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Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing

Marina V. Copetti [a,*], José Luís Pereira [b], Beatriz T. Iamanaka [c], John I. Pitt [d], Marta H. Taniwaki [c]

[a] Universidade Federal de Santa Maria, UFSM, Departamento de Tecnologia e Ciência de Alimentos, Centro de Ciências Rurais, CEP 97105-900, Santa Maria/RS, Brazil
[b] Universidade Estadual de Campinas, Unicamp, Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, C.P. 6121, CEP 13083-862, Campinas/SP, Brazil
[c] Instituto de Tecnologia de Alimentos, ITAL, C.P. 139, CEP 13070-178, Campinas/SP, Brazil
[d] CSIRO Food and Nutritional Sciences, North Ryde, NSW 2113, Australia

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ABSTRACT

This study investigated the occurrence of fungi with the potential to produce ochratoxin A (OTA), and the occurrence of OTA, in Brazilian cocoa beans. Two hundred and twenty two samples of cocoa were evaluated, taken at various stages of fermentation, drying and storage. Samples were collected from Bahia, the main cocoa producing region in Brazil. Fungi with the potential to produce OTA were isolated by direct plating of cocoa beans on Dichloran 18% Glycerol agar after surface disinfection, and identified by standard techniques. The ability of the fungi to produce OTA was estimated using the agar plug technique and TLC. The presence of OTA in cocoa samples was determined by HPLC after immunoaffinity column clean up. The most common ochratoxigenic species found were *Aspergillus carbonarius* and *A. niger* aggregate, with lower numbers of *A. melleus, A. westerdijkiae* and *Av. ochraceus*. A considerable increase in the numbers of these species was observed during drying and storage. OTA was found at all stages of cocoa processing, with the major incidence during drying and storage. The OTA levels found were in general low and there was a strong positive correlation between the presence of *A. carbonarius* and OTA contamination in the beans.

1. Introduction

In Brazil, the primary processing of cocoa is commenced by opening freshly harvested pods, placing the high water content beans in wooden boxes and allowing fermentation to proceed naturally for about 6 days. During this time microorganisms consume the sugars present in the pulp surrounding the beans, and secrete pectinolytic enzymes and other metabolic products such as ethanol and organic acids. The water activity (a_w) remains high. After fermentation, beans are transferred to drying platforms where a gradual reduction in a_w occurs. When a_w values are considered to be sufficiently low, beans are transferred to storage rooms, then later bagged and marketed.

In recent years, several studies on the occurrence of ochratoxin A (OTA) in cocoa and its products have been published (Miraglia and Brera, 2002; Burdaspal and Legarda, 2003; Amezqueta et al., 2004; Bonvehi, 2004; Tafuri et al., 2004; Gilmour and Lindblom, 2008). However, the fungi that produce OTA and their sources have been little studied, although it is known that filamentous fungi appear in cocoa beans during the later stages of fermentation and during drying. They are often associated with cocoa bean spoilage and the occurrence of off-flavours that persist in spite of industrial processing.

OTA has been detected in different crops (MacDonald et al., 1999; Taniwaki et al., 2003; Iamanaka et al., 2005; Leong et al., 2006) and is stable during most food processing stages (Boudra et al., 1995; Ferraz et al., 2010). Rejections of certain food products by EU importers due to the presence of OTA have been increasing. In temperate climates, *Penicillium verrucosum* is the source of OTA in cereals, however in the tropics the presence of OTA in food commodities has been associated with the presence of *Aspergillus carbonarius, Aspergillus niger* and *Aspergillus ochraceus* (Taniwaki et al., 2003; Magnoli et al., 2003; Pitt and Hocking, 2009). OTA was first isolated from *A. ochraceus* (Van der Merwe et al., 1965). However, the production of OTA by members of *Aspergillus* section *Nigri* has received considerable attention since the first description of OTA production by *A. niger* var. *niger* (Abarca et al., 1994) and from *A. carbonarius* (Horie, 1995).

Currently, international discussions are taking place to determine the acceptable OTA levels in cocoa and cocoa products. Recently, the European Union decided that cocoa and cocoa products are not significant contributors to OTA exposure in the diet, thus no maximum level was established for this product (Commission Regulation, 2010). The Joint FAO/WHO Expert Committee on Food Additives has established 100 μg/kg body weight as the tolerable limit for daily ingestion of this toxin (WHO (World Health Organization), 2001).

The purpose of this research was to evaluate the occurrence of fungi with the potential to produce OTA, the occurrence of OTA during the primary production of cocoa on Brazilian farms and to establish the critical steps in OTA production on cocoa beans.

