

Ozone treatment efficiency on toxigenic fungi and mycotoxins decontamination from post-harvest wheat (*Triticum aestivum* L.) grains

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Abstract

Fusarium graminearum is reported in freshly harvested wheat grains and has been associated with deoxynivalenol (DON) production - one of the most important trichothecene type B toxins. Ozone (O₃) has been an attractive decontamination method for the food industry because it decomposes to molecular oxygen without leaving residues. This study evaluated the effectiveness of O₃ gas on *F. graminearum*, *A. flavus* and *P. citrinum* growth inhibition, as well as on fungi conidia germination and hyphae mortality. In addition, we evaluated the gas effect on DON degradation. The tests were performed in pilot silos containing artificially contaminated grains (with each fungus strains and DON), which were divided into two treated Groups (40 and 60 O₃ µmol/mol), with an untreated control. Gas exposure times were 30/60/120/180 min followed by fungi and DON analysis. After 180 min of O₃ exposure (for both concentrations), *F. graminearum* growth was totally inhibited (100% reduction). Regarding *A. flavus* and *P. citrinum* growth, the same inhibition occurred only at 60µmol/mol gas concentration after 180 min of exposure. At lower concentrations (40µmol/mol) those strains growth inhibition was less, 92.2 and 74.7% total spore loads, respectively. Thus *F. graminearum* was more sensitive to that treatment showing greater conidia germination reduction (90.7%) and hyphae mortality (95.9%). DON was significantly reduced after 60 O₃ µmol/mol exposure, from 2159,08 (control) to 627,07 µg/kg after 120 min. The O₃ gas treatment was effective on inactivating toxigenic fungi growth, as well as DON degradation in wheat grains.

Keywords: Fungi, mycotoxin, ozone gas, decontamination, quality

1. Introduction

Fusarium species, especially *Fusarium graminearum* cause *Fusarium* Head Blight (FHB) in wheat. Among the mycotoxins associated with FHB, such as trichothecenes, e deoxynivalenol (DON) is the most common in wheat grains (Muthomi et al., 2008; Bensassi et al., 2010; Soleimany et al., 2012; Stankovic et al., 2012; Ennouari et al., 2013; Santos et al., 2013). The presence of DON in humans and animals through ingestion of contaminated food, can induce acute and chronic effects such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity (Rotter et al., 1996; Wijnands and Van Leusden, 2000; Pestka, 2007).

Aspergillus flavus and *Penicillium citrinum* strains also have been isolated from stored wheat grains worldwide (Berghofer et al., 2003; Riba et al., 2008; Roigé et al., 2009; Riba et al., 2010). *A. flavus* is an aflatoxins producer (AFLs) in storage grains in tropical and subtropical climates, especially AFB₁ which is the predominant and most potentially mutagenic, teratogenic and hepatocarcinogenic mycotoxin according to the International Agency for Research on Cancer (IARC, 1993). *P. citrinum* is the main citrinin producer (CTR), which was originally isolated in this fungi (Cole et al., 1986; Blanc et al., 1995). The most important

toxic properties of this mycotoxin is nephrotoxic, however, it has cytotoxic, genotoxic, mutagenic, immunotoxic and teratogenic properties (Kogika et al., 1993; Kumar et al., 2007).

According to Brazilian legislation of mycotoxins, since 2012 a maximum tolerable level (MTLs) of 2000 µg/kg is proposed for DON in whole wheat grains. The limit will be decreased over time to allow grain producers and the industry to adapt to the legislation without causing a shortage of wheat. From January 2017, DON limits for whole wheat grain will be set at 1000 µg/kg (Brazil, 2011; 2013). Now, the lower limits of DON is equal to 1750 µg/kg and it is fixed by the European Communities Commission for unprocessed durum wheat (European Commission, 2006).

On the other hand, the MTLs for AFLs in cereals and products derived from cereals is 5 µg/kg (Brasil, 2011), similar to the levels defined in Europe of 4 µg/kg (European Commission, 2006). There is no specific legislation for CTR because it is difficult to establish widely acceptable limits for this mycotoxin due to a lack of suitable analytical methods.

