Kreibich HH, Christ D, Silva J, Giovana S, Maria, Scussel VM (2016) Antifungal efficiency of ozone on cocoa beans (*Theobroma cocoa*) inoculated with *Aspergillus flavus*. Pp. 229–234. In: Navarro S, Jayas DS, Alagusundaram K, (Eds.) Proceedings of the 10th International Conference on Controlled Atmosphere and Fumigation in Stored Products (CAF2016), CAF Permanent Committee Secretariat, Winnipeg, Canada.

# Antifungal efficiency of ozone on cocoa beans (*Theobroma cocoa*) inoculated with *Aspergillus flavus*

H H KREIBICH, D CHRIST, J SILVA, S GIOVANA, MARIA, V M SCUSSEL*

*Laboratory of Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, P.O. Box 476, Florianopolis, SC, Brazil*

ABSTRACT

The antifungal properties of ozone ($O_3$) gas on *Aspergillus flavus* L. strain inoculated in dry cocoa beans (*Theobroma cocoa* L.) was investigated by applying a factorial design ($2^2$), with gas concentrations of 20, 40 and 60 µmol/mol, exposure times of 30, 105 and 180 min and 30 days storage. Trials were carried out with two main cocoa groups, viz. Control group (C –no gas treated) and $O_3$ treated group (I, II and III - for the 3 gas concentrations, respectively). The $O_3$ gas was applied into the silos through an inlet aperture, left standing inside the silos (for three exposure times) and then cocoa portions had the gas antifungal efficienc , humidity and lipid stability variation measured (both, just after application at day zero and after 30 days of storage). As expected, a fungal reduction was observed with the increasing of the $O_3$ concentration (Groups I to III) and gas exposure time. The response surface showed a 88% inhibition in spores of *A. flavus* immediately after the maximum gas concentration and time of exposure reached cocoa beans, followed by total inhibition as the time of storage increased (when compared to Group C). The cocoa moisture content (previous 6.7%) reduced after treatment (6.1%) and the $O_3$ gas conditions showed no lipid oxidation in cocoa bean during the storage period. In the present study, the most effective treatment obtained was in group III (60 µmol/mol concentration) at the longer $O_3$ exposure (180 min), as the response surface revealed 100% inhibition in spores of *A. flavu*s.

**Key words:** *Aspergillus flavu*s, Cocoa, Contamination, Ozone gas, *Theobroma cocoa*

Cocoa (*Theobroma cocoa* L.) trees are grown in very warm and humid tropical climates— environment that allows their proper growth and development of desirable fruit/seed composition. However, these conditions (high temperature and humidity) may lead to spoilage and toxigenic growth of fungi (Ostovar and Keeney, 1973; Schwan and Wheals, 2004; Kreibich et al., 2014, 2016 a,b). Apart from their deteriorative influence and consequent reduction in cocoa bean and chocolate sensory-quality, the presence of toxigenic fungi strains is also of health concern (regarding toxins formation). *Aspergillus*, *Penicillium* and *Fusarium* produce toxic secondary metabolites (mycotoxins) in food. Several of them are mutagenic, teratogenic, and carcinogenic for humans and animals (IARC 1993, Scussel 2002, 2004).

*Corresponding author e-mail: *vildescussel_2000@yahoo*.co.in

Decontamination methods that apply heat (sterilization) can cause the development of undesirable compounds, nutrient loss, toxic side reactions and changes in the physical, mechanical and chemical properties. Therefore, there has been a growing interest on developing different procedures, such as ultraviolet radiation or the application of microwaves, which are expensive treatments, lead to food alterations and low consumer acceptance (Copetti et al., 2011, Codex Alimentarius Commission 2013).