* Corresponding author.
E-mail address: marinacopetti@yahoo.com.br (M.V. Copetti).

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2. Materials and methods

2.1. Samples

Two hundred and twenty two samples of cocoa beans were collected from farms in Bahia, the main cocoa producing region in Brazil. These samples were collected from 2006 to 2008, comprising 25 samples of cocoa beans at the opening of the pods, 51 samples from different times of fermentation in wooden boxes (1 to 6 days), 81 samples from different times of sun drying on wooden floor platforms with movable roofs (1 to 12 days) and 64 samples of dried beans in storage. All the farms in this study used a wooden drying floor, which is difficult to maintain in good hygienic condition and thus resulting in a high fungal load.

2.2. Water activity determination

The water activity (a_w) of cocoa bean samples was determined in triplicate in an Aqualab Series 3TE instrument (Decagon, USA) at 25 ± 0.1 °C.

2.3. Identification of fungal species with the potential to produce OTA

Samples (about 200 g) were subsampled (50 g) and surface disinfected in a sodium hypochlorite solution (0.4%) for 2 min. A total of 33 beans (eleven particles per plate) were placed aseptically on Dichloran 18% Glycerol agar (DG18) (Pitt and Hocking, 2009). The plates were incubated for 7 days at 25 °C. After incubation, beans were inspected for fungal growth and all colonies isolated on Czapek Yeast Extract agar (CYA) (Pitt and Hocking, 2009) for subsequent identification of potentially ochratoxigenic fungi based both on macroscopic (colony diameter, colour, exudate and soluble pigment production) and microscopic characters, following appropriate keys (Klich and Pitt, 1988; Pitt, 2000; Samson et al., 2002; Frisvad et al., 2004; Samson et al., 2004). The isolation frequency of each species was expressed as the percentage of particles showing growth of that species.

Fungi identified as potential producers of OTA were inoculated onto Yeast Extract Sucrose agar (Samson et al., 2002) for 7 days at 25 °C and then the agar plug technique (Filtenborg et al., 1983) was used to evaluate the capability of isolates to produce OTA. Fungal extracts taken as plugs with a cork borer were placed on TLC plates, developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualized under UV light at 365 nm. An OTA standard (Sigma, St. Louis, USA) was used for comparison.

2.4. Analyses of OTA on cocoa beans

2.4.1. Clean-up

Ten grams of finely ground cocoa were extracted in $NaHCO_3$ (1% aqueous; 200 mL). Suspensions were 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 immunoaffinity 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).

2.4.2. HPLC parameters

A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a 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 the 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.

2.4.3. Chemical confirmation of OTA

OTA was confirmed by methyl ester formation (Pittet et al., 1996). Aliquots (about 200 μL) of sample and standard were evaporated to dryness at 40 °C under a stream of N_2 and the residue redissolved in boron trifluoride–methanol complex (20% solution in methanol; 300 μL). The solution was heated at 80 °C for 10 min and allowed to cool to room temperature. The identity of OTA was confirmed by the formation of methyl ester that gave a retention time of approximately 22 min.

2.5. Statistical analyses

Statistical analyses (correlation analyses) were carried out with the software The Unscrambler® 9.2 (Camo Process AS, Norway). Correlation coefficients (*r*) were calculated to identify possible associations between the incidence of fungi with the potential to produce OTA and OTA levels in the cocoa in all samples evaluated. Interpretation of values was performed according to Pearson's coefficient (*r*) which are: very low $0.00 \leq r \geq 0.19$; low $0.20 \leq r \geq 0.39$; moderate $0.40 \leq r \geq 0.69$; high $0.70 \leq r \geq 0.89$ and very high $0.90 \leq r \geq 1.00$ (Rodgers and Nicewander, 1988).

3. Results

3.1. Water activity of cocoa

Cocoa processing on the farm, from the opening of a cocoa pod until it is stored, results in a large reduction in a_w levels (Table 1). Most cocoa farms visited during this work used empirical methods to decide when beans were dry enough to be stored. Nineteen (29%) of the 65 stored samples where water activity was measured had an a_w above 0.70, with three having an a_w greater than 0.80.

3.2. Ochratoxigenic species

Two hundred and seventy one fungi belonging to potentially ochratoxigenic *Aspergillus* species were isolated from raw cocoa during processing stages on the farm (Table 2). These were identified as *A. carbonarius, A. niger* aggregate, *A. ochraceus, A. melleus* and *A. westerdijkiae. P. verrucosum* was not found.

