

## **Wheat grains storage protection against toxigenic fungi and mycotoxins through ozone gas and its effects on grains germination**

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### **Abstract**

Species of *Aspergillus flavus* has been isolated in stored wheat grains worldwide, which is well known aflatoxins (AFLs) producer, with AFB<sub>1</sub> the predominant and most potentially toxic. Ozone (O<sub>3</sub>) gas has been studied to reduce fungal growth (including mycotoxins decontamination) in order to preventing/control post-harvest losses. The aims of this study was to evaluate the effectiveness of O<sub>3</sub> gas treatment, against *A. flavus* strain growth, AFB<sub>1</sub> degradation and its effects on seeds germination. The tests were performed in pilot silos containing artificially contaminated (*A. flavus* and AFLs) grains which were divided into three Groups: I and II-O<sub>3</sub> gas treated (40 and 60 µmoL/moL, respectively) and III-no O<sub>3</sub> gas treated as Control. They were exposed to two periods of time (120 and 180 min) and checked for reduction of contamination by mycological tests and liquid chromatography with fluorescence detection analysis. *A. flavus* growth reduction was observed in both periods of time and concentrations applied, at the different degrees. Some growth was observed in Group I (lowest concentration:40 µmol/mol) at the longest time (180 min), however it was quite low (to 1.5x10<sup>1</sup>CFU/g) when compared to the Group III (Control: 44.3x10<sup>1</sup>CFU/g), which represents 96.6% inhibition. On the other hand, in Group II (highest concentration: 60 µmol/mol) also at longest time of exposure, that fungi strain was completely reduced / destroyed (100%). Regarding the AFB<sub>1</sub> degradation, in both gas concentrations (40 and 60 µmoL/moL) at 180 min exposure, it was significantly reduced from 345.08 and 333.80 µg/kg (Group III:Control) to 105.13 and 92.91 µg/kg, corresponding to 69.5 and 72.2% degradation. Regarding the effect O<sub>3</sub> on wheat grain behavior, no modifications was observed on germinated wheat seed coleoptile length and seminal root, up to 120 min gas exposure/60 µmol/mol concentration. Ours results showed that effective inactivation of fungi and mycotoxins was achieved far below the thresholds for germination reduction, and thereby was an effective method for wheat grain for storage protection.

Keywords: *Aspergillus flavus*, aflatoxins, ozone gas, wheat grains, food safety

### **1. Introduction**

The *Aspergillus flavus* strain have been isolated worldwide from storage wheat grains (Berghofer et al., 2003; Riba et al., 2008; Roigé et al., 2009; Riba et al., 2010). *A. flavus* is aflatoxins (AFLs) producer of storage grains in tropical and subtropical climates, together with *A. parasiticus* and *A. nomius* (Hussein and Brasel, 2001). AFLs are the most commonly occurring and widely known mycotoxin contaminants. They are highly toxic and the four most commonly found in food are AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>.

The AFLs are distinguished by their fluorescence properties. Both AFB<sub>1</sub> and AFB<sub>2</sub> form blue fluorescence and AFG<sub>1</sub> and AFG<sub>2</sub> form yellow-green fluorescence under ultraviolet light (Hussein and Brasel, 2001). The toxicity of AFLs decreases in the following order: AFB<sub>1</sub>>

AFG<sub>1</sub>> AFB<sub>2</sub>> AFG<sub>2</sub> (Scussel, 2002). Among the aflatoxins, AFB<sub>1</sub> is the predominant and most potentially mutagenic, teratogenic and hepatocarcinogenic according to the International Agency for Research on Cancer (IARC, 1993). Moreover, can cause damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma (Scussel et al., 2002; Reddy et al., 2009).

Since 2011 the Brazilian regulation has proposed the maximum levels (ML) of 5 µg/kg for AFLs in cereals and products derived from cereals (Brasil, 2011). On the other hand, lower limits of AFLs totals and AFB<sub>1</sub> of 4 µg/kg and 2 µg/kg are fixed by the European Communities Commission for cereals and products derived from cereals (European Commission, 2006).