A green method that has been studied, showing good results is the application of ozone ($O_3$) (Calvo et al., 2007; Savi and Scussel 2014; Savi et al., 2014 a,b; Savi et al., 2015; Christ et al., 2016). The $O_3$ gas, besides sanitizing stored grains and nuts, is robust and safer than the conventional procedures and acts on a large number of microorganisms (Graham et al., 2011; Giordano et al., 2011; 2012). It controls

Table 1 Factors levels applied in the cocoa ozone gas and exposure time experimental design

| Factor[a] | Symbol | Levels (2)[b] | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| $O_3$ concentration (µmol/mol) | $O_3$ | 20 | 40 | 60 |
| $O_3$ exposure time (min) | T | 30 | 105 | 180 |

[a]ozone, [b] factorial design ($2^2$)

Table 2 Levels of ozone *Aspergillus flavus* spores decontamination in dry cocoa bean at different gas and time conditions

| $O_3$[a] treatment[b] | | *Aspergillus flavus* | | | | | | Humidity | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Count[c] (×10 CFU/g) | | | | Reduction[d] (%) | | | | | |
| Concentration (µmol/mol) | Time (min) | Control* | | $O_3$ treatment | | Storage (Day) | | m.c. (%) | | $A_w$ | |
| | | Zero[δ] | 30[th] | Zero[δ] | 30[th] | Zero[δ] | 30[th] | Zero[δ] | 30[th] | Zero[δ] | 30[th] |
| 20[e] | 30[e] | 56 | 37 | 25 | 5 | 55.4 | 86.5 | 6.0 | 6.3 | 0.61 | 0.66 |
| 60[g] | 30[g] | 55 | 42 | 14 | 2 | 74.5 | 95.2 | 6.9 | 6.4 | 0.61 | 0.65 |
| 20[e] | 180[e] | 51 | 39 | 16 | 3 | 68.6 | 92.3 | 6.8 | 6.4 | 0.49 | 0.61 |
| 60[g] | 180[g] | 53 | 40 | 7 | NG** | 86.8 | 100.0 | 7.5 | 5.7 | 0.52 | 0.60 |
| 40[f] | 105[f] | 51 | 47 | 17 | 2 | 66.7 | 95.7 | 6.4 | 6.3 | 0.53 | 0.53 |
| 40[f] | 105[f] | 50 | 43 | 20 | 2 | 60.0 | 95.3 | 6.5 | 5.5 | 0.56 | 0.57 |
| 40[f] | 105[f] | 53 | 35 | 18 | 1 | 66.0 | 97.1 | 6.9 | 6.2 | 0.51 | 0.61 |

[a]ozone; [b], $2^2$ factorial design; [c]mean; [d]efficiency; mc: moisture content; aw: water activity; [e]Group I, [f]Group II; [g]Group III; *no gas treatment; **no growth; [δ]Day

fungal growth, degrading mycotoxins and pesticide residues in raw and processed foods, either at post-harvest stage or in the industry (without reducing the nutritional value) (Ong et al., 1996; Mendez et al., 2003; Tiwari et al., 2010; Mcdonough et al., 2011; Scussel et al., 2011; White et al., 2013; Savi and Scussel 2014; Savi et al., 2015).

Considering the difficulty that dry cocoa bean producers and importers face on fungi development during storage and long-term transport (ship), an experiment was conducted to study the $O_3$ gas atmosphere as a method for inactivation of *A. flavus* (at different concentration and time of exposure) during storage of dry post-fermentation cocoa beans.

## MATERIALS AND METHODS

The samples dry post-fermentation cocoa beans (14 kg from 2014 harvest) were utilized for study. Antifungal property of $O_3$ gas was investigated by applying a factorial design ($2^2$), utilizing gas concentrations (20, 40 and 60 µmol/mol), time of exposure (30, 105 and 180 min) and 30 days storage. Trials were carried out with two main Groups of cocoa ($O_3$ treated: I, II and III for each gas concentration, respectively and Control: C– not gas treated), as per Giordano et al. (2011). The humidity (m.c.) was determined by the AOAC gravimetric method 31.1.02 (AOAC, 2005), water activity (a.w.) by measuring each sample in the Aqua lab apparatus at 25°C, and rancidity according to the official method of the Ministry of Agriculture (MAPA, 1981).