Before fermentation, no species capable of producing OTA was found in cocoa pods, either healthy or wounded. During fermentation, only a few isolates belonging to *A. niger* aggregate were found, but a rapid increase in the occurrence of OTA producing fungi was observed in the later processing stages (Table 2). During sun drying, the greatest diversity and numbers of species capable of producing OTA were found. During storage, an increase in the occurrence of *A. niger* aggregate and *A. carbonarius* was observed.

Table 1
Water activity of cocoa beans at different processing stages on cocoa farms.

Stage	No. of samples	Water activity	
		Average	Range
Before fermentation	25	0.99	0.99–0.98
Fermentation	51	0.99	0.99–0.98
Drying	81	0.81	0.99–0.49
Storage	65	0.67	0.85–0.40

Table 2
Isolation frequency of ochratoxigenic species and incidence of infected cocoa beans at different processing stages [a].

	Fermentation (51 samples)		Drying (81 samples)		Storage (65 samples)	
	IF (%)	RI (%)	IF (%)	RI (%)	IF (%)	RI (%)
Aspergillus carbonarius	1.96	0–3	3.70	0–24	7.81	0–66
A. niger aggregate	3.92	0–9	14.8	0–48	26.15	0–51
A. ochraceus	0	0	2.47	0–3	0	0
A. melleus	0	0	2.47	0–6	3.13	0–3
A. westerdijkiae	0	0	2.47	0–6	0	0

[a] IF = isolation frequency % (number of samples contained a fungal species/ total of samples evaluated, %); RI = range of infection % (range of infected beans in a sample, %).

A. niger aggregate was the most common species isolated with the potential to produce OTA. However, only ten (5.2%) of the 191 isolates were able to produce OTA on YES agar. On the other hand, all 92 isolates of *A. carbonarius* and 10 isolates from *Aspergillus* section *Circumdati* (6 *A. melleus*, 2 *A. ochraceus* and 2 *A. westerdijkiae*) were able to produce OTA on YES.

In some samples collected during sun drying and from storage, more than 50% of cocoa beans were infected with *A. niger* aggregate. A high number of beans infected by *A. carbonarius* were also observed in some samples. Only a few isolates from *Aspergillus* section *Circumdati* were found, at a low infection rate.

3.3. OTA in cocoa samples

OTA assays on cocoa samples at various processing stages on the farm are given in Table 3. As the OTA incidence from the three consecutive years (2006–2008) of sampling did not show a great difference, the results are shown all together in Table 3. None of 25 samples taken before the commencement of fermentation contained OTA. Fourteen (27%) of 51 samples from fermentation contained OTA, although most samples were close to the limit of detection of the method (0.01 μg/kg). Only three samples had levels higher than 0.10 μg/kg, with a maximum of 1.70 μg/kg (Table 3). After fermentation, at the sun drying stage, OTA was detected in 41 of 81 samples (51%), and most (73%) were lower than 0.10 μg/kg. Only one sample contained 5.54 μg/kg.

In storage, both the number of OTA positive samples and the level of contamination were similar to results found during drying (Table 3). Of the 221 samples analyzed, only two had OTA values above 2 μg/kg.

3.4. OTA and ochratoxigenic fungi

Data from this work were analyzed by comparing the extent of fungal contamination, the number of ochratoxigenic fungi occurring, water activity content and level of contamination with OTA. Results of Pearson's coefficient analysis are shown in Table 4. A strong positive correlation was seen between the presence of fungi from *A. niger* aggregate and the occurrence of OTA in the samples, especially when *A. carbonarius* was present, with a correlation coefficient of 0.7.

Table 3
Ochratoxin A (OTA) contamination in cocoa beans at different processing stages [a].

Stage/number of samples evaluated		OTA>LOD n (%)	OTA>2 μg/kg n (%)	OTA (μg/kg)		
				Max.	Median	Mean
Before fermentation	25	0 (0%)	0 (0%)	<0.01	<0.01	<0.01
Fermentation	51	14 (27%)	0 (0%)	1.70	<0.01	0.05
Sun drying	81	41 (51%)	1 (1%)	5.54	0.01	0.13
Storage	65	33 (52%)	1 (2%)	4.64	0.02	0.10

[a] Limit of detection (LOD): 0.01 μg/kg; method mean recovery: 90.8%.

Table 4
Correlation coefficients of total fungi, ochratoxigenic fungi, water activity and ochratoxin A (OTA) ($p<0.001$).