One strategy for decontamination in food is ozone (O₃) gas application for reduce fungal growth and mycotoxins in wheat grains and reduce post-harvest losses. O₃ is a powerful antimicrobial agent due its potential oxidizing capacity (Khadre et al., 2001). It is attractive to the food industry because O₃ gas decomposes to molecular oxygen without leaving residues. The US Food and Drug Administration (FDA) classified O₃ for treating bottled water as *generally recognized as safe* (GRAS) (FDA, 1982), and it has been affirmed as GRAS for use in food processing (Graham, 1997). Moreover, the potent disinfectant characteristics of O₃ are recognized by the Food and Agriculture Organization (FAO, 1994). O₃ has been effectively used to control fungal growth in laboratory scale trials in food, including barley, wheat, fig and Brazil nuts (Kottapalli, 2005; Wu et al., 2006; Zorlugenç et al., 2008; Scussel et al., 2011; Beber et al., 2014; Savi et al., 2014a,b,c). It can reduce mycotoxin contamination in peanut, fig, Brazil nuts and artificially contaminated corn (Dwarakanath et al., 1968; Zorlugenç et al., 2008; McDonough et al., 2011; Scussel et al., 2011, Savi et al., 2014bc).

This study reports the effectiveness of O₃ gas treatment against *F. graminearum*, *A. flavus* and *P. citrinum* strains growth, as well as DON degradation in wheat grains. In addition, it explores the effects of O₃ gas on conidia germination and hyphae mortality.

2. Materials and Methods

2.1. Fungi strain

F. graminearum, *P. citrinum* and *A. flavus* were obtained from the Food Mycology Laboratory of Mycotoxicology and Food Contaminants (LABMICO) culture collection at the Federal University of Santa Catarina, Florianopolis, SC, Brazil.

2.2. Culture media and chemicals

Culture media: potato dextrose agar (PDA) and peptone bacteriology media were purchased from Himedia company (Curitiba, Parana, Brazil). *Chemicals*: DON standard and Evans blue dye from Sigma Aldrich Chemicals (St. Louis, MO, USA); acetonitrile, methanol and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil) with LC grade and tween 80 from Synth (Diadema, SP, Brazil). Water was obtained from a Milli-Q system 18.2 MΩ/cm. *Other materials*: immunoaffinity columns from DON-Test Vicam (Milford, MA, USA).

2.3. Instruments

LM, CH-B145-2, Olympus (Shinjuku, Tokyo, Japan); autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); rotary shaker, Marconi

(Piracicaba, SP, Brazil); microbiological incubator, Quimis (Diadema, SP, Brazil) and ultraviolet cabine, Dist (Florianopolis, SC, Brasil). For moisture content (mc) and water activity (a_w) were utilized a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil) and Aqua-Lab 4TE Decagon Devices (Sao Jose dos Campos, SP, Brazil), respectively. O₃ generator, model OP-35-5L, Interzon (Jundiai, SP, Brazil). High performance liquid chromatography (HPLC) equipment model 321, Gilson (Middleton, WI, USA) equipped with a isocratic pump model 805, manual injector (20 µL loop) and with ultraviolet-visible (UV) detector model 118 set a 218 nm. The chromatographic column used was a C₁₈ 250 x 4.60 mm, 4 µm particle size Fusion-RP 80, Phenomenex (Madrid Avenue, Torrance, USA).

2.4. Samples artificial contamination before ozone treatment

About 50 Kg of wheat grains were collected from vertical silos (wheat stored for three months) from the 2012 harvest, from the Brazilian Agricultural Research Corporation (Embrapa Wheat). Samples were received after cleaning and drying (up to a maximum of 60°C) in the storage unit, packed in a polyethylene bag and stored at 4°C. For mycological analysis, a solution (5 mL) of Tween 80 containing 1×10^3 spores/mL of *F. graminearum*, *A. flavus* or *P. citrinum* were sprayed on wheat grains (25g). For mycotoxin analysis, wheat grains were spiked using 100 µL of DON standard solutions (1 mg/mL). The samples presented 20% mc. The wheat grains were submitted to drying in an oven (105°C ± 5°C) up to constant weight through gravimetric method (AOAC, 2005).

