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The effect of cocoa fermentation and weak organic acids on growth and ochratoxin A production by *Aspergillus* species

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ABSTRACT

The acidic characteristics of cocoa beans have influence on flavor development in chocolate. Cocoa cotyledons are not naturally acidic, the acidity comes from organic acids produced by the fermentative microorganisms which grow during the processing of cocoa. Different concentrations of these metabolites can be produced according to the fermentation practices adopted in the farms, which could affect the growth and ochratoxin A production by fungi. This work presents two independent experiments carried out to investigate the effect of some fermentation practices on ochratoxin A production by *Aspergillus carbonarius* in cocoa, and the effect of weak organic acids such as acetic, lactic and citric at different pH values on growth and ochratoxin A production by *A. carbonarius* and *Aspergillus niger* in culture media. A statistical difference ($\rho<0.05$) in the ochratoxin A level in the cured cocoa beans was observed in some fermentation practices adopted. The laboratorial studies demonstrate the influence of organic acids on fungal growth and ochratoxin A production, with differences according to the media pH and the organic acid present. Acetic acid was the most inhibitory acid against *A. carbonarius* and *A. niger*. From the point of view of food safety, considering the amount of ochratoxin A produced, fermentation practices should be conducted towards the enhancement of acetic acid, although lactic and citric acids also have an important role in lowering the pH to improve the toxicity of acetic acid.

1. Introduction

Chocolate flavor is influenced by the acidity of cocoa beans used for their manufacture. Beans described as characteristically acidic give chocolate a fruity or raisin-like flavor, and are used for improvement in blending special formulae. On the other hand, when acidity is excessive it becomes objectionable, since it gives an undesirable, sour, acid flavor to the chocolate. Thus, excess acidity can limit the use of cocoa in chocolate manufacture (Lopez, 1983).

Cocoa cotyledons are not naturally acidic, the acidity comes from a natural fermentation the beans go through. The inoculation of fermentative microorganisms occurs by chance, resulting from cocoa bean manipulation after harvesting. The microorganisms come from the workers' hands, knives used to open the pods, insects, unwashed baskets used to transport the beans and dried mucilage remaining on the walls of fermentation boxes from previous fermentations (Schwan and Wheals, 2004).

A microbial fermentation is required to initiate the formation of precursors of cocoa flavor. Early in the fermentation, several species of yeasts proliferate, leading to production of ethanol and secretion of pectinolytic enzymes (Schwan and Wheals, 2004; Gálvez et al., 2007). This is followed by a phase in which bacteria appear, principally lactic-acid and acetic-acid bacteria. Since, in general, there is no microbial culture employed as starter inoculum for cocoa fermentation and different methods of fermentation can be adopted, variations in the microbial growth pattern in turn affect the metabolites produced in the pulp (Lopez, 1983; Papalexandratou et al., 2011). Lactic and acetic acids derived from fermentative microorganisms together with citric acid naturally present in the cocoa pulp are the main weak organic acids influencing the acidity and thus the flavor of cured cocoa beans (Jinap and Dimick, 1990; Holm et al., 1993).

There are differences in acidic characteristics of cocoa beans produced in different countries. Cocoa beans from Brazil and Malaysia have been known to be excessively acidic (pH 4.2) compared to West African beans (pH 4.8) (Jinap and Dimick, 1990), and different

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experiments have been conducted trying to find a way to correct this defect. They include techniques increasing the fermentation time aiming for a bigger production of acetic acid that is volatile in spite of lactic acid (Lopez, 1983) and removing part of the pulp to reduce the amount of carbohydrate available for microbial fermentation (Lopez, 1979; Schwan and Lopez, 1988). A partial (20% of the bean weight) removal of cocoa pulp gave an accelerated fermentation, with a more rapid progression in the microbial succession and rise in pH value to 5.5 (Schwan and Lopez, 1988).

In parallel, investigations have shown that the levels of ochratoxin A (OTA), a nephrotoxic mycotoxin with carcinogenic, immunosuppressive and teratogenic properties (IARC, 1993) which can be present in cocoa and cocoa products like chocolate, tend to be low in samples from Brazil (Copetti et al., 2010). This low OTA contamination could be related to the acidity and especially acetic acid present in the beans.