The strategies of chemical decontamination in food, as ozone (O<sub>3</sub>) gas application to reduce fungal growth and mycotoxins in wheat grains, are increasingly needed in order to reduce post-harvest losses and protection the consumer health. O<sub>3</sub> is a powerful antimicrobial agent due its potential oxidizing capacity (Khadre et al., 2001). That gas has attractive aspect in the food industry, is due to its decomposition to molecular oxygen without leaving residues. For instance, the US Food and Drug Administration (FDA) classified O<sub>3</sub> as *Generally Recognized as Safe* (GRAS) (FDA, 1982) to be used in bottled water and in food processing (Graham, 1997). Moreover, the potent disinfectant characteristics of O<sub>3</sub> is recognized by the Food and Agriculture Organization (FAO, 1994).

O<sub>3</sub> has been effectively used for control fungal growth at laboratory and small silos trials in food, as barley, wheat, fig, Brazil nuts and wheat (Kottapalli, 2005; Wu et al., 2006; Zorlügenç et al., 2008; Scussel et al., 2011; Beber et al., 2014; Savi et al., 2014a,b,c) and reduce mycotoxin contamination in peanut, fig, Brazil nuts, wheat and in field trials for artificially contaminated corn (Dwarakanath et al., 1968; Zorlügenç et al., 2008; McDonough et al., 2011; Scussel et al., 2011, Savi et al., 2014b,c).

Based on that, the aim of this study was to evaluate the effectiveness of O<sub>3</sub> gas treatment, against *A. flavus* strain growth, AFB<sub>1</sub> degradation and its effects on wheat seeds germination.

## 2. Materials and Methods

### 2.1. Fungi strain

*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 (Curitiba, Parana, Brazil). *Chemicals:* AFB<sub>1</sub> standard from Sigma Aldrich Chemicals (St. Louis, MO, USA); acetonitrile, methanol (both LC grade) and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil). Water was obtained from a Milli-Q system 18.2 MΩ/cm. *Other materials:* immunoaffinity columns from NeoColumn Aflatoxin DR (Direct Read), AOAC RI 081002 (2010) Neogen Corporation (Lansing, MI, USA).

### 2.3. Instruments

Light microscopy, 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) was utilized a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil). Others equipment were an O<sub>3</sub> generator, model OP-35-5L, Interzon (Jundiai, SP, Brazil).

High performance liquid chromatography (HPLC) equipment model 321, Gilson (Middleton, WI, USA) equipped with an isocratic pump model 805 and with fluorescence detection - FLD (excitation 335 nm and emission 440 nm). Injector (20 µl loop), Rheodyne (California, USA). The chromatographic column used was a C<sub>18</sub> 250 x 4.60 mm (length x inner diameter), 5 µm particle size, Phenomenex (Madrid Avenue, Torrance, USA).

#### *2.4. Samples artificial contamination before ozone treatment*

About 50 kg of wheat grains were collected from vertical silos (stored for three months) in 2012 post-harvest, from Brazilian Agricultural Research Corporation (Embrapa Wheat). Samples were received after cleaning and drying (up to a maximum of 60°C) steps 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 1x10<sup>3</sup> spores/mL of *A. flavus* was sprayed on wheat grains (25 g). For mycotoxin analysis, wheat grains were spiked using 100 µL of AFB<sub>1</sub> standards solutions (2.5 µg/mL). The samples presented 14% mc. In order to know the mc (2 g), the wheat grains were submitted to drying in an oven (105 ± 5°C) up to constant weight through gravimetric method according to 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 only two apertures: one for the input of O<sub>3</sub> 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 350 g of wheat grains. The O<sub>3</sub> gas generated was applied to pilots silos, divided in control group (no O<sub>3</sub> gas) and treated group (40 and 60 µmoL/moL) and were exposed of 120 and 180 min. The O<sub>3</sub> gas generator system followed the procedures detailed by Giordano et al. (2012) and Savi et al. (2014a) 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 O<sub>3</sub> generator was calibrated to reach a concentration of 40 or 60 µmoL/moL. The O<sub>3</sub> 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 O<sub>3</sub> gas produced was introduced through a tube into input aperture of each chamber. The control chamber (without O<sub>3</sub> gas) was ventilated with "room air" in the same flow (1 L/min).

The O<sub>3</sub> gas concentration measurement was performed by the iodinometric test. For this, the O<sub>3</sub> concentration was measured in each chamber by the titration method on the outlet of the O<sub>3</sub> 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*

The enumeration technique was applied to evaluate the fungi total load (Silva et al., 2010). Twenty five grams of each artificially contaminated wheat sample were added to 225 ml of 0.1% peptone dissolved in water under sterile conditions. The mixture was stirred on a rotary shaker for 2 min and the dilutions of 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup> were performed. Aliquots of 0.1 ml of each dilution were spread (in duplicate) on the surface of the PDA medium containing

chloramphenicol 100 mg/l and incubated for up 7 days at 28°C in the dark. The results were expressed in colony forming units per gram (CFU/g).

