

Short communication

## Filamentous fungi producing ochratoxin a during cocoa processing in Cameroon

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### Abstract

Ochratoxin A (OTA) is the main mycotoxin occurring in cocoa. A study was conducted in Cameroon to assess how filamentous fungi and toxigenesis were affected by the type of cocoa post-harvest treatment (boxes or heaps). The filamentous fungi isolated were almost identical when fermentation was carried out in boxes or heaps, with the presence of abundant black *Aspergillus* filamentous fungi: *A. niger* and *A. carbonarius*. Filamentous fungi were more abundant at the end of the harvesting season. Factors affecting bean integrity (poor handling, deferred processing) resulted in a qualitative and quantitative increase in contamination, when the total number of filamentous fungi could reach a maximum value of  $5.5 \pm 1.4 \times 10^7$  CFU g<sup>-1</sup> and black *Aspergilli* a maximum value of  $1.42 \pm 2.2 \times 10^7$  CFU g<sup>-1</sup>. A toxigenesis study showed that *Aspergillus carbonarius* was the main OTA-producing strain isolated. Its maximum production could reach 2.77 µg g<sup>-1</sup> on rice medium. *Aspergillus niger* strains did not always produce OTA and their toxigenesis was much lower. Fermented dried cocoa from poor quality pods was the most contaminated by OTA: up to 48 ng g<sup>-1</sup>.

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### 1. Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite produced by several species of *Aspergillus* and *Penicillium* genera. It attracts particular attention through the damage it does to the organism of humans and animals (Abarca et al., 1998). It has nephrotoxic (Mantle and McHugh, 1993), immunotoxic, teratogenic and carcinogenic properties (Kuiper-Goodman and Scott, 1989; Kuiper-Goodman, 1996; Höhler, 1998). OTA has been associated with Balkan Endemic Nephropathy (BEN) and tumour development in the urinary tract (Mantle and McHugh,

1993). Following experiments on animals, the International Agency for Research on Cancer (IARC, 1993) classed OTA as carcinogenic for humans (group 2B).

Ochratoxin A is mainly produced by *Aspergillus carbonarius*, *A. niger* and *A. ochraceus* in tropical zones, and by *Penicillium verrucosum* and *P. nordicum* in temperate zones (Pitt et al., 2000; Abrunhosa, et al., 2001; O'Callaghan et al., 2003). Studies have been conducted to determine the degree of OTA contamination in several foodstuffs and drinks (Thirumala-Devi et al., 2001; Pittet et al., 1996; WHO, 2001; Gareis and Scheuer, 2000; Skaug, 1999; Blanc et al., 1998; Hurst and Martin, 1998; Jorgensen, 1998). Given its existence in several consumer products, consumer exposure to OTA is increasing. In order to protect consumers, the European Union has drawn up a standard defining tolerable contamination limits. Other products, such as cereals, coffee and wine are already covered by international regulation (European

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Commission, 1995; Règlement (CE) n°472/2002), which is not yet the case for cocoa. Given the extent of cocoa consumption worldwide, the European Union will not delay in defining maximum contamination limits.

World cocoa production is estimated at 3 592 000 tons (ICCO, 2006). Fermentation is the main stage in cocoa post-harvest processing. It is generally carried out in a traditional manner by spontaneous fermentation. First of all, there is colonization by yeasts, followed by lactic bacteria, and then by acetic bacteria, which are finally replaced by aerobic sporulated bacilli (Schawn and Wheals, 2004; Thompson et al., 2001; Lopez and Dimick, 1995; Lehrian and Patterson, 1983). Recent studies dealt with yeast (Jespersen et al., 2005) and bacteria (Camu et al., 2007) populations associated with cocoa fermentations. Their succession and respective implication during fermentation were investigated using molecular-based methods and the understanding of the process was subsequently improved (Nielsen et al., 2007). However, very few studies exist on cocoa filamentous fungi during technological treatments. The main species isolated during natural fermentation of cocoa in Indonesia comprise *Penicillium citrinum*, *Kloeckera apis*, *Saccharomyces cerevisiae*, *Candida tropicalis*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum* and *Acetobacter pasteurianus* (Ardhana and Fleet, 2003). In the Dominican Republic, a predominance of yeasts of the genera *Kloeckera* and *Candida* is found at the beginning of fermentation, followed by *Lactobacillus pentosus*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus brevis* as the lactic bacteria and *Acetobacter lovaniensis* as the main acetic bacterium (Lagunes-Gálvez et al., 2007). Whilst several studies have been carried out on the evolution of filamentous fungi in coffee and its relation with OTA content during post-harvest processing (Suárez-Quiroz et al., 2004; Wilkens and Jörissen, 1999; Studer-Rohr et al., 1995; Micco et al., 1989), that is not the case for cocoa. A microbiological analysis on cocoa samples from 9 producing countries led to the isolation of *Aspergillus fumigatus* and *Rhizomucor pusillus* (Niles, 1981). There have been no studies on filamentous fungi and OTA-producing species in cocoa depending on the type of post-harvest processing.