It is well established that the pH by itself has little influence on inhibition of fungal growth and OTA production by *Aspergillus carbonarius* and *Aspergillus niger* (Esteban et al., 2005, 2006; Kapetanakou et al., 2009; Pitt and Hocking, 2009). However the effect of weak organic acids has not yet been investigated.

In this work two independent experiments were carried out to investigate the effect of some fermentation practices on OTA production by *A. carbonarius* in cocoa and the effect of weak organic acids such as acetic, lactic and citric at different pH values on growth and OTA production by *A. carbonarius* and *A. niger* in culture media.

2. Materials and methods

2.1. Field study of influence of fermentation on ochratoxin production

2.1.1. Ochratoxigenic fungi

Two ochratoxigenic *A. carbonarius* (ITAL 792cc and ITAL 1375cc) were randomly selected for this study among isolates recovered from cocoa beans. Previously the isolates had their identification carried out according to Pitt and Hocking (2009) methodology, after growing the fungi for 7 days in Czapek yeast autolysate (CYA) agar and Malt extract agar (MEA) at 25 °C. The identity of the isolates was confirmed through secondary metabolite profile analysis (Smedsgaard, 1997).

A mixed inoculum of these two isolates (10^5 conidia/mL) was aseptically prepared after growing each fungus in CYA for 7 days at 25 °C, suspending the colonies of both fungi in peptone water 0.1% and mixing.

2.1.2. Cocoa fermentation

The experiment was conducted at the Cocoa Crop Executive Commission (CEPLAC) located in Itabuna municipality, Bahia state, Brazil. About 500 kg of fully mature and healthy cocoa pods most of Trinitario hybrid were harvested and broken open with machetes. The beans were manually removed out of the pods, separated from placenta and mixed.

The beans were divided in two batches for the fermentation process: (i) beans with full pulp and (ii) beans where 20% pulp had been removed by a mechanical pulp extractor (Fig. 1).

Both types of cocoa beans were submitted to two different curing processes: (i) conventional fermentation in a box for 7 days after which the beans are removed and dried in the sun on platforms until they reduce their moisture content to 6–7% and (ii) combined fermentation–drying whereby the beans are placed on sun drying platforms until they reduce their humidity to 6–7% (Fig. 1).

To investigate the steps where the OTA is produced when ochratoxigenic fungi are present, triplicate portions of 6 kg were inoculated with an *A. carbonarius* mix (1.67 mL/kg) before being subjected to the fermentation process. The remaining uninoculated triplicate portions were used as negative control (Fig. 1).

The fermentation boxes had 6 holes of 10 mm for natural drainage of the pulp juices (sweating) generated by fermentation. The aeration of the cocoa mass was achieved by transferring the contents into a similar box every 48 h.

Sampling points were: (i) before fermentation processes, (ii) at the end of conventional fermentation, (iii) at the end of drying and (iv) at the end of combined fermentation–drying.