2.5. Ozone gas treatment in the storage silos

The silos with capacity of 25 x 10 cm (length x diameter) were made with vinyl polychloride tubes containing two apertures: one for the input of O₃ gas and one for the output. The grains artificial contaminated were packed in the top part of the silos, on the polyamide screen surface. The bottom part of the silos was filled with 350g of wheat grains. The O₃ gas generated was applied to pilots silos, divided in control group (no O₃ gas) and treated group (40 and 60 µmol/mol) and were exposed of 30 up to 180 min. For conidia germination analysis and morphological alterations observation, all the fungi were placed within storage silos and received the O₃ gas treatment with the concentration of 60 µmol/mol by 120 min, separately. The O₃ gas generator system followed the procedures detailed by Giordano et al. (2012) and Savi et al. (2014) with minor modifications, as described along this section: the compressed air pump was connected to an air impurities remover to filter the room air. The impurities removed were solid particles and humidity. Afterwards, the air filtered was conducted to the adjusted flow meter for 1 L/min and then the O₃ generator was calibrated to reach a concentration of 40 or 60 µmol/mol. The O₃ generator used (5 a 60 µmol/mol) was the corona discharge process, produced by electrical discharge by means of the air passage or pure oxygen between the two electrodes. The O₃ gas produced was introduced through a tube into input aperture of each chamber. The control chamber (without O₃ gas) was ventilated with “room air” in the same flow (1 L/min).

The O₃ gas concentration measurement was performed by the iodometric test. For this, the O₃ concentration was measured in each chamber by the titration method on the outlet of the O₃ generator. The gas was bubbled into a potassium iodide solution (50 mL), acidified with 2.5 mL of sulfuric acid 1 N (pH below 2.0). The solution was titrated with sodium thiosulfate 0.005 N using a starch solution as indicator, according to APHA (1999).

2.6. Mycological analysis after ozone treatment

Fungi total count: The enumeration technique was applied to evaluate the fungi total load (Silva et al., 2010). Each artificially contaminated wheat sample were added to 0.1% peptone dissolved in water under sterile conditions. The mixture was stirred on a rotary shaker for 2

min and the dilutions were performed. Aliquots of each dilution were spread on the surface of the PDA medium containing chloramphenicol 100 mg/L and incubated for up to 7 days at 28°C in the dark. The results were expressed in colony forming units per gram (CFU/g).

Conidia germination: the treated fungi colonies were transferred to a 0.89 % NaCl and 0.1% Tween 80 solution tube and shaker stirred for fungi conidia detachment. The conidia solution (100µL) was placed on the surface of a microscope slide containing culture medium PDA. This slide was transferred to into of a sterile Petri dish containing moist cotton and incubated for 28°C in the dark, during 15 hours. After, a drop of lactophenol cotton blue was placed on the growth to stop the conidia germination and to perform the counting (Marques et al., 2004). Finally, was checked 100 conidia in each area of the microscope slide, utilizing LM at 400x and counted germination and no germination conidia.

Mortality: the Treated and Control hyphae were soaked in 0.05% Evans blue dye solution and left stand for 5 min. After, the hyphae were washed three times with PBS to remove the excess of dye. The resultant sediment was verified in LM at x400 magnification. The hyphae were observed according to their staining differences (Evans blue staining: dead hyphae and natural color: intact hyphae) (Semighini and Harris, 2010). For quantification were counted at 100 fungal hyphae in each area of the microscope slide.