High contamination frequencies have been found in cocoa samples and by-products. Burdaspal and et Legarda (2003) showed that OTA was found in 99.7 % of chocolate and cocoa powder samples. Contamination of 81.3% was also described in cocoa by-products by Miraglia and Brera (2002). Tafuri et al. (2004) found OTA contamination of between 0.22 and 0.77  $\mu\text{g kg}^{-1}$  in 10 samples of cocoa powder found on the Italian market. A study involving 46 cocoa samples of different origins found that 63 % of samples were contaminated by OTA, with an average contamination of 1.71  $\mu\text{g kg}^{-1}$  (Amézqueta et al., 2004). A maximum content of 100  $\mu\text{g kg}^{-1}$  was obtained with cocoa contaminated artificially (Hurst and Martin, 1998). Shelling by hand helped to reduce contamination levels in cocoa beans by more than 95% (Amézqueta et al., 2005).

The purpose of our study was to list and identify the fungi that colonize cocoa beans at different stages of processing, depending on the type of post-harvest process, and to study their potential for producing OTA.

## 2. Materials and methods

### 2.1. Cocoa

The cocoa pods (*Theobroma cacao* L.) were harvested by hand during the 2005 cocoa season in the Kumba region of Cameroon.

### 2.2. Cocoa fermentation

Two types of fermentation were studied: box fermentation, where the beans were placed in wooden boxes measuring 45 cm  $\times$  45 cm  $\times$  45 cm, and heap fermentation where the beans were tipped onto banana leaves placed on the ground. Fermentation was carried out in each case using 50 kg of beans. The heap was then covered with other banana leaves. After harvesting in the field, the pods were opened either immediately or later. Depending on the type of fermentation, pod condition or delay in pod opening, four treatment variants were investigated (Fig. 1): heap fermentation of beans from whole pods that had been opened immediately (T1), box fermentation of beans of the same type (T2), heap fermentation of beans from whole pods opened after 10 days (T3) heap fermentation of beans from damaged pods also opened after 10 days (T4). In the last case, the pods were partially opened after harvesting. Natural drying (in the sun) was carried out for between 5 and 10 days. Three fermentations of each type were performed during the cocoa season: at the beginning (September–October), in the middle (October–November) and at the end of the season (November–December).

### 2.3. Sampling

Cocoa samples were taken at different stages of processing (Fig. 1). They involved unfermented beans (A), fermented

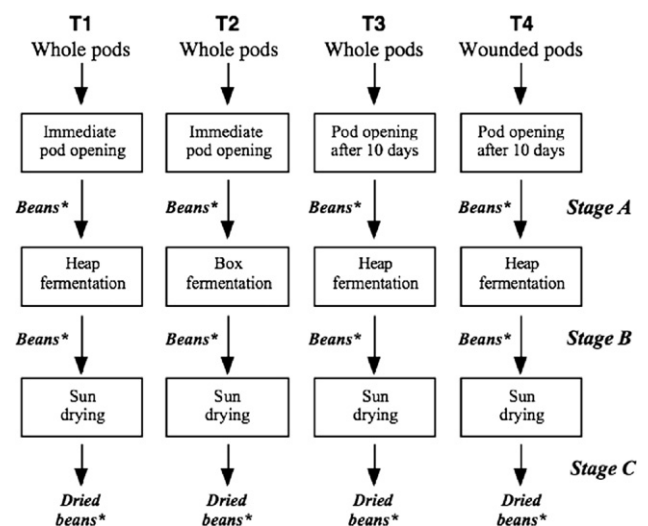


Fig. 1. Cocoa post-harvest processing (\* sampling). T1: Heap fermentation of beans from whole pods with immediate pod opening. T2: Box fermentation of beans from whole pods with immediate pod opening. T3: Heap fermentation of beans from whole pods with pod opening deferred by 10 days. T4: Heap fermentation of beans from wounded pods with pod opening deferred by 10 days. A, B, C: Sampling stages.

undried beans (B), and fermented sun-dried beans (C). In each case, 300 g cocoa samples were taken for microbiological analyses. OTA quantification was performed on dry beans (dry matter >90%). The samples taken at stages A and B were dried for 48 h in the oven at 30 °C to reproduce natural drying, bypassing any contamination occurring at that stage.

