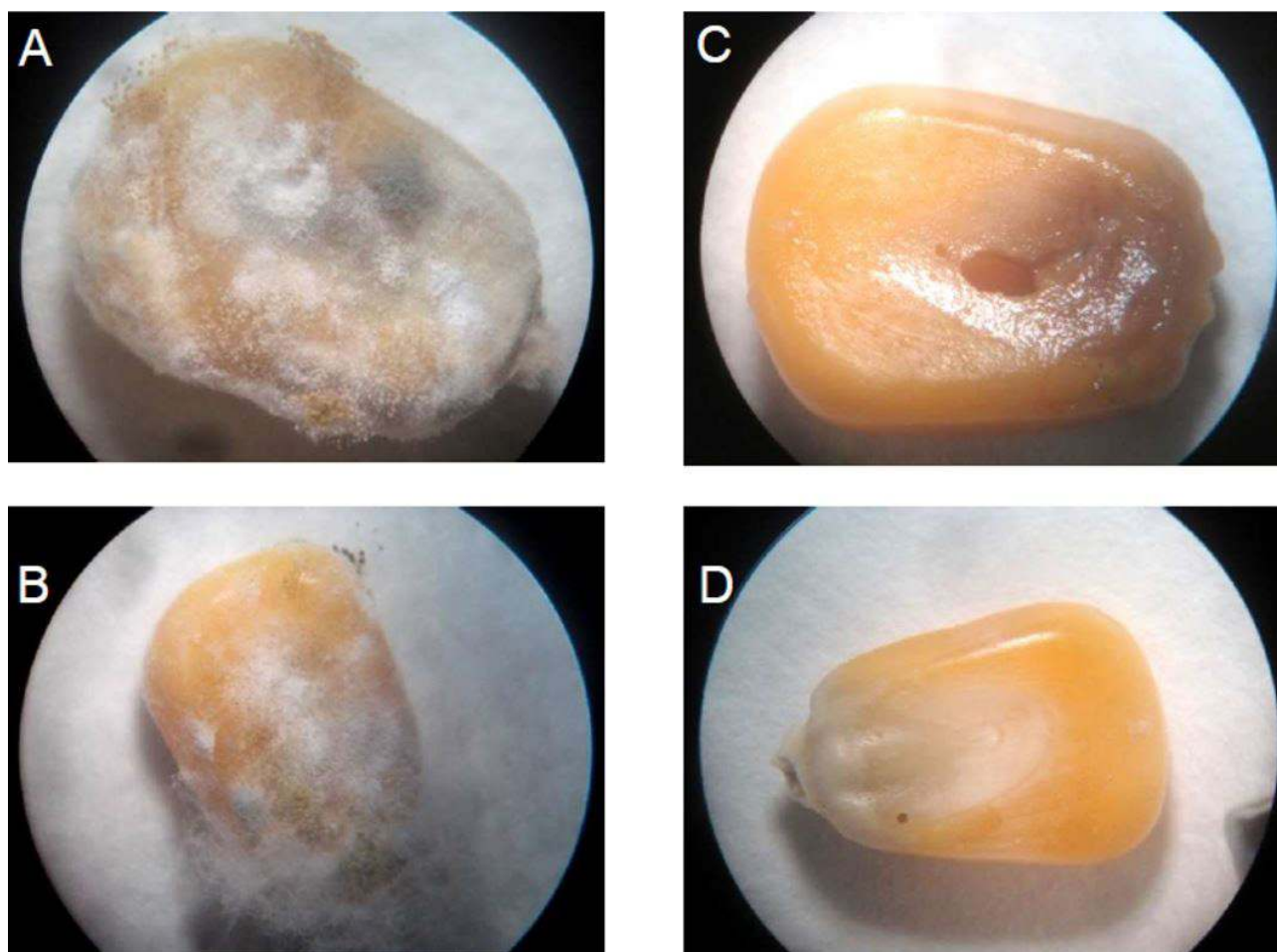


Figure 6. Maize grains observed under a stereomicroscope after exposure to atmospheric air (A and B) and ozone gas (C and D) for a 50 h period.

obtain reductions of 3.0 cycles $\log \text{cfu g}^{-1}$ in the total fungal count, 64.3% in the deoxynivalenol levels and 48.0% in the total aflatoxin levels. By comparing the results reported by these authors and those obtained in the present work, it was concluded that the ozone efficiency for fungus inactivation depended mainly on the fungus to be inactivated and the product to be treated (type and moisture content).

Due to its powerful and wide spectrum of antimicrobial activity, ozone has often been investigated in studies involving microbicidal action, fungi, bacteria, viruses, protozoa and fungal and bacterial spores (KHADRE et al., 2001). It must be pointed out that the microbial inactivation by ozone results from the interaction of a number of factors involved in a complex process (GREENE et al., 2012). According to these authors, ozone acts on various components of the cell wall and cell membrane and on components of the cell contents, including enzymes and nucleic acids. This action is attributed to the activity of molecular ozone and free radicals released during the fast decomposition process (GUZEL-SEYDİM et al., 2004). The rupture or lysis of the cell envelope, which leads to the flow of the cytoplasmic contents, is referenced by many researchers

as the climactic outcome of microbial cell death (SCOTT; LESHER, 1963; GUZEL-SEYDİM et al., 2004; PASCUAL et al., 2007; CULLEN et al., 2009).

4 Conclusions

The ozonation process reduced both the total fungal count and the rate of incidence of *Aspergillus* spp and *Penicillium* spp in maize grains exposed to ozone gas at a concentration of 2.14 mg L^{-1} for a 50 h period. These results confirmed that ozone gas has a fungicidal effect and can be used in the control of the genera *Aspergillus* and *Penicillium* in maize grains under the conditions reported.

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