2.7. DON analysis after ozone treatment

Whole wheat grains samples were analyzed using an immunoaffinity columns for clean-up step and LC/UV for detection, according to Vicam protocol DON Test, No. G1005 USA (Vicam, 2013), with some modifications. Briefly, each artificially contaminated wheat sample were ground in a laboratory mill and set into an industrial blender jar with 30 ml of LC grade water. The mixture was blended for 30 seconds, followed by twice filtration and cleaning by immunoaffinity column. The toxin was slowly eluted with 100% LC grade methanol. The eluate was evaporate to dryness using a heating block at 40 °C with gentle nitrogen stream and the dry residue was then redissolved with 100 µL of mobile phase acetonitrile:water (10:90, v/v). The extract (20 µL) was injected onto the LC/UV System set at a wavelength of 218 nm. The mobile phase was delivered in a flow constant rate of 0.6 ml/min. DON levels quantification was performed by measurement of peak area at DON retention time compared with the standard solutions used for calibration curve (0.150 to 15 µg/ml) with a correlation $r = 0.996$. Recovery was determined by spiking DON-free samples of wheat with DON concentrations of 250 and 1.500 µg/Kg in the same day and same HPLC conditions.

2.8. Statistical analysis

The obtained data were analyzed using analysis of variance (ANOVA) followed by Bonferroni post-test. The data were expressed as mean \pm standard deviation and the values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Ozone gas effect on wheat grain mycological analysis

In our study, *F. graminearum* growth was significantly reduced (15×10^1 and 5.8×10^1 CFU/g) after 30 min of O₃ exposition in concentrations of 40 and 60 µmol/mol, when compared to the Control (55×10^1 CFU/g), which represents a 91.75 and 96.81% spores inhibition. After 180 min of O₃ exposition in the same concentrations, the *F. graminearum* growth was totally inhibited (Figure 1a). *A. flavus* and *P. citrinum* growth was totally inhibited only after 180 min of O₃ exposition in the concentration of 60 µmol/mol. In the concentration of 40 µmol/mol the growth was significantly smaller (10 and 22×10^1 CFU/g) when compared to the Control (128 and 87×10^1 CFU/g), respectively (Figure 1b e 1c). In

study *in vitro* performed in our laboratory was verified that after O₃ exposition (60 µmol/mol) for 60 min the strain of *F. graminearum* did not present growth until the 8th day of incubation. For the strain of this fungi that received O₃ gas exposure for 40 min, the growth was significantly smaller (38 mm) when compared to the control (53 mm) at the end of the incubation (Savi et al. 2014). *P. citrinum* also was not able of grow in culture medium PDA after received 120 min of O₃ gas exposition (60 µmol/mol). Moreover, in other times of O₃ gas exposure (40, 60 and 90 min), its growth was significantly smaller (19, 16 and 10 mm) than Control (23 mm). On the other hand, *A. flavus* was more resistant to treatment and grown on culture medium after O₃ gas exposure in different times. Nevertheless, the growth was significantly smaller after 120 min of O₃ gas exposure (60 mm) than control (78 mm).