#### 2.4. Microbiological analyses

The filamentous fungi population was enumerated by inoculation on the surface of PDA medium (Biokar Diagnostics, Beauvais, France). The inoculum was obtained by soaking 15 cocoa beans in 90 mL of a peptone water solution (0.1% w/v; Biorad, Marnes la Coquette, France) for 10 min (Hocking and Pitt, 1980). The result was expressed in CFU g<sup>-1</sup> for the total filamentous fungi and for “black *Aspergillus*”. In order to determine the infection percentage and increase numbering sensitivity, direct plating was carried out at the same time, aseptically placing cocoa beans (3 beans per dish) on the surface of dishes containing PDA medium (Hocking and Pitt, 1980). The dishes were incubated at 25 °C for 5 to 7 days and the result was expressed as the percentage of infected beans. The filamentous fungi isolates were selected randomly according to phenotypic criteria (3 isolates for each phenotypic group). Isolates were identified according to morphological criteria (Samson et al., 1995). The identification of *Aspergillus* and *Penicillium* filamentous fungi was confirmed using molecular techniques by the Fungi and Yeasts Culture Collection at the Catholic University of Leuven in Belgium (BCCM™/MULC Culture Collection). For each sample, the frequency of *A. carbonarius* and *Aspergillus niger* isolates was estimated in relation to total filamentous fungi.

#### 2.5. Study of OTA production

For each strain isolated, a suspension (3 × 10<sup>6</sup> of conidia mL<sup>-1</sup>) was made up by scraping a PDA culture dish with a saline solution containing 0.01% Tween® 80 (P1754, Sigma-Aldrich, L’Isle d’Abeau, France). Five mL of the suspension were deposited in the centre of a dish of PDA medium which was incubated at 25 °C. After 20 days incubation, direct extraction was carried out from 3 agar discs taken from the centre of the colony. Extraction was carried out in 2.5 mL of solvent (methanol/formic acid 25:1 v/v) for 15 min in an ultrasound bath.

In order to test production on cocoa, 50 g of cocoa beans (verified OTA-free) were inoculated with 8 mL of a suspension of 50 × 10<sup>6</sup> conidia mL<sup>-1</sup> and incubated at 25 °C for 20 days. Extraction was carried out in an acetonitrile/water solution (60:40 v/v) for 40 min. Solvents were from Sigma-Aldrich (L’Isle d’Abeau, France) (HPLC grade). OTA production was also sought on rice using the FDA method (Tournas et al., 2001).

In all cases, OTA was quantified on extracts by HPLC with fluorimetric detection (Shimadzu LC-10 ADVP, Japan) (Nakajima et al., 1997). The operating conditions were as follows: 100 µL injection loop, C18 reverse phase HPLC column, ODS 5 µm (Supelco, Interchim, Montluçon, France) with an identical pre-column thermostatically controlled at 35 °C, an isocratic flow of 1 mL/min, an excitation wavelength of 333 nm and an emission

wavelength of 460 nm. Contents were calculated from a calibration curve established from a standard (1 µg mL<sup>-1</sup>; ref PD 226 R. Biopharm Rhône Ltd, Glasgow, UK).

#### 2.6. OTA quantification in cocoa beans

The dried cocoa bean samples were frozen at -80 °C, then ground. Fifty grams of ground beans were extracted in 200 mL of solvent (acetonitrile/water, 60/40, v/v). Four mL of filtered extract were diluted in 44 mL of phosphate buffer. The mixture was purified on an immunoaffinity column (Ochraprep, Rhône Diagnostics, Scotland). OTA was eluted by 3 mL of methanol and evaporated till dry in a nitrogen stream at 70 °C. The residue was resuspended in 1 mL of the mobile phase (water/acetonitrile/acetic acid, 51:48:1 v/v). Quantification was by HPLC using the previously described method.