### 2.7. Aflatoxin analysis

Briefly, 12.5 g of each artificially contaminated wheat sample were ground in a laboratory mill and set into an industrial blender jar with 2.5 g of NaCl and 62.5 ml of LC grade methanol 70%. The mixture was blended for 2 minutes, followed by filtration. The filtrate (7.5 ml) was diluted in 15 ml of 10 mM PBS (phosphate buffered saline), followed by filtration and cleaning of 7.5 ml of this filtrate by immunoaffinity column (AFLs DR HPLC) in a flow rate of one drop per second. After washing the column with 10 ml of LC grade water, the toxin was slowly eluted with 0.5 ml of 100% LC grade methanol and 0.5 ml of LC grade water. 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 (water:methanol:acetonitrile (600:200:200 v/v/v) added 119 mg potassium bromide and 47.6 µL nitric acid). The extract (20 µL) was injected onto the LC/FLD System. The mobile phase was delivered in a flow constant rate of 1 ml/min. AFLs levels quantification was performed by measurement of peak area at AFLs retention time compared with the standard solutions used for calibration curve (0.035 to 4 µg/ml for AFB<sub>1</sub> with a correlation r = 0.990). Recovery was determined by spiking AFLs-free samples of wheat with AFLs concentrations of 2.5 and 4.0 µg/ml in the same day and same HPLC conditions.

### 2.8. Seed germination

The wheat seed germination was examined before and after O<sub>3</sub> treatment according to the method proposed by the International Seed Testing Association (1985). The seeds were allowed to germinate between two blotter paper layers at 25 to 27°C for 8 days and, consequently, the germination percentage was calculated. The tests were repeated four times and the averages were recorded.

### 2.9. Statistical analysis

The obtained data were analyzed using analysis of variance (ANOVA) followed by Bonferroni post-test or Dunnett's Multiple Comparison Test as appropriated. The data were expressed as mean ± standard deviation and the values of p<0.05 were considered statistically significant.

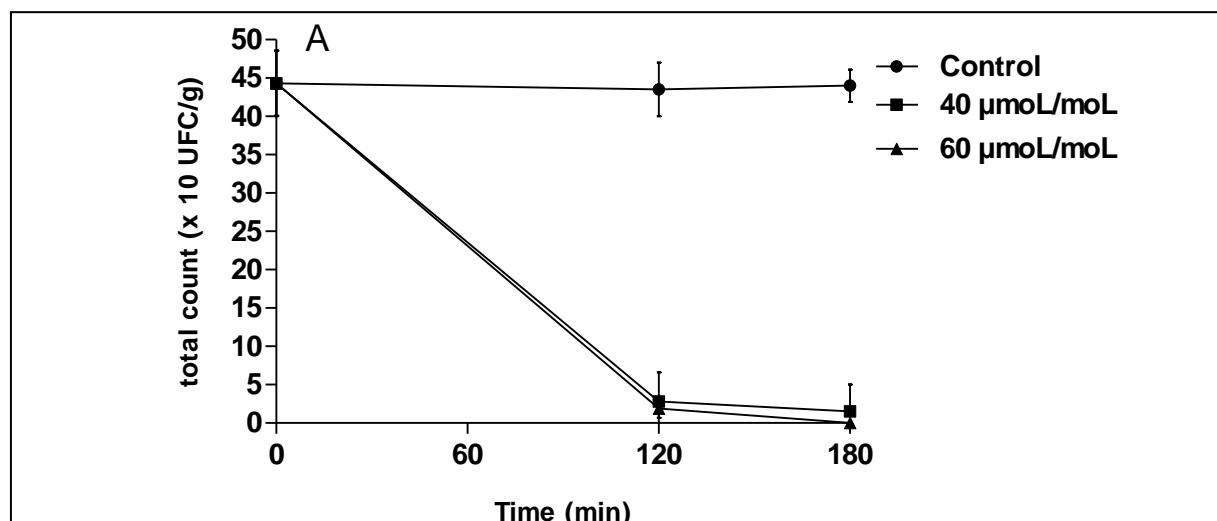
## 3. Results and Discussion

### 3.1. Ozone gas effect on mycological analysis

*A. flavus* growth reduction was observed in both periods of time and concentrations applied. In Group I (the lowest concentration: 40 µmol/mol) at the longest time (180 min) it was observed high growth reduction (to 1.5x10<sup>1</sup>UFC/g) when compared to the Group III (Control: 44.3x10<sup>1</sup>UFC/g), which represents 96.6% inhibition. On the other hand, in Group II (the highest concentration: 60 µmol/mol) also at the longest time of exposure, that fungi strain growth was completely reduced/destroyed (100%) (Fig. 1).