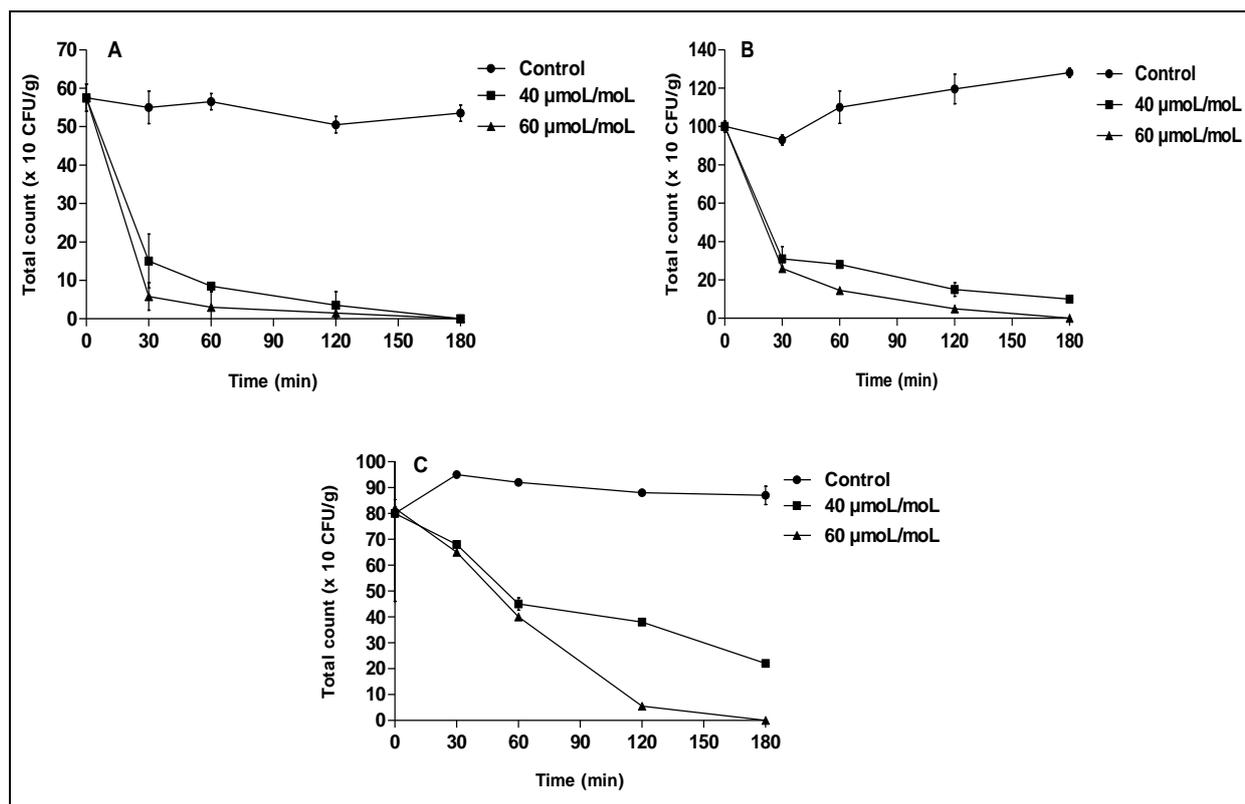


Figure 1 Ozone gas effects (40 and 60 µmol/mol in 30 to 180 min of exposure) on (A) *F. graminearum*, (B) *A. flavus* and (C) *P. citrinum*, data are shown as total count. All treatments were statistically significant when compared to control group ($p < 0.05$) by Bonferroni post-test.

Other results also showed efficiency of O₃ treatment on this fungi genera. In *Fusarium* contaminated wheat grains, Kotapalli et al. (2005) verified that the O₃ treatment reduced 24 to 36% of fungi growth after 15 min of O₃ exposition in concentrations of 11 and 26 mg/g. This results are according with our study, showing the efficiency of O₃ gas treatment towards *Fusarium* sp. in wheat grains. In Brazil nuts, the O₃ treatment in concentration of 31 µmol/mol in 5 hours exposition was able to successfully reduced completely *A. flavus* and *A. parasiticus* (Initial: $6,7 \times 10^4$ CFU/g), since day one after application. On the other hand, they were still able to grow, at the lower O₃ concentrations (10; 14 µmol/mol), however only in the first days of storage and at reduced number though (from $6,7 \times 10^4$ to $3,1 \times 10^3$; $1,9 \times 10^3$ CFU/g, respectively) (Giordano et al., 2012). Lower concentrations of O₃ (0.3-1.5 µmol/mol) also caused inhibition of the mycelial growth and sporulation of *Penicillium*

genera on citrus fruit. *In vitro* radial growth of *P. italicum*, during a 5-day incubation period at 20°C was significantly reduced by a previous 0.3 ± 0.05 $\mu\text{mol/mol}$ O_3 exposure at 5°C for 4 days (Palou et al., 2001).

3.2. Ozone gas effects on conidia germination

When fungus is exposed to extreme adverse conditions of growth (i.e., chemical treatments, low temperature and high water presence) including long term storage (Aregger, 1992; Savi et al., 2014), the number of conidia germination can be reduced. This occurred in the current work, when conidia germination of all tested fungi were significantly inhibited by the O_3 gas exposure (Figure 2).

