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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 flavu* 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* .

Key words: *Aspergillus flavu* , 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 flavu* spores decontamination in dry cocoa bean at different gas and time conditions

O_3[a] treatment[b]		*Aspergillus flavu*						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 conten t; $_w$, 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. flavu* (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).

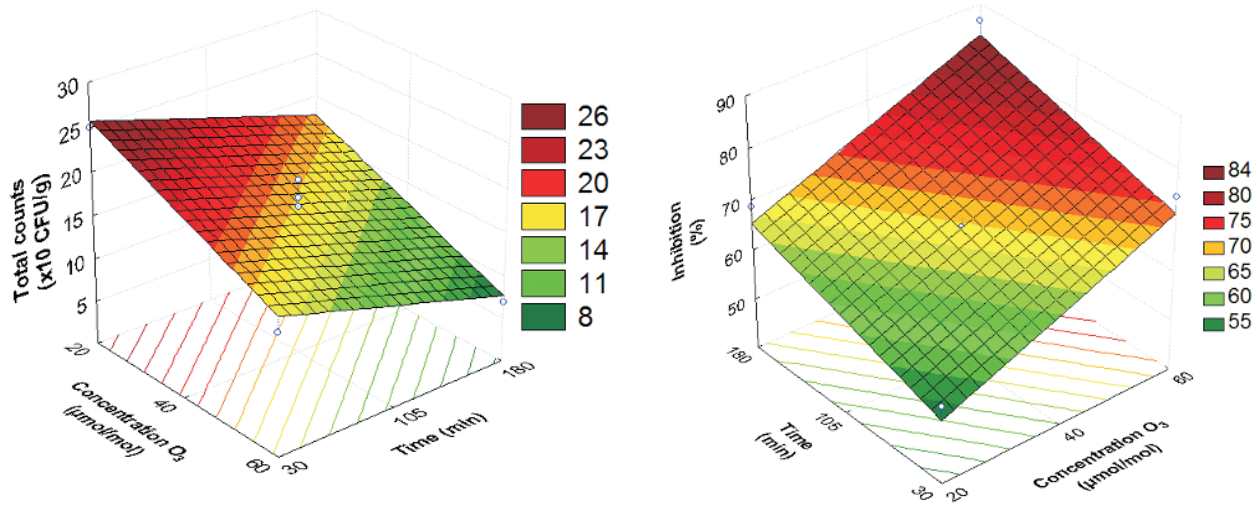
The main effects and the interactions of variables on responses, determining the significant factors ($P < 0.1$) and adjusting a model (Eq. 1) to correlate variables and their responses. The significant coefficients of the model were evaluated by the "t" test, and the data were subjected to an analysis 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.



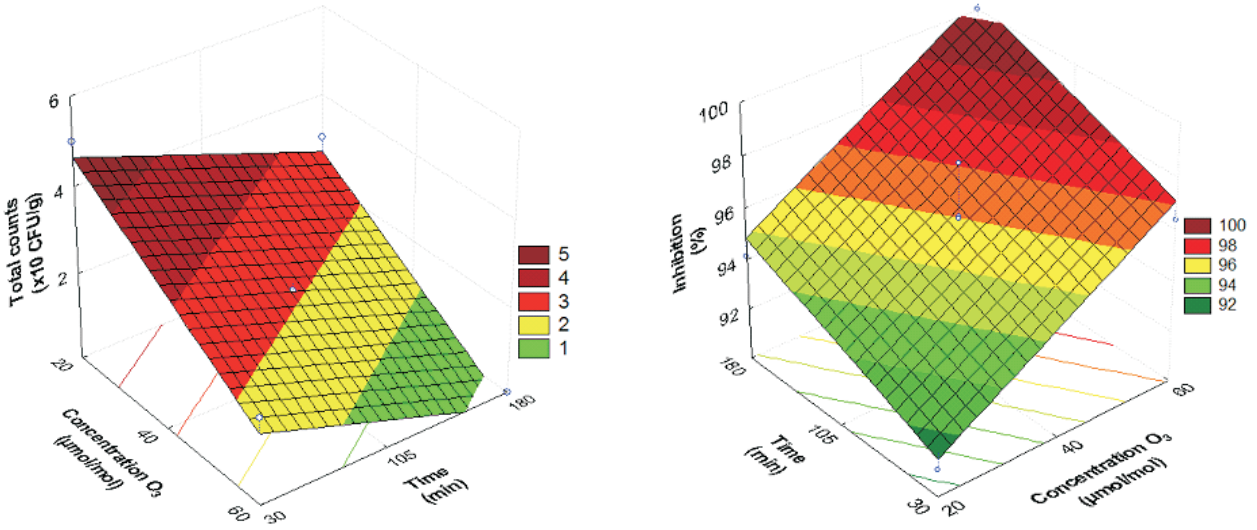
(a) (b)

Fig. 1. Response surface of O_3 concentration (20, 40, 60 µmol/mol) and time of exposure (20, 105, 180 min) in cocoa *Aspergillus flavu* 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. fl vus

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.

Efficienc : The fungal spore inactivation efficienc 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. flavu* 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 coeffi ient (r^2) of 0.82. The surface response represents the model



(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 flavu* inoculated on (*a*) total load (CFU/g) inhibition and (*b*) total reduction (%) at day 30.

(Fig 1b). 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.

Efficienc : 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. flavu* in cocoa.

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