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The effect of ozone treatment on species of *Fusarium* growth in malting barley (*Hordeum vulgare* L.) grains

K.C. Piacentini*, G.D. Savi and V.M. Scussel

Mycotoxicology and Food Contaminants Laboratory (LABMICO), Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, P.O. Box 476, Florianopolis, SC, CEP 88034-001, Brazil; karim.piacentini@hotmail.com

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RESEARCH ARTICLE

Abstract

Fusarium is a large genus of filamentous fungi widely distributed around the world and often associated with contamination of mycotoxins, representing one of the most prevalent sources of food contamination, with strong, acute or chronic negative impacts on human health. The complete elimination of fungi-contaminated commodities may not be indeed achievable, but a reduction in these is essential for final product quality as well as for consumer health. For this reason, the objective of this study was to evaluate the efficacy of the ozone (O_3) treatment in *Fusarium* species and, in addition, to investigate the effect of O_3 in malting barley seed germination. The O_3 gas was applied to pilot cylinders, divided into control, without O_3 gas application, and treated groups, with concentrations of 40 and 60 mg/kg. These were exposed for 30, 60, 120 and 180 min. The species that demonstrated the greatest reduction after the O_3 treatment was *Fusarium poae*, represented by 93.3%, followed by *Fusarium graminearum*, with 92.6%. Germination of the malting barley seed was not affected.

Keywords: *Fusarium* species, ozone, malting barley, treatment

1. Introduction

Fusarium is a large genus of filamentous fungi widely distributed around the world (Backhouse *et al.*, 2001), representing one of the most prevalent sources of food contamination. *Fusarium graminearum* is the species most frequently responsible for scabs in grains, especially in malting barley (Osborne and Stein, 2007). Furthermore, other species, such as *Fusarium verticilioides*, *Fusarium poae*, *Fusarium oxysporum* and *Fusarium semitectum* can also be found (Gonzalez Pereyra *et al.*, 2011; Ivic *et al.*, 2011; Maenetje and Dutton, 2007; Schwarz *et al.*, 2001) and may interfere in the quality of the grains and the final product.

Barley infection by these pathogenic fungi negatively affects crop health and leads to loss of yield, decreasing germination capacity and worsening of grain malting quality (Oliveira *et al.*, 2012). Although micromycetes of *Fusarium* spp. are known as 'field fungi', they can also grow during storage under favourable conditions (Oliveira *et al.*, 2012; Vaughan *et al.*, 2005). These are responsible for raw and processed grains deterioration and can cause discoloration and reduction of nutritional values, especially when exposed to optimal environment conditions as high temperature and humidity. These fungi have often been associated with contamination of mycotoxins as deoxynivalenol (DON), fumonisins (FBs) and zearalenone (ZEA), which are known to frequently contaminated malting barley grains (Nielsen *et al.*, 2014; Piacentini *et al.*, 2015a). Some mycotoxins are responsible by cause disease and death in humans and other animals. For example, the exposition of DON can cause acute and chronic effects such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity. The FBs are associated to several mycotoxicoses, including equine leukoencephalomalacia, swine pulmonary oedema and experimental rats kidney and liver cancers; and fumonisin B_1 is classified as possible carcinogen to humans. In addition, ZEA is also a *Fusarium* metabolite with potent estrogenic activity (IARC, 1993; Scussel *et al.*, 2011a).

The mycotoxins may effectively occur at different stages of the brewing process and thus be transferred from malt into final products. The occurrence of these mycotoxins in beer, for example, has been examined recently (Bauer *et al.*, 2016; Piacentini *et al.*, 2015b).

The complete elimination of fungi-contaminated commodities may not be indeed achievable (FAO/WHO, 2003), but a reduction is essential for final product quality as well as for the consumer.

Nowadays, new decontamination technologies are used, highlighting the ozonation of food products. Ozone (O_3) is a powerful antimicrobial agent due to its potential oxidising capacity (Khadre and Yousef, 2001). It is currently used as a disinfectant for microorganisms and viruses, odour and taste removal, colour and decomposition of organic matter (Cataldo, 2008; Christ *et al.*, 2016; Karaca and Velioglu, 2009; Karaca *et al.*, 2010).