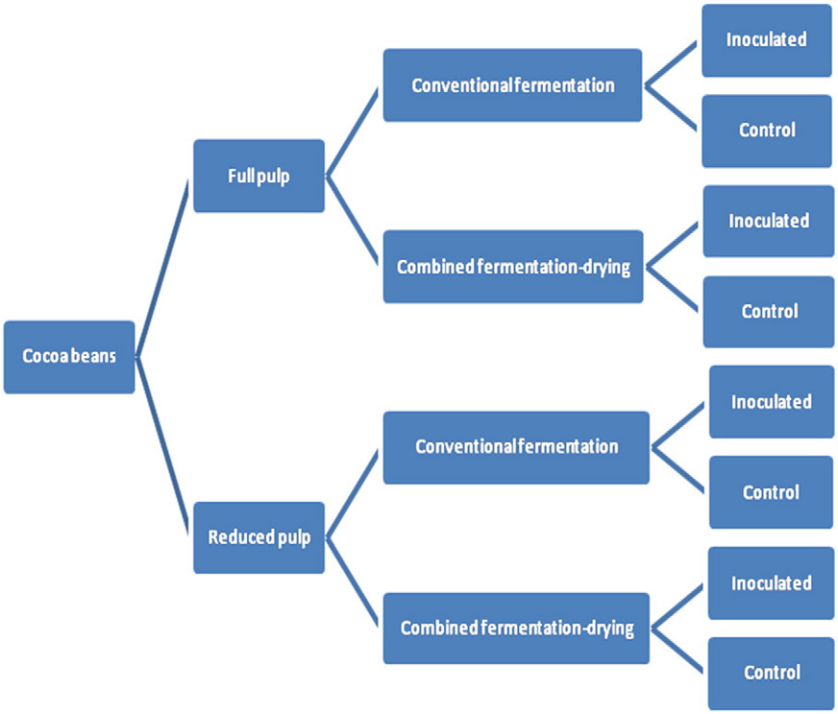

Fig. 1. Processing of cocoa beans according to the pulp status, type of fermentation and the presence of *Aspergillus carbonarius* inoculum. The experiment was carried out in triplicate.

Moistened samples were dried in an oven with air circulation at 45 °C before grinding.

2.1.3. Determination of ochratoxin A in cocoa samples

OTA analyses were performed by HPLC according to the method described by Copetti et al. (2010).

2.1.3.1. Clean-up. Ten grams of finely ground cocoa were extracted in $NaHCO_3$ (1% aqueous; 200 mL). The suspension was blended (2 min) at high speed (10,000 rpm) using an Ultra-Turrax homogenizer (Polytron, Switzerland). Homogenized solutions were filtered through Whatman No. 4 filter paper and Whatman A-H glass microfiber filter (Whatman, England). Filtrate (20 mL) was diluted in phosphate buffered saline (20 mL) plus Tween 20 (0.01%) and applied to an Ochraprep immunoafinity column (R-Biopharm Rhône Ltd, Scotland) at a flow rate of 2–3 mL/min. The column was then washed with distilled water (20 mL), and OTA eluted with acidified methanol (methanol: acetic acid, 98: 2, v/v; 4 mL) into an amber vial. After evaporation to dryness at 40 °C under a stream of N_2, the dry residue was redissolved in mobile phase (0.3 mL).

2.1.3.2. HPLC parameters. A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with fluorescence detection set at 333 nm excitation and 477 nm emission. A Shimadzu CLC G-ODS (4×10 mm) guard column and Shimadzu Shimpack (4.6×250 mm) column were employed. The mobile phase was acetonitrile:water: acetic acid (51:47:2, v/v/v) and the flow rate was 1 mL/min. An OTA standard was used for construction of a five point calibration curve of peak areas versus concentration (μg/L). The injection volume was 100 μL for both standard solution and sample extracts.

2.2. Laboratorial study of influence of weak organic acid on ochratoxin production

2.2.1. Ochratoxigenic fungi

Species of ochratoxigenic *A. carbonarius* (ITAL 792cc and ITAL 1375cc) and *A. niger* (ITAL 1240cc) were isolated from cocoa and maintained at 5 °C on CYA (containing per liter: 1 g K_2HPO_4, 5 g yeast extract, 20 g agar, 30 g sucrose, 3 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.005 g $CuSO_4 \cdot 5H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$) until use.

2.2.2. Culture conditions

CYA medium was used as control medium. For modified media, sucrose was half or totally substituted by a weak organic acid (acetic, citric or lactic) and its respective sodium salt by maintaining the same amount of carbon per liter of medium (30 g sucrose is equivalent to 31.5 g acetic acid, 33.6 g citric acid, 31.5 g lactic acid, 43.1 g sodium acetate, 51.5 g trisodium citrate, 39.2 g sodium lactate). Media had their pH adjusted to 4.2, 4.8, 5.5, 5.8 or 7.0 and were sterilized by autoclaving for 30 min at 110 °C. Cultures were grown in Petri dishes for 7 days at 25 °C. The cultures were three-point inoculated in triplicate.