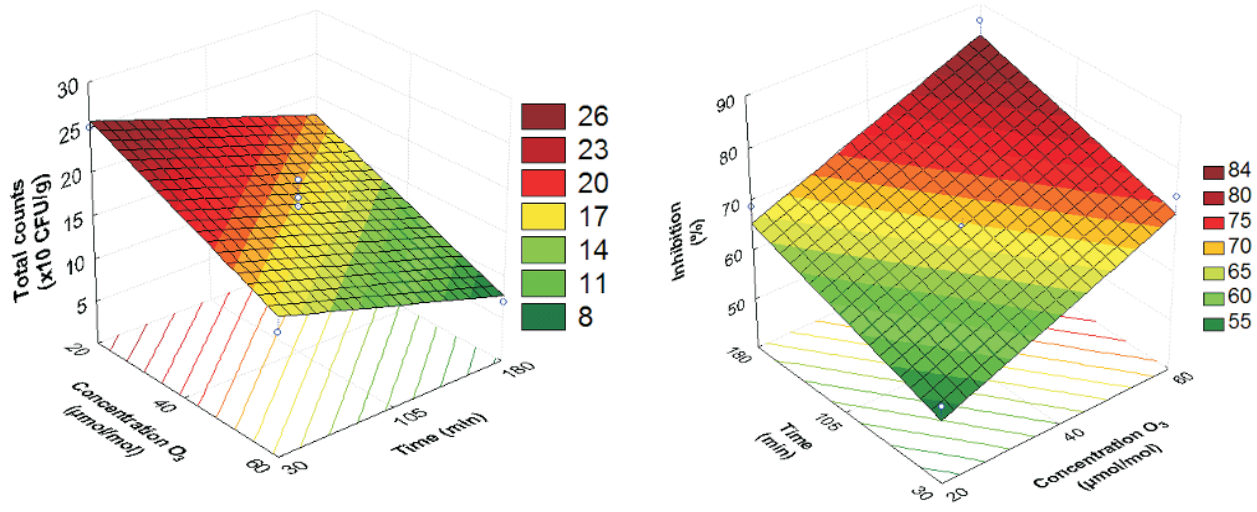
The main effects and the interactions of variables on responses, determining the significant factors ($P < 0.1$) and adjusting a model (Eq. 1) to correlate variables and their responses. The significant coefficients of the model were evaluated by the "t" test, and the data were subjected to an analysis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

$$y = b_0 + b_i X_i + b_j X_j + b_j X_i X_j \quad \text{(Eq.1)}$$

where $b_0$, 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.

## RESULTS AND DISCUSSION

The data obtained after $O_3$ treatment showed that the gas reduced the load of fungal spores and inactivated the spores. It was dependent on the concentration and time of exposure applied. Effect of $O_3$ gas on total fungal load on cocoa beans (Groups I, II and III) was evaluated at day 0 and after 30 days of storage, together with humidity (m.c., aw) under the factorial design ($2^2$), and are shown in Tables 1 and 2 and Fig. 1 and 2.