A number of surveys have been carried out with O_3 to control the growth of several fungi in food or grains, in a laboratory environment (Kottapalli, 2005; Scussel *et al.*, 2011b; Wu *et al.*, 2006; Zorlugenç *et al.*, 2008). The idea presented in these studies is to reduce fungi and mycotoxin contamination in peanuts, figs, Brazil nuts and wheat, and in field trials researching artificially and naturally contaminated corn (McDonough *et al.*, 2011; Savi *et al.*, 2014; Scussel *et al.*, 2011b; Zorlugenç *et al.*, 2008).

The major advantage of O_3 treatment is related to the molecular oxygen decomposition without leaving residues. The US Food and Drug Administration (FDA) classifies O_3 for treating bottled water as 'generally recognised as safe' (GRAS) (FDA, 1982), and it has been affirmed as GRAS for use in food processing (Graham, 1997). Moreover, the Food and Agriculture Organization (FAO, 2014) recognises the potent disinfectant characteristics of O_3.

Based on this, the objective of this study was to evaluate the efficacy of the O_3 treatment in five species of *Fusarium* (*F. graminearum*, *F. verticilioides*, *F. poae*, *F. oxysporum*, and *F. semitectum*) and, in addition, to investigate the effect of O_3 in malting barley seed germination.

It is necessary to mention that there are no studies evaluating the O_3 treatment efficiency in some of the *Fusarium* species studied. This technique and the surveys related to it, can be promising to the industry in order to decrease the fungi contamination in the grains used for brewing, and also to avoid the production of mycotoxins.

2. Materials and methods

Materials

Culture media

Potato dextrose agar (PDA), malt extract agar (MEA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil). Czapek-dox, 25% glycerol nitrate (GN25), Czapek yeast extract (CYA) media and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil).

Instruments

To accomplish the mycological tests the following equipment was required: autoclave, Phoenix (Araraquara, SP, Brazil); rotary shaker, Marconi (Piracicaba, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil) and microbiological incubator, Quimis (Diadema, SP, Brazil). In addition, the O_3 gas generator (5-60 μM/M), corona discharge, Interzone (Jundiai, SP, Brazil); impurities remover (Alvorada, RS, Brazil); flow meter, Protec (Sao Paulo, SP, Brazil) were obtained. Finally, to analyse the moisture content (mc) the following equipment was used: a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil).

Fungi strains

Fusarium species were obtained from the culture collection of the Laboratory of Mycotoxicology and Food Contaminants in the Federal University of Santa Catarina (Florianopolis, SC, Brazil). The isolated strains were sub-cultured on PDA, MEA, GN25 and CYA media and the species identification were performed through microcultive in carnation leaf agar (Weber and Pitt, 2000). The isolates were examined under the light microscope (100× and 400× magnification) and the species confirmation were carried out according to the taxonomic keys and guides available (Nelson *et al.*,1983; Pitt and Hocking, 1997).

Samples

About 30 kg of malting barley grains were collected from vertical silos in 2014, from the Brazilian Agricultural Research Corporation (Embrapa Wheat). Samples were received after cleaning and drying (maximum 60 °C) in the storage unit. Then, they were packed in a polyethylene bag and stored at 4 °C for analysis at the laboratory. In order to know the mc (2 g) of the malting barley grains, they were submitted to drying in an oven (105±5 °C) up to constant weight by means of gravimetric method (AOAC, 2005) and showed average values of 13%.

Methods

Samples artificial contamination

The malting barley samples (25 g) were contaminated with a solution of Tween 80 (10 ml) containing 3.3×10^5 ml of *F. semitectum* spores, 7×10^5 ml of *F. verticilliodes* spores, 1×10^4 ml of *F. graminearum* spores, 8.5×10^4 ml of *F. oxysporum* spores and 4.1×10^5 ml of *F. poae* spores. The spores of each species were counted by means of the Neubauer chamber. The malting barley grains after contamination had a mc equal to 16%.

Ozone gas treatment

Laboratory silo preparation

The laboratory pilot cylinders used were 25×10 cm (length×diameter) with two apertures: one for the input of O_3 gas (bottom) and one for the output (top). The cylinders were filled with 350 g of malting barley grains. At the top (above grains), a polyamide screen surface was placed in order to support the grains and on which the mycological (25 g) analyses would be performed after O_3 gas application.

The O_3 gas was briefly applied to pilot cylinders, divided into control (no O_3 gas) and treated groups (40 mg/kg and 60 mg/kg). They were exposed for 30 min, 60 min, 120 min and 180 min, in a room at 25±0.5 °C. Based on these analyses, the most effective concentration against the *Fusarium* species (60 mg/kg for 180 min) was chosen for malting barley seed germination analysis.