2.2.3. Ochratoxin A analyses

Ochratoxin A analyses were performed by HPLC following an adaptation of the method described by Smedsgaard (1997) for analyses of fungal secondary metabolites. After incubation colony diameters were measured, and 5 plugs from each colony were placed in a 1.5 mL glass vial and extracted. Extraction was done using ultrasonication for 55 min with a solution of ethyl acetate:dichloromethane:methanol (3:2:1, v/v/v) with 1% (v/v) formic acid. The eluted product was transferred to a new vial, the solvents evaporated and the dried extract re-dissolved in 500 μL methanol. After filtering through a 0.45 μm PFTE filter, the extract was injected into the HPLC apparatus.

2.2.3.1. HPLC parameters. The parameters used were the same described for the item 2.1.4, but the injection volume was 3 μL for both standard solution and sample extracts.

3. Results

3.1. Influence of fermentation practices on OTA production in cocoa

OTA was not detected in the beans before fermentation and only levels near the limit of detection were detected in the control samples. The content of OTA introduced by inoculation of *A. carbonarius* on beans before the beginning of fermentation was around 3 ng/g (Table 1).

Considering the beans inoculated with *A. carbonarius*, a continuous increase in OTA levels through the cocoa processing stages was observed, both in the beans with full pulp and those with reduced pulp ($\rho<0.05$) (Table 1).

A statistical difference ($\rho<0.05$) in the OTA content of beans at the end of drying (cured beans) was observed between the beans with full pulp and those with reduced pulp, regardless of the processing method, i.e. conventional fermentation or combined fermentation–drying (Table 1).

The OTA values found in the full pulp beans were quite similar in both processes ($\rho>0.05$). However the final product of the full pulp beans subjected to combined fermentation–drying was not technologically acceptable because an excessive residual pulp remained surrounding the beans, making them stick together in blocks.

The samples with reduced pulp and subjected to combined fermentation–drying had the highest OTA contamination ($\rho<0.05$) (Table 1). These dried fermented beans with reduced pulp were the only ones where it was possible to see active growth of *A. carbonarius* (visibly moldy) after 3 days on drying platforms.

3.2. Influence of weak organic acids and pH on fungal growth and OTA production in culture media

3.2.1. Fungal growth

When *A. carbonarius* or *A. niger* were cultivated in the traditional CYA medium, having sucrose as carbon source, the growth was not influenced by the differences in the medium pH.

The growth of *A. carbonarius* and *A. niger* was affected by the presence of lactic acid as the only source of carbon. Under this condition, both fungi grew better at low pH, and a complete suppression of growth was not achieved at the highest pH 7.0. This decrease in

Table 1
Ochratoxin A production in cocoa beans inoculated with *Aspergillus carbonarius* related to the processing stage and pulp content.

	Ochratoxin A (ng/g)		
	Before fermentation	End of fermentation	End of drying
Normal amount of pulp			
Conventional process	$2.93^{a*,C**}\pm0.01°$	$4.43^{a,B}\pm0.11$	$6.46^{c,A}\pm0.41$
Combined fermentation–drying	$2.93^{a,B}\pm0.01°$	–	$6.63^{c,A}\pm0.51$
Control	ND	$0.04^{b,A}\pm0.01$	$0.03^{d,A}\pm0.01$
Pulp content 20% reduced			
Conventional process	$3.02^{a,C}\pm0.07°$	$5.02^{a,B}\pm0.61$	$7.91^{b,A}\pm0.39$
Combined fermentation–drying	$3.02^{a,B}\pm0.07°$	–	$9.44^{a,A}\pm0.62$
Control	ND	$0.02^{b,A}\pm0.01$	$0.03^{d,A}\pm0.01$

*Values with the same letter in the same column show no significant difference ($\rho>0.05$).
**Values with the same capital letter in the same line show no significant difference ($\rho>0.05$).
° = OTA contamination introduced with the inoculum.
ND = Not detected (less than 0.01 ng/g).

Table 2
Colony diameter of *Aspergillus carbonarius* or *Aspergillus niger* in response to organic acids and/or sucrose as carbon source at different pH after 7 days at 25 °C.