### 3. Results

#### 3.1. Changes in filamentous fungi species during cocoa processing

Table 1 gives the identification of the total filamentous fungi in cocoa samples from the different fermentation operations. They mostly belonged to the genera *Penicillium*, *Aspergillus*, *Mucor*, *Geotrichum*, *Trichoderma*, *Rhizopus*, and *Fusarium*, with some species known to produce OTA (*A. niger*, *A. carbonarius*). There was no significant difference for the isolated filamentous

Table 1  
Identification of the filamentous fungi isolated during technological treatments

Treatment	Sampling stage	Filamentous fungi isolated
T1	A	–
	B	<i>A. versicolor</i> , <i>Mucor</i> spp, <i>A. niger</i> , <i>Geotrichum</i> spp, <i>A. fumigatus</i> , <i>Fusarium</i> spp, <i>Rhizopus nigricans</i>
	C	<i>A. tamarii</i> , <i>A. fumigatus</i> , <i>Rhizopus nigricans</i> , <i>A. niger</i>
T2	A	–
	B	<i>A. versicolor</i> , <i>A. fumigatus</i> , <i>A. tamarii</i> , <i>Rhizopus nigricans</i> , <i>Fusarium</i> spp, <i>A. niger</i>
	C	<i>Rhizopus nigricans</i> , <i>A. fumigatus</i> , <i>A. tamarii</i> , <i>A. niger</i>
T3	A	<i>P. crustosum</i> , <i>Fusarium</i> spp, <i>A. tamarii</i> , <i>P. sclerotiorum</i> , <i>A. flavus</i> , <i>A. fumigatus</i> , <i>Mucor</i> spp, <i>Rhizopus nigricans</i> , <i>A. niger</i>
	B	<i>Rhizopus nigricans</i> , <i>Mucor</i> spp, <i>A. fumigatus</i> , <i>Syncephalastrum racemosum</i> , <i>A. tamarii</i> , <i>P. sclerotiorum</i> , <i>A. flavus</i> , <i>Geotrichum</i> spp, <i>Trichoderma</i> spp, <i>A. niger</i>
	C	<i>Geotrichum</i> spp, <i>Mucor</i> spp, <i>A. fumigatus</i> , <i>A. flavus</i> , <i>A. tamarii</i> , <i>A. niger</i>
T4	A	<i>Scopulariopsis</i> spp, <i>A. niger</i> , <i>Syncephalastrum racemosum</i> , <i>A. fumigatus</i> , <i>Rhizopus nigricans</i> , <i>Mucor</i> spp, <i>P. crustosum</i> , <i>A. carbonarius</i>
	B	<i>A. versicolor</i> , <i>Rhizopus nigricans</i> , <i>Mucor</i> spp, <i>Scopulariopsis</i> spp, <i>Syncephalastrum racemosum</i> , <i>A. niger</i> , <i>P. crustosum</i>
	C	<i>P. crustosum</i> , <i>P. sclerotiorum</i> , <i>Fusarium</i> spp, <i>Scopulariopsis</i> spp, <i>Rhizopus nigricans</i> , <i>A. flavus</i> , <i>Trichoderma viride</i> , <i>A. niger</i> , <i>A. carbonarius</i>

A, B, C, T1–T4 are defined on Fig. 1.

fungi depending on fermentation type (heap or box). Pod integrity and, to a lesser degree, a delay in pod opening affected the qualitative diversity of the filamentous fungi. Wounded pods revealed high *A. carbonarius*, *A. niger* and *Fusarium* spp proliferation in the pod openings.

Table 2 gives the quantification of total filamentous fungi and black filamentous fungi isolated during the technological treatments. Filamentous fungi were found for all the conditions, but they varied in number depending on the sampling stage. Stage B had the highest contamination rate for both total filamentous fungi and for black *Aspergillus*. Maximum concentrations of  $5.5 \pm 1.4 \times 10^7$  CFU g<sup>-1</sup> and  $1.4 \pm 0.2 \times 10^7$  CFU g<sup>-1</sup> were obtained for those two categories respectively. It should be noted that the number of black *Aspergillus* filamentous fungi (*A. carbonarius* and *A. niger*) was not identical throughout the cocoa harvest season. Those *Aspergillus* were less frequent at the beginning of the season, and were only encountered at stage B (after fermentation), except in wounded pods. Cocos from the middle and end of the season were more contaminated, irrespective of the sampling stage, and that contamination increased

in line with pod damage. Identification of the isolated strains revealed that *A. niger* was the predominant strain (90 to 100% of black *Aspergillus*). *A. carbonarius* was mostly found in unfermented beans (A) from wounded pods.