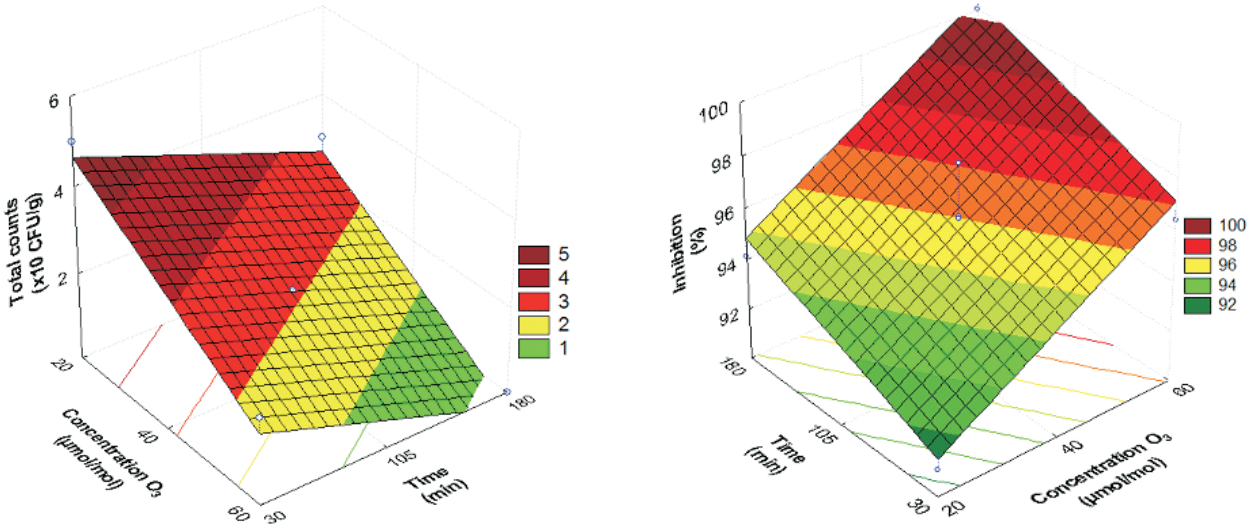

(a) (b)

Fig. 1. Response surface of $O_3$ concentration (20, 40, 60 µmol/mol) and time of exposure (20, 105, 180 min) in cocoa *Aspergillus flavus* inoculated on (*a*) total load (*CFU/g*) inhibition and (*b*) total reduction (*%*) at day zero.

*Effect of $O_3$ gas on total load of A. flavus*

*CFU/g reduction (Day zero)*: The effect of $O_3$ gas on the fungi spore inactivation (CFU/g) showed good reduction in spores since the effect at day zero, was significan . An increase in the concentration of $O_3$ from 20 to 60 µmol/mol decreased fungi spores (Table 2). Increase in the cocoa bean ozonation time (from 30 to 180 min) also showed fungi reduction. The mathematical model (Eq. 1) to estimate the total counts in CFU/g depending on the $O_3$ concentration ($b_i$=5.0), the exposure time ($b_j$=4.0) and mean ($b_0$=16.7) was found to be predictive test by "F", with a correlation coefficient ($r^2$) of 0.89. The surface response (Fig 1a) represents the model.

*Efficiency*: The fungal spore inactivation efficiency of the $O_3$ was significant. Increasing the gas concentration (from 20 to 60 µmol/mol) increased the fungal strain degradation of 18.7%. Similarly, increasing the exposure time of ozone (from 30 to 180 min) decreased *A. flavus* degradation, i.e. 86.8% when compared to Group C (Table 2). The mathematical model for estimating the gas efficiency depending on the $O_3$ concentration ($b_i$=9.4), the exposure time ($b_j$=6.4) and mean ($b_0$=68.3) was found to be a predictive by the test "F", with a correlation coefficient ($r^2$) of 0.82. The surface response represents the model (Fig 1b).




(a) (b)

Fig. 2. Response surface of $O_3$ concentration (20, 40, 60 µmol/mol) and time of exposure (20, 105, 180 min) in cocoa *Aspergillus flavus* inoculated on (*a*) total load (CFU/g) inhibition and (*b*) total reduction (%) at day 30.

Our results confirm the findings of Ciccarese et al. (2007), Scussel et al. (2011), Alencar et al. (2012), El-Desouky et al. (2012), Giordano et al. (2012), Savi et al. (2014b) in other food types (peanuts, Brazil nuts, pea and wheat).

*CFU/g reduction* (*day* 30): The effect of $O_3$ concentration (-3.0) and exposure time (-2.0) on fungal spore inactivation was significant. Increase in the concentration of $O_3$ from 20 to 60 µmol/mol, resulted in a reduction from 4.0 × 10 to 1.0 × 10 CFU/g, (3 × 10 CFU/g spores destruction) and then with an increase of the ozonation time (from 30 to 180 min), there was also a reduction from 3.5 × 10 to 1.5 × 10 CFU/g. The mathematical model for estimating the total counts depending on the $O_3$ concentration ($b_i$=1.5), the exposure time ($b_j$=1.0) and mean ($b_0$=2.14) was found to be predictive test by "F", with a $r^2$ of 0.88. The surface response (Fig 2a) represents the model.