Isolates	pH	Diameter (mm) in 7 days at 25 °C versus the carbon source[a]						
		AA	CA	LA	S	AA + S	CA + S	LA + S
A. carbonarius	4.2	0	70	70	70	0	70	70
ITAL 1375cc	4.8	0	65	70	70	0	70	70
	5.5	0	37	65	70	0	70	70
	5.8	0	18	55	70	28	70	70
	7.0	3	0	20	70	22	0	70
A. carbonarius	4.2	0	70	70	70	0	70	70
ITAL 792cc	4.8	0	65	70	70	0	70	70
	5.5	0	44	70	70	0	70	70
	5.8	0	17	58	70	27	70	70
	7.0	3	0	29	70	24	0	70
A. niger	4.2	0	70	64	70	0	70	70
ITAL 1240cc	4.8	0	70	66	70	0	70	70
	5.5	0	63	65	70	21	70	70
	5.8	2	59	63	70	39	70	70
	7.0	16	24	52	70	44	58	70

[a] AA = acetic acid; CA = citric acid; LA = lactic acid; S = sucrose.

growth rate related to pH was not observed when both lactic acid and sucrose were present in the media.

The behavior of *A. carbonarius* and *A. niger* when citric acid substituted sucrose was the same shown by lactic acid. The higher the medium pH, the smaller was the diameter of colony produced, with complete cessation of *A. carbonarius* growth at pH 7.0. When both citric acid and sucrose were available, the fungi did not suffer any influence on growth up to pH 5.8. At pH 7.0 *A. carbonarius* also had their growth completely inhibited and a slight reduction on growth of *A. niger* was verified.

Acetic acid was the weak organic acid with the most notable influence on growth parameters (Table 2). When the sucrose was completely substituted by acetic acid, the growth of both isolates of *A. carbonarius* was entirely suppressed, only germinating at pH 7.0. Under this circumstance, *A. niger* was able to germinate at pH 5.8 and formed small colonies at pH 7.0. When half of the sucrose was substituted by acetic acid, *A. carbonarius* was able to grow at pH 5.8 and 7.0 and *A. niger* at pH 5.5 and above. It demonstrates a more inhibitive action of acetic acid on *A. carbonarius* than *A. niger*.

3.2.2. Ochratoxin A production

The maximum productions of OTA by all tested fungi in culture media were achieved in the absence of weak organic acids. Toxin production by *A. carbonarius* was favorable at high pH, whereas the converse was true for *A. niger*, i.e. more OTA produced at low pH values (Fig. 2).

When weak organic acids were the sole carbon source available in the culture media, a strong depletion in OTA production was observed. When fungal growth occurred, acetic acid yielded in 99% reduction in maximum OTA production, whereas the range of reduction produced by citric and lactic acid was 87–99% and 80–99%, respectively. The toxin levels followed the same patterns observed for fungal growth on citric and lactic acids, namely, lower pH supported greater OTA production (Fig. 2).

In the presence of a weak organic acid and with the availability of sucrose in the substrate, the lower inhibition of OTA production was in general produced by the lactic acid. On the other hand, acetic acid produced the strongest reduction on OTA level, supporting the mycological observations of growth suppression (Fig. 2).

A reduction in OTA production was also observed in the presence of sucrose. Acetic, citric and lactic acid, respectively, reduced OTA production by 88–99%, 46–72% and 18–75%. Thus acetic acid yielded the greatest reduction in OTA, and lactic acid, the least, which mirrors the trends of growth inhibition (Fig. 2).