Direct plating confirmed high cocoa bean contamination by filamentous fungi (Table 3). The contamination rates by total filamentous fungi was 100% irrespective of treatment type. Black filamentous fungi were found after fermentation and drying (when pods were opened immediately) and at all stages of processing when pod opening was delayed, and with wounded pods. These results confirmed the counting results.

### 3.2. OTA production by filamentous fungi

The ability of strains of the main species of filamentous fungi isolated (*A. fumigatus*, *A. tamarii*, *A. versicolor*, *A. carbonarius*, *A. niger*, *Penicillium sclerotiorum*, *P. paneum*, *P. crustosum*) to produce OTA was first tested for a large number of strains (310) on PDA medium. Production was then quantified on rice medium (Tournas et al., 2001) and on cocoa medium for the

Table 2  
Quantification of total and black *Aspergilli* isolated during technological treatments

	Sampling stage	Total filamentous fungi (CFU g <sup>-1</sup> )	Black <i>Aspergilli</i> (CFU g <sup>-1</sup> )	<i>A. carbonarius</i> (% of black <i>Aspergilli</i> )	
Beginning of season (September–October)	T1	A	nd	nd	–
		B	$4.0 \pm 1.4 \times 10^7$	$5.2 \pm 1.8 \times 10^6$	0
		C	$3.7 \pm 0.5 \times 10^6$	nd	–
	T2	A	nd	nd	–
		B	$4.5 \pm 0.8 \times 10^7$	$1.3 \pm 0.2 \times 10^6$	0
		C	$4.0 \pm 1.9 \times 10^4$	nd	–
	T3	A	$2.6 \pm 0.7 \times 10^5$	nd	–
		B	$5.0 \pm 0.8 \times 10^7$	$8.7 \pm 4.2 \times 10^5$	–
		C	$3.5 \pm 2.6 \times 10^4$	nd	0
	T4	A	$1.6 \pm 0.6 \times 10^5$	$1.4 \pm 0.5 \times 10^5$	75
		B	$5.5 \pm 1.4 \times 10^7$	$2.2 \pm 0.2 \times 10^6$	0
		C	$1.0 \pm 0.4 \times 10^6$	$1.3 \pm 0.57 \times 10^5$	0
Mid-season (October–November)	T1	A	nd	nd	–
		B	$7.7 \pm 1.9 \times 10^6$	$2.5 \pm 0.6 \times 10^6$	0
		C	$5.2 \pm 0.7 \times 10^6$	$3.5 \pm 0.5 \times 10^6$	0
	T2	A	nd	nd	–
		B	$2.3 \pm 0.5 \times 10^6$	nd	–
		C	$4.2 \pm 2.0 \times 10^4$	$2.8 \pm 1.3 \times 10^4$	0
	T3	A	$3.2 \pm 0.4 \times 10^6$	$5.5 \pm 0.7 \times 10^5$	0
		B	$2.0 \pm 0.3 \times 10^7$	$3.4 \pm 0.5 \times 10^6$	0
		C	$4.9 \pm 0.7 \times 10^5$	$4.9 \pm 0.7 \times 10^6$	0
	T4	A	$6.0 \pm 1.7 \times 10^6$	$1.6 \pm 0.5 \times 10^6$	38
		B	$2.0 \pm 0.3 \times 10^7$	$1.4 \pm 0.2 \times 10^7$	0
		C	$7.8 \pm 0.8 \times 10^6$	$4.1 \pm 0.5 \times 10^6$	0
End of season (November–December)	T1	A	nd	nd	–
		B	$7.7 \pm 1.9 \times 10^6$	$1.5 \pm 0.2 \times 10^6$	0
		C	$5.2 \pm 0.7 \times 10^6$	$2.0 \pm 0.2 \times 10^5$	0
	T2	A	nd	nd	–
		B	$2.3 \pm 0.5 \times 10^6$	$3.0 \pm 0.8 \times 10^6$	0
		C	$4.2 \pm 2.0 \times 10^4$	$4.6 \pm 0.7 \times 10^4$	0
	T3	A	$1.8 \pm 0.9 \times 10^5$	$9.0 \pm 4.0 \times 10^3$	0
		B	$5.4 \pm 0.5 \times 10^7$	$2.7 \pm 0.3 \times 10^6$	0
		C	$3.1 \pm 0.5 \times 10^6$	$2.5 \pm 0.4 \times 10^5$	0
	T4	A	$2.9 \pm 0.4 \times 10^7$	$7.0 \pm 0.9 \times 10^6$	50
		B	$2.5 \pm 0.3 \times 10^7$	$4.0 \pm 0.5 \times 10^6$	0
		C	$2.5 \pm 0.5 \times 10^6$	$3.0 \pm 0.6 \times 10^5$	0