Parameter	OTA	a_w
OTA	1.000	−0.154
Water activity	−0.154	1.000
Total fungi contamination (all fungi isolated)	0.102	−0.292
Aspergillus carbonarius	0.702	−0.196
A. niger aggregate	0.402	−0.155
A. section *Circumdati*	−1.55e-02	−6.02e-03

4. Discussion

Our results indicate that the critical point for relevant fungi to infect cocoa beans and commence producing OTA is during the drying stage, when the beans start to lose water. The decrease of a_w reduces the number of competitors due to the high sensitivity of bacteria and yeasts to low water availability (Beuchat, 1987). In addition, the dispersal of fermented beans on wooden drying platforms facilitates contact with fungi which can act as initial inoculum, and increases the oxygen tension essential for the growth of these fungi. The wooden drying floor used in most of the farms made good hygiene conditions difficult during the drying. Some farms are evaluating alternatives such as cement and plastic platforms which are more readily and effectively cleaned. More studies are needed comparing different drying platforms and focusing on fungal contamination, as in the drying stage a sharp increase in the numbers and species of *Aspergillus* capable of producing OTA was observed. The recommended moisture content of dried cocoa beans for safe storage is 8% (Minifie, 1999). The farms should have appropriate equipment to measure the moisture content instead of using empirical methods.

Our results indicate that *A. carbonarius* is the main source of OTA in cocoa. Of 92 isolates of this species, 100% were found to be capable of OTA production. *A. niger* aggregate was found in a higher number (191 isolates), but only 5.2% produced this toxin. *Aspergillus* section *Circumdati* was also found, though much less commonly, but they also had the potential to produce OTA. Other reports have pointed out *A. carbonarius* as the main source of OTA in grapes and grape products throughout the world (Abarca et al., 2003; Iamanaka et al., 2005; Leong et al., 2006) and in robusta coffee (Joosten et al., 2001).

A recent study carried out in Cameroon by Mounjouenpou et al. (2008) reported *A. niger* as the main species producing OTA in cocoa beans under conditions similar to those reported in our study. Mounjouenpou et al. (2008) reported that 70% of the *A. niger* isolates could produce OTA. This high percentage has not been confirmed by other studies carried out worldwide with this species (Taniwaki et al., 2003; Belli et al., 2004; Iamanaka et al., 2005; Leong et al., 2007; Magnoli et al., 2007; Amezqueta et al., 2008).

Mounjouenpou et al. (2008) reported that *A. carbonarius* was isolated only in samples from wounded pods opened 10 days after harvest. In our study, OTA was found during fermentation of a batch containing wounded pods and *A. carbonarius* was already present. Nine samples of dried cocoa beans imported from Sierra Leone, Equatorial Guinea and Ecuador were examined by Sanchez-Hervas et al. (2008), who reported 138 isolates of black *Aspergillus* species, with 60% of individual cocoa beans infected. The ability of these isolates to produce OTA was also high, as 44.7% of *A. niger* isolates and 100% of *A. carbonarius* isolates were positive, with higher amounts of OTA produced by *A. niger* aggregate in pure culture when compared with *A. carbonarius*.

The correlation we found between the presence of *A. carbonarius* and OTA indicates the importance of this species as a source of OTA in cocoa and other tropical and subtropical raw materials, as stated in earlier studies (Pitt and Hocking, 2009; Magnoli et al., 2003; Taniwaki et al., 2003; Belli et al., 2004; Samson et al., 2004; Martínez-Culebras and Ramón, 2007).

OTA levels found in cocoa beans from storage evaluated in this study (52% positive samples; mean 0.25 μg/kg) were lower than those observed and reported by Dongo et al. (2008) in Nigerian cocoa. In that study, 54 (91.5%) of 59 samples were contaminated with OTA, with an average of 40 μg/kg and maximum of 277 μg/kg. Our results are more comparable with those of Bonvehi (2004) with OTA positive in 76% of 21 samples, with an average of 0.45 μg/kg and a maximum of 3.5 μg/kg and those of Amezqueta et al. (2004), with 63% positive among 46 samples analyzed, with an average of 1.71 μg/kg and a maximum of 15 μg/kg. Samples for analyses were taken from a wide geographical range of sources in both of these studies.

Other reports have described a lower occurrence of OTA in cocoa beans from America and the Pacific when compared to other cocoa producing countries (Raters and Matissek, 2000; Gilmour and Lindblom, 2008).

Results found in this study suggest that, in general, OTA contamination in Brazilian cocoa is low, although the occurrence of fungi able to produce OTA increases during drying and storage. *A. carbonarius* is the main species responsible for OTA production on cocoa beans, although other ochratoxigenic species from the genus *Aspergillus* have also been isolated.

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