*Efficiency*: After 30 days of storage, the viable spores reduced 5.6% (Concentration $O_3$) and 3.3 (Time), (significant for the "t" test). Increasing the concentration of $O_3$ from 20 to 60 µmol/mol increased the effi iency of 5.6%. In addition, also with the longer ozonation exposure time (from 30 to 180 min), it registered spore inactivation efficiency (increasing 3.3%), i.e.100% degradation compared to Group C (Table 2). These findings confirm the results of Ciccarezi et al. (2007), Giordano et al. (2011, 2012), Alencar et al. (2012), El-Dzouky et al. (2012), Savi et al. (2014b) in nuts and grains. The mathematical model for estimating the efficiency depending on the concentration of $O_3$ ($b_i$=2.8), the exposure time ($b_j$=1.7) and mean ($b_0$=95.9) was found to be predictive test by "F", with a $r^2$ of 0.88. The surface response (Fig 2b) represents the model.

*Humidity and lipid stability*

*Humidity*: The mc of the cocoa beans after treatment with $O_3$ reached a mean of 6.7% (min 6.0; max 7.5). After 30 days of storage, cocoa had a mean m.c. of 6.1% (min 5.5; max 6.4), being statistically similar for all treatments ($P$<0.1).This variation in mc of 0.6% after the $O_3$ treatment and 30 days of storage, respectively, is probably because of the environment temperature that somewhat reduced (min 19; max 25°C) and the relative humidity variations (min 64; max 82%) by the time the experiment was carried out, which established a new equilibrium of mc. There was no statistical difference among the m.c. throughout the $O_3$ treatments, which corroborates the fact that there was no m.c. influence on the cocoa bean treatments responses. Similar to the m.c. of the cocoa, $a_w$ value of 0.55 after the $O_3$ treatment and 0.6 after 30 days of storage, did not differ between treatments in both periods of time ($P$ <0.1).

*Lipid stability to $O_3$*: The data obtained with Kreiss test indicated that $O_3$ treated cocoa showed no lipid oxidation. The analysis was repeated after 30 days of storage, and the samples, again showed no rancidity development. Others studies on $O_3$ decontamination utilizing products containing high lipid contents (Brazil nuts and peanuts) also reported no changes in lipids stability (Scussel et al., 2011; Giordano et al., 2012; Alencar et al., 2011; Chen et al., 2014).

## CONCLUSION

*Aspergillus flavus* can be efficiently destroyed by the $O_3$ gas under the conditions of 60 µmol/mol and 180 min. No lipid oxidation was recorded within the 30 days after the $O_3$ application. The effect of increasing $O_3$ concentration was higher than the increase on exposure time for the destruction of *A. flavus* in the cocoa beans substrate. Perhaps this is the first work that has assessed the effect of $O_3$ on *A. flavus* in cocoa.

## REFERENCES

Alencar ER, Faroni LRD, Martins MA, Costa AR, Cecon PR (2011) Decomposition kinetics of gaseous ozone in peanuts. Eng. Agric **31**: 930–9.

Alencar ER, D'antonino LRF, Ferreira SNF, Da silva WA, Carvalho, MC (2012) Efficacy of ozone as a fungicidal and detoxifying agent of aflatoxins in peanuts. Journal of Science Food Agriculture **4**: 899–905.

AOAC - Association Official Method of Analysis of AOAC Internacional (2005) (In) Thiex, NJW (ed). Officia Methods of Analysis. 18ed Maryland.

Ardhana MM, Fleet GH (2003) The microbial ecology of cocoa bean fermentations in Indonesia. International Journal of Food Microbiology **86**: 87–99.