A, B, C, T1–T4 are defined on Fig. 1.

nd: not detectable (quantification limit 10 CFU g<sup>-1</sup>).

Table 3  
Variation in contamination rate by total and black filamentous fungi during cocoa processing (Infected beans/total beans)

	Treatment	Sampling stage	Rate of contamination by filamentous fungi	Rate of contamination by black Aspergilli
Beginning of season (September–October)	T1	A	0/6	0/6
		B	6/6	0/6
		C	6/6	2/6
	T2	A	0/6	0/6
		B	6/6	5/6
		C	6/6	2/6
	T3	A	6/6	0/6
		B	6/6	0/6
		C	6/6	0/6
	T4	A	6/6	4/6
		B	6/6	3/6
		C	6/6	3/6
Mid-season (October–November)	T1	A	0/6	0/6
		B	6/6	2/6
		C	6/6	5/6
	T2	A	0/6	0/6
		B	6/6	0/6
		C	6/6	2/6
	T3	A	6/6	2/6
		B	6/6	3/6
		C	6/6	5/6
	T4	A	6/6	4/6
		B	6/6	3/6
		C	6/6	5/6
End of season (November–December)	T1	A	0/6	0/6
		B	6/6	0/6
		C	6/6	2/6
	T2	A	0/6	0/6
		B	6/6	0/6
		C	6/6	2/6
	T3	A	6/6	1/6
		B	6/6	4/6
		C	6/6	4/6
	T4	A	6/6	2/6
		B	6/6	3/6
		C	6/6	5/6

A, B, C, T1–T4 are defined on Fig. 1.

Table 4  
OTA production by identified filamentous fungi

Isolate	OTA production (ng g <sup>-1</sup> )		
	Rice medium after 20 days	Cocoa medium 8 days	Cocoa medium after 20 days
<i>A. carbonarius</i> 1	573.4	39.2	50.6
<i>A. carbonarius</i> 2	2772	84.5	110.7
<i>A. niger</i> 1	nd	0.5	nd
<i>A. niger</i> 2	3.5	0.1	0.2
<i>A. niger</i> 3	3.6	nd	0.05

A, B, C, T1–T4 are defined on Fig. 1.

nd: not detectable (detection limit 0.03 ng g<sup>-1</sup>).

*A. carbonarius* 1: Beginning of season, treatment T4, sampling stage A.

*A. carbonarius* 2: Mid-season, treatment T3, sampling stage C.

*A. niger* 1: Beginning of season, treatment T4, sampling stage A.

*A. niger* 2: Beginning of season, treatment T2, sampling stage B.

*A. niger* 3: End of season, treatment T4, sampling stage B.

most toxigenic strains (Table 4). No OTA production was found with *A. fumigatus*, *A. tamarii*, *A. versicolor*, *P. sclerotiorum*, *P. paneum*, *P. crustosum*. Of the fifty-three *A. carbonarius* isolates, only two were studied in detail. Irrespective of the culture medium (rice or cocoa), those isolates revealed strong toxigenesis which varied depending on the culture medium. OTA production was greater on rice medium with a content of 573.4 to 2772 ng g<sup>-1</sup> after twenty days' culture. On cocoa medium, values of 39.2 to 84.5 ng g<sup>-1</sup> after 8 days' culture and 50.6 to 110.7 ng g<sup>-1</sup> after 20 days' culture were obtained. Of the

Table 5  
Correlation between OTA content in fermented dried cocoa, treatment type and associated filamentous fungi