Ozone application

The O_3 gas generator system followed the procedures detailed by Giordano *et al.* (2012) with minor modifications. First, the compressed air pump was connected to a device responsible for clearing the air impurities and, consequently, getting rid of solid particles and humidity. The filtered air was then driven to the adjusted flow meter at 1 l/min and the O_3 generator was calibrated to reach a concentration of 40 or 60 mg/kg.

The O_3 production by the generator (5-60 mg/kg) used the corona discharge process, in which an electrical discharge caused by the passage of air or pure oxygen (O_2) between the two electrodes generates the conversion of O_2 to O_3. Therefore, the O_3 gas produced was injected through a tube into the input aperture of each test chamber while the control chambers were ventilated with 'room air' at the same flow rate (1 l/min).

Measuring the ozone concentration

The O_3 gas concentration was measured by the iodinemetric titration test from the output of the O_3 generator. The gas was bubbled into potassium iodide solution (50 ml) acidified with 2.5 ml of sulphuric acid 1 N (pH below 2.0) and titrated with sodium thiosulfate (0.005 N) using a starch solution as the indicator, according to the American Public Health Association (1999).

Mycological analyses after ozone treatment

The enumeration technique was applied to evaluate the total fungi 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 in a rotary shaker for 2 min and dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained. Aliquots of 0.1 ml of each dilution were spread (in duplicate) over the surface of PDA medium plates containing 100 mg/l chloramphenicol 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).

Seed germination

The malting barley seed germination was examined before and after O_3 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 25-27 °C for 8 days and the percentage of germination was calculated. Germination rate is estimated by the formula: germination percentage = seeds germinated/ total seeds × 100. The tests were repeated four times and the averages were recorded.

Statistical analysis

All data were analysed taking into account the analysis of variance (ANOVA) and, additionally, considering the Bonferroni as post-test. Here, the main results were expressed in terms of mean ±standard deviation and values of $P<0.05$, $P<0.01$ and $P<0.001$ were considered statistically significant.

3. Results

Mycological analysis after ozone treatment

Figure 1 displays information about the O_3 gas effects on *Fusarium* species at different times of exposure and at different concentrations. Firstly, it is clear that all species had a decrease in their availability after the O_3 treatments (min reduction 8.2%, max reduction 93.3%), as observed in Supplementary Figure S1.