In a study *in vitro*, performed previously in our laboratory (Savi et al., 2014b), *A. flavus* presented more resistance to the treatment, nevertheless, the growth was significantly reduced after 120 min of O<sub>3</sub> gas exposure (60 mm) when compared to the Control group (78 mm). This study *in vitro* showed that O<sub>3</sub> exposition reduced the conidia germination and caused hyphae morphological alterations of the fungi.

Kells et al. (2001) observed O<sub>3</sub> efficacy against *A. parasiticus* in storage corn grains, showing that the number of viable *A. parasiticus* conidia on the grain surface was reduced around 63% when grain was exposed to 50 μmol/mol for 3 days. In study of Giordano et al. (2012), the O<sub>3</sub> gas treatment in concentration of 31 μmol/mol during 5 hours of exposure was successfully able to complete reduction *A. flavus* and *A. parasiticus* (initial: 6.7x10<sup>4</sup> CFU/g) in Brazil nuts, since day one after application.



**Figure 1** Ozone gas effect on *A. flavus* growth at different concentrations and exposure times (40 and 60 μmol/mol; 120 and 180 min). All treatments were statistically significant when compared to Control Group ( $p<0.05$ ) by Bonferroni post-test.

### 3.2. Ozone gas effect on mycotoxin analysis

The LC/FLD method for AFB<sub>1</sub> chromatographic separation and the validation parameters (linearity, limit of detection - LOD, limit of quantification - LOQ, reproducibility, repeatability and recovery) showed to be quite adequate. Under the chromatographic conditions applied, the retention time (R<sub>t</sub>) was 19±0.5 min for AFB<sub>1</sub>. Linearity was confirmed using the calibration curve, which was linear at 0.035-4 μg/mL for AFB<sub>1</sub>, with a correlation factor equal to 0.990. The LOD/LOQ (signal to noise ratio = 3/10) were 0.26/3.1 μg/kg for AFB<sub>1</sub>. The mean recovery of the extraction method considering the concentrations equal to 2.5 and 4.0 μg/mL was 87 and 90%.

Regarding the AFB<sub>1</sub> degradation, in both gas concentrations (40 and 60 μmol/mol) at 180 min exposure, it significantly reduced from 345.08 and 333.80 μg/kg (Group III:Control) to 105.13 and 92.91 μg/kg, corresponding to 69.5 and 72.2% degradation, respectively (Table 1).

Recently, in a study proposed by Luo et al. (2014), authors only verified that AFB<sub>1</sub> was easily degraded by O<sub>3</sub> in corn. When corn, with 13.47% mc, was exposed to O<sub>3</sub> at the concentrations of 40, 65 and 90 μmol/mol for 40 min, the AFB<sub>1</sub> degradation rates were 41.1, 56.2 and 88.1%, respectively. In another study which analysed the mechanism of AFB<sub>1</sub> degradation (McKenzie et al., 1997), the O<sub>3</sub> firstly reacts across the C8 and C9 double bond of the furan ring of AFB<sub>1</sub> through electrophilic attack based on the Criegee mechanism, and then produces intermediate products. The destruction of the C8 and C9 double bond on the furan ring means that the toxicity of AFB<sub>1</sub> has been reduced or even disappeared.

The efficiency on O<sub>3</sub> treatment against fungi and mycotoxins have been reported in the scientific literature, nevertheless, there are few studies with respect to O<sub>3</sub> gas treatment on

AFLs degradation in cereals. Ours studies showed that O<sub>3</sub> gas can be an efficient decontamination method for AFB<sub>1</sub> degradation in grains storage.