	Treatment	OTA in beans (ng g <sup>-1</sup> )	Main associated filamentous fungi	
Beginning of season	T1	A	Nd	
		B	0.05±0.01	<i>A. versicolor</i> , <i>A. niger</i> <sup>a</sup>
		C	0.02±0.02	<i>A. tamarii</i>
	T2	A	Nd	/
		B	0.04±0.02	<i>A. versicolor</i> , <i>A. niger</i> <sup>a</sup> , <i>A. fumigatus</i> , <i>P. paneum</i>
		C	0.15±0.01	<i>A. tamarii</i>
	T3	A	0.02±0.01	<i>P. sclerotiorum</i>
		B	0.17±0.04	<i>A. versicolor</i>
		C	0.09±0.01	<i>A. fumigatus</i> , <i>A. tamarii</i>
	T4	A	12.14±0.10	<i>A. carbonarius</i> <sup>b</sup> , <i>A. niger</i> <sup>a</sup>
		B	0.41±0.04	<i>A. versicolor</i>
		C	0.16±0.02	<i>A. niger</i> <sup>a</sup> , <i>P. sclerotiorum</i> , <i>P. crustosum</i>
Mid-season	T1	A	nd	
		B	0.13±0.02	<i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
		C	0.03±0.00	<i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
	T2	A	nd	/
		B	nd	<i>A. tamarii</i> , <i>P. paneum</i>
		C	0.01±0.00	<i>A. niger</i> <sup>a</sup> , <i>A. tamarii</i> , <i>P. paneum</i>
	T3	A	0.03±0.00	<i>A. tamarii</i> , <i>A. niger</i> <sup>a</sup> , <i>P. sclerotiorum</i>
		B	0.25±0.20	<i>A. tamarii</i> , <i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
		C	0.05±0.00	<i>A. niger</i> <sup>a</sup>
	T4	A	22.20±0.91	<i>A. carbonarius</i> <sup>b</sup> , <i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
		B	1.14±0.07	<i>A. niger</i> <sup>a</sup> , <i>A. tamarii</i>
		C	1.01±0.02	<i>A. niger</i> <sup>a</sup>
End of season	T1	A	nd	
		B	0.06±0.01	<i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
		C	0.12±0.01	<i>A. niger</i> <sup>a</sup>
	T2	A	nd	/
		B	0.27±0.01	<i>A. niger</i> <sup>a</sup> , <i>A. tamarii</i> , <i>P. paneum</i>
		C	0.06±0.00	<i>P. paneum</i> , <i>A. niger</i> <sup>a</sup>
	T3	A	0.08±0.01	<i>A. niger</i> <sup>a</sup> , <i>P. paneum</i>
		B	nd	<i>P. paneum</i>
		C	0.06±0.03	<i>A. tamarii</i> , <i>A. niger</i> <sup>a</sup>
	T4	A	48.01±0.75	<i>A. carbonarius</i> <sup>b</sup> , <i>A. niger</i> <sup>a</sup> , <i>P. crustosum</i>
		B	4.18±0.24	<i>A. niger</i> <sup>a</sup> , <i>P. crustosum</i>
		C	5.40±0.41	<i>A. niger</i> <sup>a</sup>

A, B, C, T1–T4 are defined on Fig. 1.

<sup>a</sup> Toxigenic.

<sup>b</sup> Very toxigenic.

145 *A. niger* isolates, only 3 were studied in detail. Compared to *A. carbonarius*, the toxigenesis of the three *A. niger* strains was much lower. On rice medium, OTA production was from 0.03 to 3.6 ng g<sup>-1</sup> as opposed to 0.03 to 0.2 ng g<sup>-1</sup> on cocoa medium after 20 days' culture.

### 3.3. OTA quantification in fermented dried cocoa

Fermented dried cocoa from the different fermentation operations was analysed to check for the existence of OTA. Table 5 gives the OTA contents found, along with the toxigenic flora associated with those cocoa beans.

Whatever the sampling stage, bean contamination by OTA was low for box and heap fermentation when the beans came from intact pods. In that case, OTA contents were between nd (not detectable) and 0.27 ng g<sup>-1</sup>, which remained below 2 ng g<sup>-1</sup> (limit defined for wine) and 5 ng g<sup>-1</sup> (limit defined for roasted coffee). The toxigenic microflora associated with beans contaminated by OTA mostly consisted of *A. niger*. When pods were wounded, a maximum content of 48.02 ng g<sup>-1</sup> was observed, which was well over tolerable rates. The toxigenic species associated with those beans were *A. carbonarius* and *A. niger*.