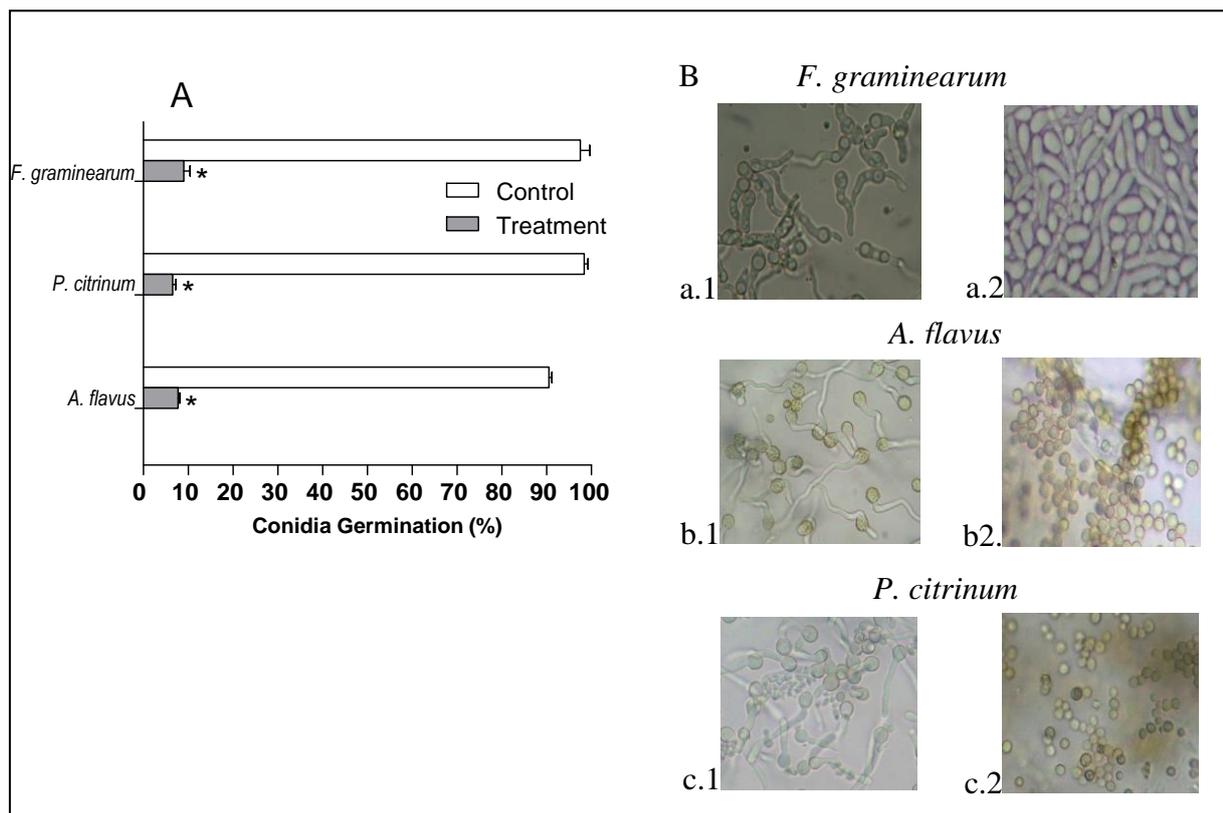


Figure 2 Effects of ozone gas (60 $\mu\text{mol/mol}$, 120 min exposure) on fungi conidia germination. (A) Data are shown as average values and standard deviation of conidia germination percentage. Symbols indicate statistically significant when compared to Control group $*p < 0.05$. (B) Conidia germination of Groups Control (a.1, b.1 and c.1) and Treated (a.2, b.2 and c.2).