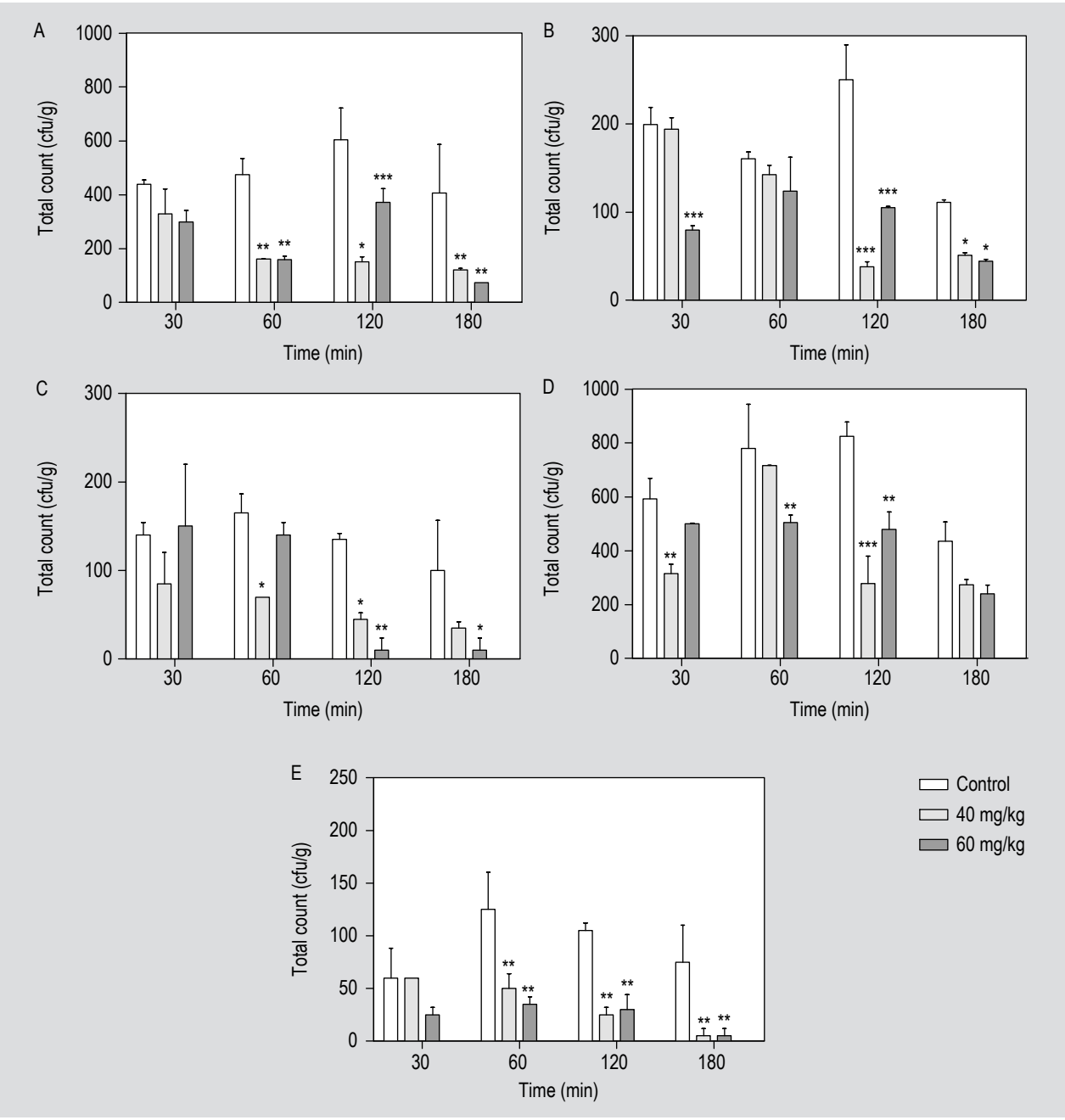

Figure 1. Ozone gas effects on the *Fusarium* species: (A) *Fusarium semitectum*, (B) *Fusarium verticilioides*, (C) *Fusarium graminearum*, (D) *Fusarium oxysporum*, and (E) *Fusarium poae*, during 30 to 180 min at 40 mg/kg and 60 mg/kg (P*<0.05; ** *P*<0.01; *** *P*<0.001).**

Considering *F. semitectum*, this species was significantly reduced after 60, 120 and 180 min in both concentrations, 40 mg/kg and 60 mg/kg of O_3 treatment, compared with the control group (Figure 1A). In the case of *F. verticilliodes*, there was a significant reduction at 30, 120 and 180 min of viable spores, highlighting 120 min (P<0.001) and 180 min (P<0.05) in both concentrations (Figure 1B).

In contrast, for *F. graminearum*, the O_3 treatment was significantly effective at 60, 120, 180 min, emphasising 120 min in both concentrations (40 mg/kg: P<0.05; 60 mg/kg: P<0.01). This represents a 66.7 and 92.6% reduction, respectively. Furthermore, only at 180 min at 60 mg/kg (P<0.05) did the availability of the spores show a decrease, representing a 90% reduction (Figure 1C).

Regarding *F. oxysporum*, there were significant reductions of the viable spores at 30, 60 and 120 min, with a more accentuated decrease after 120 min of exposure at 40 mg/kg (P<0.001), representing a 66.3% reduction (Figure 1D).

Finally, the last *Fusarium* species treated in malting barley was *F. poae*. The exposure time and concentration that showed the most significant reduction of the viable spores were at 60, 120 and 180 min, at both concentrations. All reductions were statistically significant ($P<0.01$), compared to the control group. It is necessary to mention that this species demonstrated the greatest decrease (93.3%), compared to the other species (Figure 1E).

Malting barley seed germination

Concerning malting barley seed germination after the O_3 treatment, no effect was observed after 180 min of exposure at a concentration of 60 mg/kg. Figure 2 shows the data of seminal root and coleoptile length of the control and O_3 treated groups. In other words, the seed germination capacity was as expected, a minimum of 95%.

4. Discussion

The current study was similar to research proposed by Mylona *et al.* (2014) concerning *F. verticillioides*, inoculated conidia in maize. The results demonstrated that in both O_3 gas concentrations applied (100 and 200 mg/kg), the total of fungal cfu/g was significantly reduced. In the same study, the treatments were also carried out *in vitro*, and the exposure showed an immediate effect (100%) after 30 min of treatment at 200 mg/kg.