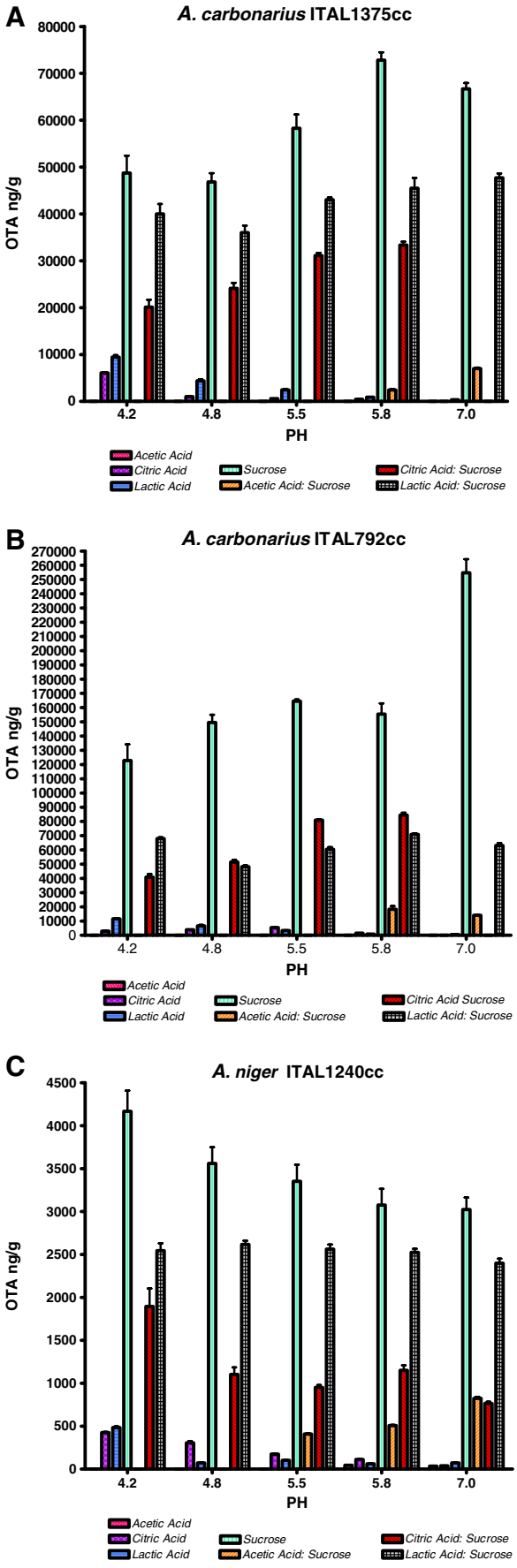

Fig. 2. (A–C). Ochratoxin A production (ng/g) by *Aspergillus carbonarius* or *Aspergillus niger* in response to organic acids and/or sucrose as carbon source at different pH.

In the presence of sucrose the effect of pH on OTA production according to the acid and fungi can be considered. For citric acid the OTA production by *A. carbonarius* was greater at the higher pH values, while higher amounts were produced by *A. niger* at pH 4.2. The mycotoxin production in the presence of lactic acid by *A. niger* was almost constant at all pH values and a slight reduction was observed in pH 4.8 by *A. carbonarius*. In the pH where growth occurred in the presence of acetic acid, the OTA production was greater at the higher pH values for *A. niger* and *A. carbonarius* ITAL 1375cc and the opposite was observed for ITAL 792cc (Fig. 2).

A variation in the capability to synthesize OTA was observed between the isolates studied. *A. carbonarius* ITAL 792cc was the most ochratoxigenic, achieving a maximum of about 250,000 ng/g produced, 3.5 times more OTA than that produced by the isolate ITAL 1375cc and about 60 times more than *A. niger* ITAL 1240cc.

4. Discussion

The present study has focused on *A. carbonarius* and *A. niger*, since they are the main fungi responsible for OTA production on cocoa (Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008; Gilmour and Lindblom, 2008; Copetti et al., 2010).

4.1. Experiments in cocoa fermentation

The observation of increased OTA content through the processing stages of cocoa indicates that synthesis of OTA by *A. carbonarius* is active at all stages, including the fermentation. It corroborates the observations of Gilmour and Lindblom (2008), who stated that OTA production in cocoa can start during fermentation if ochratoxigenic fungi are present, and toxin levels increase during the drying stage.