3.3. Ozone gas effects on hyphae mortality

In addition to reduce fungi reproductive capacity, the chemical treatments can induce cellular damage typical by O_3 , such as the induction of cell death (Faoro and Iriti, 2009). In the current work, the O_3 gas exposure lead to hyphae cell death of all fungi tested and consequently fungi growth reduction in different proportions. *F. graminearum* was more sensitive to treatment with greater reduction (fungi growth, conidia germination and hyphae mortality) (Figure 3).

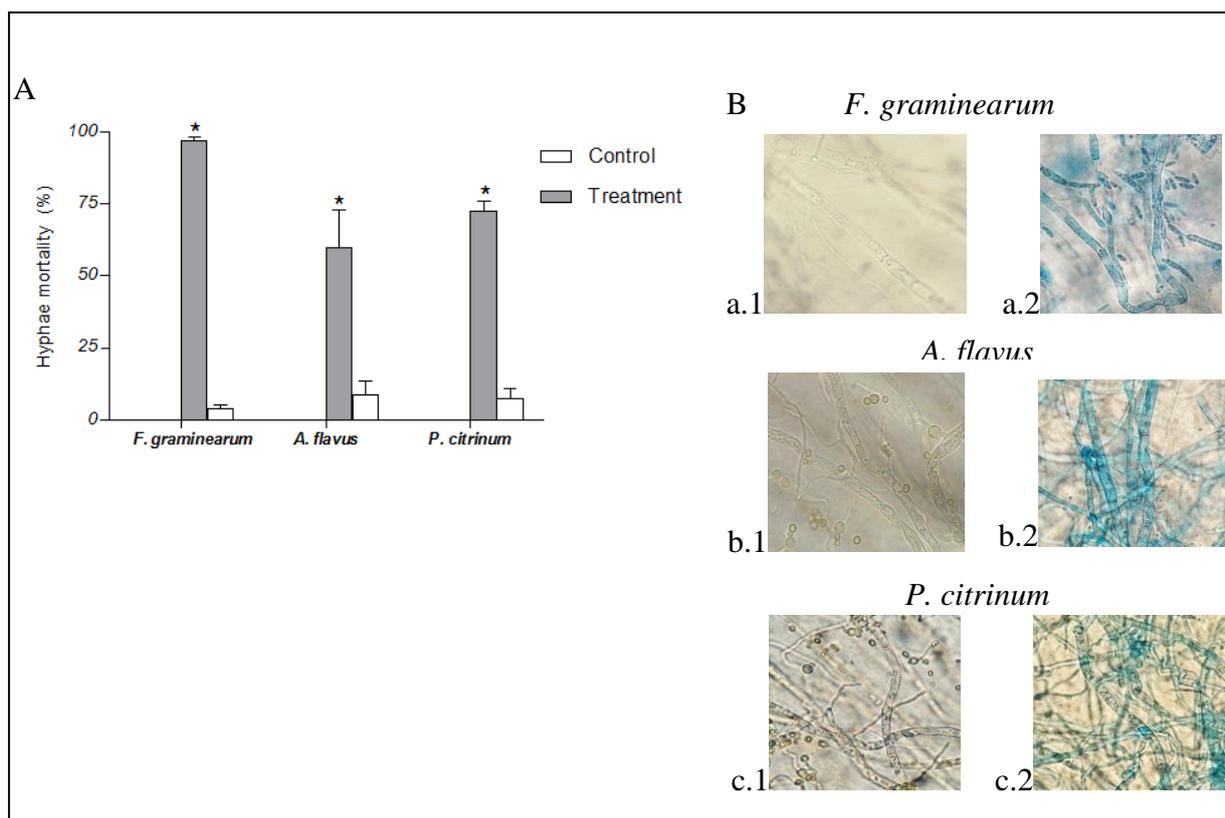


Figure 3 Hyphae mortality of the ozone gas treated fungi (60 $\mu\text{mol/mol}$, 120 min exposure): (A) showing data as average and standard deviation (%) and (B) Evans blue stain distribution: (a) *F. graminearum*, (b) *A. flavus* and (c) *P. citrinum*, [Control: no treatment - without coloration (hyphae alive - a.1, b.1 and c.1); Ozone gas treatment: hyphae coloration (hyphae dead - a.2, b.2 and c.2), showing hyphae alterations]. LM images at x400.