Calvo L, Muguerza B, Cienfuegos-jovellanos, E (2007) Microbial inactivation and butter extraction in a cocoa derivative using high pressure $CO_2$. The Journal of Supercritic Fluids **1**: 80–7.

Chen RF, Li PW, Zhang W, Ding XX, Zhang Q, Li M, Wang YR, Xu BC (2014) Effect of ozone on aflatoxins detoxification and nutritional quality of peanuts. Food Chemistry **146**: 284–8.

Christ D, Savi GD, Scussel VM (2016) Effectiveness of Ozone Gas in Raw and Processed Food for Fungi and Mycotoxin Decontamination - A Review. Journal of Chemistry Biology and Physical Science **6**: 326–48, 2016.

Ciccarese F, Sasanelli N, Ciccarese A, Ziadi T, Mancini L (2007) Seed disinfestation by ozone treatments.IOA Conference and Exhibition, October 29 – 31, at Valencia, Spain.

CODEX ALIMENTARIUS COMMISSION (2013) Proposed draft code of practice for the prevention and reduction of ochratoxin A contamination in cocoa. Joint FAO/WHO Food Standards Program,

FAO, Rome (ftp://ftp.fao.org/codex/meetings/cccf/cccf7/cf07_09e. pdf). Accessed on 18/09/2014

Copetti MV, Pereira JL, Iamanaka BT, Pitt JI, Taniwaki MH (2010) Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing. International Journal of Food Microbiology **143**: 67–70.

Copetti MV, Pereira JL, Iamanaka BT, Fungaro MH Taniwaki MH (2011) Aflatoxigenic fungi and aflatoxin in cocoa. International Journal of Food Microbiology **2**: 141–4.

EL-Desouky TA, Sharoba AL, El-Mansy HA, Naguib K (2012) Effect of ozone gas on degradation of aflatoxin B1 and *Aspergillus flavus* fungal. Journal of Environmental and Analytical Toxicology **2**: 128–33.

Giordano BNE, Simão V, Scussel VM (2011) Effect of $O_3$ gas on Brazil nut mycoflora and aflatoxin reduction. (In) Controlled Atmosphere and Fumigation in Stored Products, Chengdu, China. Proc. of 8th Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products. Chengdu, China, 214–20.

Giordano BNE, Nones J, Scussel VM (2012) Susceptibility of the in-shell Brazil nut mycoflora and aflatoxin contamination to ozone gas treatment during storage. Journal of Agriculture Science **8**: 1–10.

Graham T, Zhang P, Woyzbun E et al. (2011) Response of hydroponic tomato to daily applications of aqueous ozone via drip irrigation. Science Horticulture **129**: 464–71.

IARC (1993) Monographs on the evaluation of the carcinogenic risks to humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon, France: International Agency Research Cancer **56**: 489–521.

Kreibich HH, Savi GD, Moecke EHS, Scussel VM (2014). Easter eggs and other cocoa (*Theobroma cocoa* L.) products quality: insects, mites, fungi and packaging *versus* critical control points. International Journal of Applied Science Technology **5**: 177–89.

Kreibich HH, Moecke EHS, Scussel VM (2016a) Cocoa (*Theobroma cocoa* L.) beans processing and storage conditions control for safe chocolate products. Journal of Chemistry Biology and Physical Science **6**: 513–26.

Kreibich HH, Christ D, Maria GS, Silva JR, Savi GD, Scussel VM (2016b) Decontamination of Cocoa Beans (Theobroma cocoa L.) Inoculated with *Aspergillus fla us* by Ozone Gas. Journal of Chemistry Biology and Physical Science **6**: 560–70.

Leug MCK, Diaz-Llano G, Smith TK (2006) Mycotoxins in pet food: a review on worldwide prevalecence and preventative strategies. Journal of Agriculture Food Chemistry **54**: 9.623-35.

MAPA-Brazilian Ministry of Agriculture (1981) Oxidative rancidity analysis <http://www.agricultura.gov.br. Accessed on 18/03/2015.