Differences observed between the OTA produced in beans with normal pulp and the ones with pulp content reduced when fermented in a box previous to their placement on drying platforms and the ones directly conducted to drying platforms to ferment while drying could be explained by the effect of the pulp. The presence of normal pulp (80% water, 10–13% sugar and 1% citric acid) (Roelofsen, 1958) in the beans when placed directly on sun drying platforms form a compact mass, moistened and rich in fermentable sugars. The high sugar content in the pulp represents a rich substrate available for fermentative microorganisms, and the acidity caused by the presence of citric acid will select acidophilic groups, like yeasts and acid lactic and acetic bacteria (Schwan and Lopez, 1988). Besides this, the pulp can act as a physical barrier reducing the oxygen penetration, and propitiate a more anaerobic environment favoring the production of alcohol by yeasts through the fermentative process. This alcohol will be used in the microbial succession as substrate for acetic acid bacteria (Schwan and Wheals, 2004) producing as much acetic acid as alcohol if available as substrate for oxidation. The low availability of oxygen and high pressure of carbon dioxide are also important factors limiting the growth of filamentous fungi, since most fungi have their growth affected under conditions of microaerophilia (Taniwaki et al., 2009).

On the other hand, when the pulp is partially removed, smaller amounts of citric acid, water and sugar are present and a higher aeration between the beans occurs. This situation reduces the yeast fermentation and therefore the amounts of alcohol and acetic acid produced.

Thompson et al. (2001) understand the combined fermentation–drying as convenient, but emphasize that if not properly managed this processing tends to produce underfermented beans with the additional danger of undesirable fungal growth. The growth of fungi in cocoa beans is always a cause for concern since there is the possibility of toxin production (Mounjouenpou et al., 2008; Copetti et al., 2010, 2011a) and the stage on sun drying platforms is considered as critical because of the presence of potentially toxigenic species and the water content of beans (Copetti et al., 2011b).

4.2. Experiment with weak organic acids

When the studied fungi were grown in media free of weak organic acids they were able to grow and produce high amounts of OTA at all pH levels tested. Besides this, the maximum OTA production by the two *A. carbonarius* strains studied happened at different pH levels. It corroborates with the observations of Esteban et al. (2005, 2006) that *A. carbonarius* and *A. niger* can grow and produce OTA in a pH range of 2.0 to 10.0.

The inhibitory effect on growth caused by acetic acid (higher in lower pH both in presence or absence of sucrose) and citric and lactic acids (higher in higher pH and only in absence of sucrose) suggest that, differently to that observed for acetic acid, the effect of citric and lactic acid appears to be more because of the restriction of energy supply than due to the toxicity of these last weak organic acids. This may possibly occur because the weak acids show a dynamic equilibrium, pH-dependent, between molecular acids and their respective charged ions (Table 3). This undissociated state is able to freely penetrate the microbial cell membrane and once inside the cell, the more neutral pH causes weak acid molecules to dissociate into anions and protons (Theron and Lues, 2011).

The anion lactate could be used through gluconeogenesis to provide energy for the metabolism of the fungi. In a similar way, the citrate anion should not exert a toxic effect on the fungi since it can easily be metabolized via the tricarboxylic acid cycle, supplying energy. It is also a precursor for amino acid synthesis through citrate oxidation (Niederpruem, 1965). This was reflected both in the fungal growth and amounts of OTA produced.

On the other hand, the mode of inhibition of acetic acid is not completely clear. It is generally attributed to the release of protons, acidification of the cytoplasm and dissipation of the membrane pH gradient, disrupting normal cell physiology (Theron and Lues, 2011). An experiment carried out by Stratford et al. (2009) demonstrates that acetic acid causes a large and rapid fall in the internal pH of *A. niger* conidia, consistent with its action as a classic weak acid preservative. Nevertheless, the accumulation of anions could also exert a toxic effect (Russell, 1992).

The chelation capacity of citric acid molecules could explain the complete inhibition of *A. carbonarius* at pH 7.0 both in the absence and in the presence of sucrose. At this pH less than 1% of the acid is undissociated, consequently 99% of the three COO^- radicals are free to bind divalent metal ions, especially Mg^{2+} and Ca^{2+} (Brul and Coote, 1999; Nielsen and Arneborg, 2007). Potassium, magnesium, iron, zinc, manganese, copper and molybdenum appear to be essential for all fungi, while calcium is essential for some, but not all fungi (Lilly, 1965). The divergence of *A. niger*, which had its growth reduced but was still able to grow at pH 7.0 could be due to different necessities of calcium, since this ion is not essential for *A. niger* nutrition (Steinberg, 1948).