3.4. DON analysis

The LC/UVD method for DON detection and the validation parameters (linearity, limits of detection and quantification (LOD and LOQ), reproducibility, repeatability and recovery) obtained showed to be quite adequate. Under the chromatographic conditions used, the retention time (Rt) of DON was about 17 ± 0.5 min. Linearity was confirmed using the calibration curve for each DON concentration. It was linear in the range from 0.15 to 15 $\mu\text{g/ml}$, with a correlation coefficient, r equal to 0.996. The LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) was 0.06 and 0.12 $\mu\text{g/g}$ (corresponding to: 66.7 and 119.1 $\mu\text{g/kg}$), respectively. The recovery experiments were determined by blank wheat grains spiked with DON at concentrations of 250, 1000 and 1500 $\mu\text{g/kg}$ (analysis in triplicates), which showed a yield equal to 87 ± 9 , 96 ± 6 and $93 \pm 3\%$ respectively. The mean recovery of the extraction method was of $92 \pm 4\%$.

The O_3 treatment can completely degrade or cause chemical changes in mycotoxins, reducing its biological activity in terms of toxicity (Mckenzie et al. 1998; Lemke et al. 1999). After 60 $\mu\text{mol/mol}$ O_3 treatment, the DON levels (627.07 $\mu\text{g/kg}$) were significantly reduced when compared to control group (2159.08 $\mu\text{g/kg}$) after 120 min of exposure (Table 1). Young (1986) previously investigated the efficiency O_3 in storage grains. When whole wheat kernels naturally contaminated (ca. 1 $\mu\text{g/g}$ DON) were treated with O_3 dry, there was no reduction in DON levels. When vacuum oven dried ground unhusked corn artificially inoculated by *F.*

graminearum (ca. 1000 µg/g DON) was treated with dry O₃, the half-life of DON disappearance was 2.5 h. These differences between the wheat and corn results may also have been due to a matrix effect. The corn was ground and thus porous whereas O₃ may not have been able to penetrate the whole wheat kernels so easily. Young et al. (2006) also studied the degradation of trichothecenes with aqueous O₃, bubbling the gas in water by 30 min and verified a DON total degradation at concentration of 25 ppm. However, the use of O₃ gas in storage is more feasible than aqueous O₃ due to the humidity of grain that should be constant to avoid contaminants as insects, fungi and mycotoxins, limiting the use of aqueous O₃.

The possible mechanism of DON degradation by O₃ is that the O₃ molecule undergoes 1–3 dipolar cyclo addition with a double bond. This leads to the formation of ozonides (1,2, 4-trioxolanes) from alkenes and O₃ with aldehyde or ketone oxides as decisive intermediates, all of which have finite lifetimes (Criegee, 1975; Cullen et al. 2009). The O₃ treatment was efficient for reduction of DON levels in artificially contaminated wheat grains and this is important to maintain the levels of DON below of MTL 1000 µg/kg for storage whole wheat grains, according with the legislation expected in 2017 (Brazil, 2013).

Table 1 Deoxynivalenol concentration in wheat grains after ozone gas treatment.

Toxin	Ozone gas				
	Group	n	Concentration (mg/L)	Time of exposure (min)	Mean (µg/kg)
Deoxynivaenol	C	3	NA		2159.08
	I	3	40	120	2014.83
	II	3	60		627.07

4. Conclusions

The O₃ gas treatment was effective on inactivating *F. graminearum*, *A. flavus* and *P. citrinum* growth, as well as DON degradation in wheat grains, especially at 60 µmoL/moL concentration. O₃ gas is internationally recognized as safe and does not leave residues in food, therefore, could be a promising method of decontamination in industries and storage units during the wheat grain storage, in order to avoid contamination and ensure food security to the consumer.

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