Mcdonough MX, Campabadal CA, Mason LJ, Maier DE, Denvir A, Woloshuk C (2011) Ozone application in a modified screw conveyor to treat grain for insect pests, fungal contaminants, and mycotoxins. Journal of Stored Products Research **3**: 249–54.

Mendez F, Maier DE, Mason LJ, Woloshuk CP (2003) Penetration of ozone into columns of stored grains and effects on chemical composition and processing performance. Journal of Stored Products Research **1**: 33–44.

Mounjouenpou P, Gueule D, Fontana-Tachon A, Guyot B, Tondje PR Guiraus JP (2008) Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. International Journal of Food Microbiology **128**: 234–41.

Ong KC, Cash, JN, Zabik, MJ et al. (1996) Chlorine and ozone washes for pesticide removal from apples and processed apple sauce. Food Chemistry **2**: 153–60.

Ostovar K, Keeney PG (1973) Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cocoa beans. Journal of Food Science **38**: 611–7.

Pitt, JI, Hocking, AD (2009) Fungi and Food Spoilage, third eds, Springer, New York.

Ribeiro NCA, Bezerra JL, Lopez A (1986) Micobiota na fermentação do cacau no estado da Bahia Brasil Rev Theobroma **16**: 47–55.

Sanchez-Hervas M, Gil JV, Bisbal F, Ramon D, Martinez-Culebras PV (2008) Mycobiota and mycotoxin producing fungi from cocoa beans. International Journal of Food Microbiology **3**: 336–40.

Savi GD, Scussel VM (2014) Effects of ozone gas exposure on toxigenic fungi species from *Fusarium*, *Aspergillus* and *Penicillium* genera. Ozone-Science. Eng **2**: 144–52.

Savi GD, Piacentini KC, Bittencourt KO, Scussel VM (2014a) Ozone treatment efficiency on *Fusarium graminearum* and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum* L.) quality and germination. Journal of Stored Products Research **59**: 245–53.

Savi GD, Piacentini KC, Scussel VM (2014b) Ozone treatment efficiency in *Aspergillus* and *Penicillium* growth inhibition and mycotoxin degradation of stored wheat grains (*Triticum aestivum* L.). Journal of Food Processing and Preservation **3**: 1–9.

Savi GD, Piacentini KC, Scussel VM (2015) Reduction in residues of deltamethrin and fenitrothion on stored wheat grains by ozone gas. Journal of Stored Products Research **61**: 65–9.

Schwan RF Wheals AE (2004) The microbiology of cocoa fermentation and its role in chocolate quality. Critical Reviews in Food Science and Nutrition **44**: 1–17.

Scussel, VM (2004) AFls and food safety: recent South American perspectives Journal of Toxicology **23**: 179–216.

Scussel VM (2002) Mycotoxins in Storage Grains. In: Lorini, I, Miike, LH, Scussel, VM Armazenagem de Grãos Chap 9.2 ed. Bio Geniziz, 693-737, Campinas, SP, Brazil.

Scussel VM, Giordano, BN, Simao, V, Manfio, D, Galvão, S, Rodrigues, MNF (2011) Effect of oxygen-reducing atmospheres on the safety of packaged shelled brazil nuts during storage. International Journal of Analytical Chemistry **1**: 9, doi:org/10.1155/2011/813591.

Selma MV, Allende A, Lopez-Galvez F, Consea MA, Gil MI (2008a) Disinfection potential of ozone, ultraviolet-C and their combination in wash water for the fresh-cut vegetable industry. Food Microbiology **6**: 809–14.

Tiwari BK, Brennan CS, Curran T, Gallagher E, Cullen PJ, O'Donnel, CP (2010) Application of ozone in grain processing. Journal of Cereal Science **3**: 248–55.

White SD, Murphy PT, Leandro LF, Bern CJ, Leeuwen J (2013). Mycoflora of high-moisture maize treated with ozone. Journal of Stored Products Research **55**: 84–9.

Wilson DM, Payne GA (1994) Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In: Eaton DL, Groopman JD (eds). The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance. Academic Press, San Diego, pp 383–406.