The increase of acetic acid toxicity reflected by decreasing and complete inhibition of fungal growth parallel with the reduction of pH can be better visualized in *A. niger*, since this species was less sensitive to this acid. Both isolates of *A. carbonarius* presented an

Table 3
Percentage of undissociated[a] weak organic acid related to the pH.

	Pk	pH				
		4.2	4.8	5.5	5.8	7.0
Acetic acid	a = 4.75	78	47	15	8	0.5
Citric acid	a = 3.09, b = 4.74, c = 5.41	60	43	20.1	12.4	0.8
Lactic acid	a = 3.86	31.5	10.3	2	1	0.1

[a] Calculated by Henderson–Hasselbalch equation.

unexpected slight reduction in the diameters of colonies when pH increased from 5.8 to 7.0 in the presence of sucrose and acetic acid, although the conidial production was more intense at pH 7.0, possibly reflecting a higher fungal mass at this pH even though the colonies were smaller.

In spite of the absence of interference by citric and lactic acids in the presence of sucrose on the colony diameter of the fungi in 7 days, a reduction in the production of OTA was observed. It is possible to infer that the primary metabolism apparently is not affected but the secondary metabolism is diminished with the carbohydrate supply reduction. A possible explanation is that the decrease of sucrose available in the modified media can interfere in the synthesis of phenylalanine via the pentose phosphate pathway, which is part of the OTA molecule.

No previous data on the influence of weak organic acids on the growth of the species evaluated in this experiment at different pH, nor concerning OTA production, have been found in literature. However, the inhibitory effect of acetic acid on the growth of some species of filamentous fungi (Buchanan and Ayres, 1975; Vivier et al., 1992; Kang et al., 2003) and on aflatoxin production (Buchanan and Ayres, 1975) has already been reported.

According to the observations of the two experiments reported here, it became clear that fermentation practices and the weak organic acids present have an important role in OTA accumulation in cocoa.

Generally, the cocoa pulp initial pH is around 3.2 because of the large presence of citric acid, whereas in the unfermented cotyledons the pH is 6.5. This difference in the pH value is equalized after 3–4 days due to migration of the acid produced by fermentation of the pulp to the interior of the cotyledons, and to about pH 4.8 at the end of the fermentation process (Thompson et al., 2001). This final pH is usually lower in beans from Brazil (pH 4.2), but when the beans are partially depulped, an increase in the final pH is observed (pH 5.5–5.8) (Schwan and Lopez, 1988; Jinap and Dimick, 1990).

Nevertheless, some authors have described a lower incidence of ochratoxin A in beans from the Americas and Pacific, as compared to beans grown in Africa (Raters and Matissek, 2000; Amezqueta et al., 2004; Gilmour and Lindblom, 2008; Copetti et al., 2010), which could be related to the pH of the beans associated with the presence and action of weak organic acids, especially acetic acid, on ochratoxigenic fungi at low pH, as shown by this experiment. This supposition is supported by a study carried out by Jinap and Dimick (1990) where the acids produced during the cocoa fermentation of samples from different origin were measured in the dried beans. These researchers reported a huge difference in the amounts of acetic acid present in the beans from Brazil and Malaysia (about 80% of total acids) when compared to the African beans (about 55% of total acids), attributing to acetic acid the responsibility for the high acidity in cocoa beans. The same authors evaluated some samples from Malaysia that had their pulp content reduced before fermentation resulting in a final pH 5.5–5.8. These samples showed the acetic acid pattern similar to that of African beans.

Due to the influence of the main weak organic acids produced during cocoa fermentation on fungal growth and OTA production by *A. carbonarius* and *A. niger* fermentation practices conducted for the enhancement of acetic acid could minimize the problem of OTA contamination in cocoa. Evidently the adoption of good manufacturing practices during all steps of cocoa curing is important as they can prevent the contact of cocoa beans with toxigenic fungi and consequently the accumulation of mycotoxins.

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