Additionally, in a previous study by Savi and Scussel (2014) *in vitro* showed good efficacy O_3 gas treatment against *F. verticillioides* spores and slight inhibition of mycelial growth with some morphological changes in hyphal permeability.

The same idea can be seen with *F. graminearum*, which showed a significant reduction in a study proposed by Kottapalli *et al.* (2005) in malting barley, which represented 24-36% at a concentration of 11 and 26 mg/g of O_3 gas, respectively, for 15 min. Furthermore, in another *in vitro* study, it was verified that *F. graminearum* exposed for 60 min at 60 mg/kg of O_3 gas did not show any growth until the eighth day of incubation (Savi and Scussel, 2014).

The O_3 application in efficient concentration and time of exposure is promising to avoid the fungal growth and to help to reduce the risk of mycotoxin production. To our knowledge, no previous studies in the literature have examined the efficacy of O_3 treatment in *F. semitectum*, *F. oxysporum* and *F. poae*. In summary, the data discussed earlier on O_3 exposure showed some promising results in all *Fusarium* species studied in malting barley, emphasising high levels of decontamination compared to some studies.

Considering seed germination, other surveys have reported similar results with O_3 treatments, which showed no effects on malting barley and wheat germination (Savi *et al.*, 2014; Wu *et al.*, 2006). In addition, the germination capacity can only be influenced by high concentrations of O_3 at long treatment times (Allen *et al.*, 2003).

5. Conclusions

The experimental results revealed that the O_3 was capable of effectively inactivating all the *Fusarium* species studied associated with malting barley. The species that demonstrated the greatest reduction after the O_3 treatment was *F. poae*, represented by 93.3%, at 60 mg/kg at 180 min. Taking into account the malting barley germination after the O_3 exposures, no effects were observed in the grains.

According to the current study, it is possible to mention that the O_3 is a strong antimicrobial agent and can be a promising technique, with several potential applications in the food industry, mainly for grains.

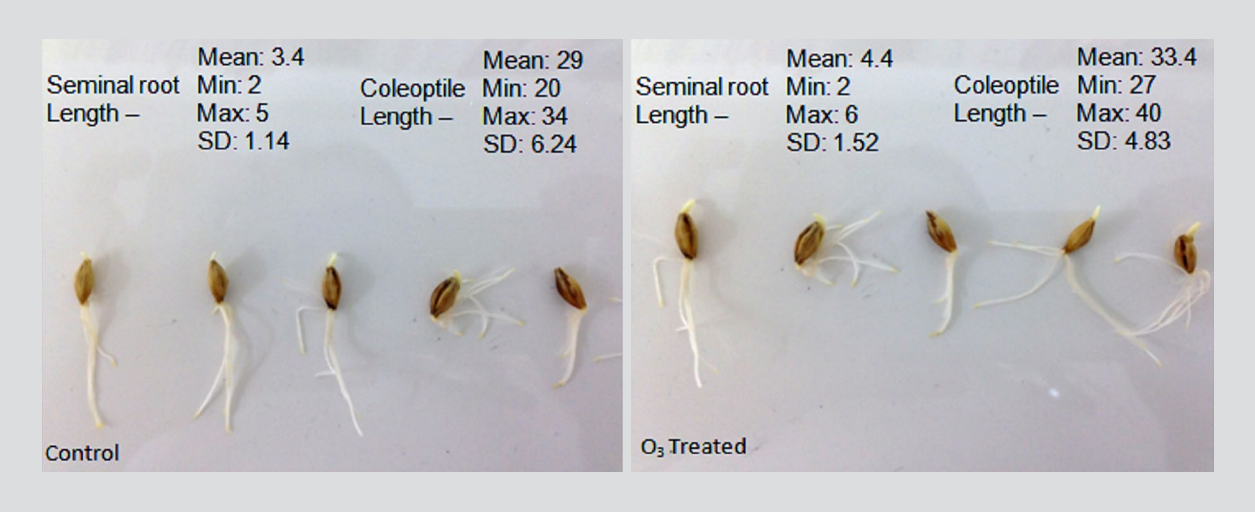

Figure 2. Ozone gas effects on the malting barley seed germination showing no effect in the seminal root and coleoptile length.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/QAS2016.0881.

Figure S1. Fungi total count (cfu/g) of *Fusarium* genera growth on PDA medium of the control and O_3 treated groups.

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