**Table 1** Aflatoxin degradation by ozone gas at two concentrations in 180 min of exposure.

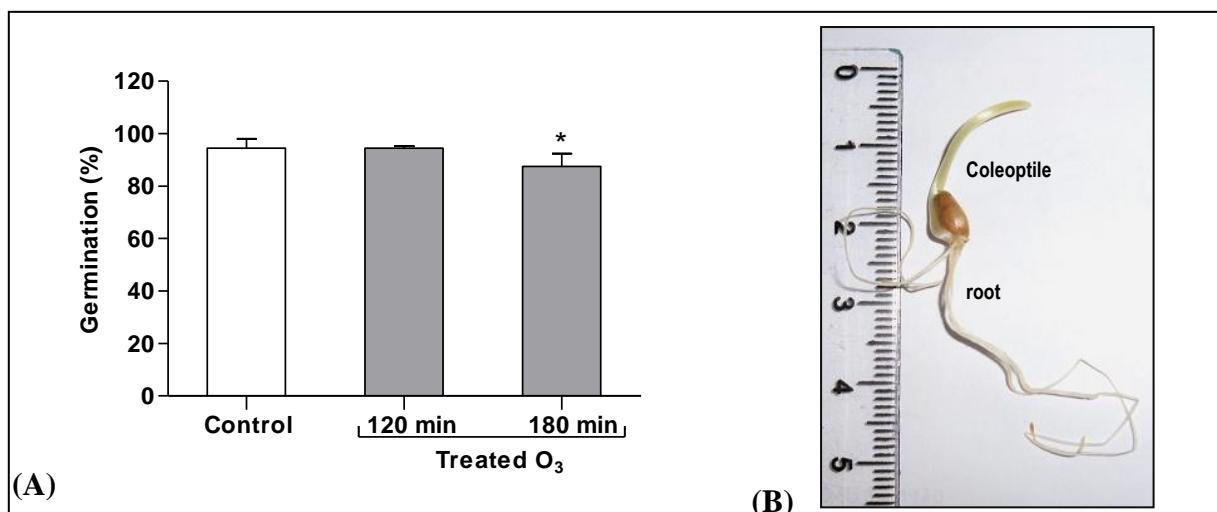
Ozone Treatment			AFB <sub>1</sub>	
Group	Concentration ( $\mu\text{mol/mol}$ )	Exposition time (min)	Mean ( $\mu\text{g/kg}$ )	Reduction (%)
Control	C <sub>I</sub>	no	NA	345.08
Treated	I	40	180	105.13
Control	C <sub>II</sub>	no	NA	333.80
Treated	II	60	180	92.91

\* Symbols indicate statistically significant when compared with Control Group p<0.05 by Dunnett's Multiple Comparison Test NA not applicable

### 3.3. Ozone gas effect on seed germination

Regarding the effect O<sub>3</sub> on wheat grain behavior, it was observed some germination capacity reduction (12.5%), no modifications on germinated wheat seeds coleoptile length and seminal root up to 180 min gas exposure/ 60  $\mu\text{mol/mol}$  concentration. After O<sub>3</sub> gas treatment considering 60  $\mu\text{mol/mol}$  after 120 min of exposition, no effect on wheat germination was observed at all (Fig. 2).

According to Wu et al. (2006), by applying different O<sub>3</sub> doses at 0.016, 0.065, 0.16 and 0.33 mg (g wheat)<sup>-1</sup> min<sup>-1</sup>, no effect on wheat germination was observed even after 60 min of ozonation. The wheat germination after applied O<sub>3</sub> dose of 0.98 mg (g wheat)<sup>-1</sup> min<sup>-1</sup> ozonation was barely affected in the last 15 min. However, considering 20 and 30 min of ozonation, the germination rate reduced to 85.4 and 80.0 %, respectively. In turn, after 45 min of ozonation, the wheat germination was reduced to 61.3 %.



**Figure 2** Wheat seed (A) germination (%) after 60  $\mu\text{mol/mol}$  ozone treatment for 120 min and 180 min. (B) root and coleoptile dimensions (symbols indicate statistically significant when compared with control group \*p<0.05 by Tukey test) and (B) germinated after 60  $\mu\text{mol/mol}$  ozone treatment for 180 min.

These results are in accordance with our study, showing that effective inactivation of fungi and mycotoxins had already been achieved far below the thresholds for germination reduction, and thereby showed to be an effective method for stored wheat grains protection.

#### 4. Conclusions

The O<sub>3</sub> gas treatment was effective on inactivating *A. flavus* growth, as well as degrading AFB<sub>1</sub> (more toxic among the AFLs) in wheat grains, especially for application during 120 min at 60 µmol/mol concentration (not alterations of seed germination). Considering the results, the treatment with O<sub>3</sub> gas could be an effective method for the food industry in the grains storage to avoid *A. flavus* growth and its mycotoxin degradation.

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