## 4. Discussion

Filamentous fungi hold an important position in the ecology of cocoa beans during fermentation (Roelofsen, 1958; Schawn and Wheals, 2004). The main filamentous fungi isolated during our work were *A. fumigatus*, *A. tamarii*, *A. versicolor*, *A. carbonarius*, *A. niger*, *P. sclerotiorum*, *P. paneum* and *P. crustosum*, *Mucor* spp, *Rhizopus* spp, *Fusarium* spp and *Trichoderma* spp. Our results differed from those quoted in the literature for the filamentous fungi associated with fermented beans (Marvalhas, 1966) or dried beans (Ciferri, 1931; Bunting, 1928; Dade, 1928). In those publications, *A. fumigatus*, *Aspergillus glaucus*, *Mucor* spp and *Penicillium* spp were isolated. Marvalhas (1966) isolated *P. citrinum* from fermented beans. According to the literature, filamentous fungi associated with fermentation and drying are different. With the exception of *A. fumigatus*, and *Mucor* spp, the other species found are not described as being associated with cocoa.

Irrespective of pod condition and type of fermentation (boxes or heaps), a large increase in filamentous fungi species was found after fermentation (stage B). That might have been explained by the existence of sweet mucilage, which is highly conducive to filamentous fungi development. However, drying helped to reduce the flora, as susceptible species disappeared to the benefit of soil-born and more generally environmental flora which led to contamination during solar drying. *A. niger* was found under all conditions, from fresh bean to fermented and dried bean. Contamination by *A. carbonarius* was particularly found in unfermented beans from damaged pods with deferred pod opening. That high contamination may have been due to the fact that when pods were partially opened, the cocoa beans came into direct contact with the air and ground. With fermentation, there was competition between the different species present and

those with rapid growth (*Mucor*, *Rhizopus* spp) managed to colonize first to the detriment of *Aspergillus* and *Penicillium*.

A study of OTA production by the isolated filamentous fungi revealed that *A. fumigatus*, *A. tamarii*, *A. versicolor*, *P. sclerotiorum*, *P. paneum*, and *P. crustosum* did not produce OTA. Contrary to our results, the literature indicates that *A. fumigatus* and *A. versicolor* can produce OTA in cereals (Rizzo et al., 2002). *P. sclerotiorum* has also been reported to produce OTA (Frisvad et al., 2004). It is likely that differences exist between strains of different origin, with a substrate effect.

Our results showed that only *A. niger* and especially *A. carbonarius* were able to produce OTA in cocoa. All the *A. carbonarius* isolates produced OTA, whereas 70 % of *A. niger* isolates were toxigenic but only with a low production level (Table 4). This is an interesting unusual result considering that Taniwaki et al. (2003) found that 75 % of *Aspergillus ochraceus* strains and 3 % of *A. niger* isolates produced OTA in coffee. In grapes, *A. carbonarius* were also the main OTA-producing species: 97% of the *A. carbonarius* isolates and 3 % of the *A. niger* aggregate isolates were OTA-positive (Lasram et al., 2007). These results illustrated well the biodiversity of the black Aspergilli according to their natural environment.

OTA production by isolated species varied depending on the substrate. It was greater on rice medium than on cocoa medium (Table 4). Our results tallied with those of other authors which revealed the effect of the substrate on mycotoxin production (Kokkonen et al., 2005).

As shown in grapes by Belli et al. (2006), the high OTA level in cocoa beans produced by heap fermentation from wounded pods, with pod opening deferred by 10 days (T4), could be correlated to the presence of *A. carbonarius*. Treatment T4, during which most *A. carbonarius* strains were isolated, thus gave the most contaminated cocoa.

However, the statistical significance of these results has to be improved by studies during other cocoa seasons in areas under different environmental conditions.

Nielsen et al. (2005) have successfully studied the yeast population dynamics during Ghanaian cocoa fermentations using denaturing gradient gel electrophoresis. In the same way, molecular-based methods could be useful tools to study the black Aspergilli genetic biodiversity in relation with their toxinogenesis.

It is known (Esteban et al., 2006) that drying and storage conditions play a major role in the presence of OTA. Our results show that contamination prior to processing also greatly influences end-quality and that good pod condition and immediate pod opening can partly reduce the risks.

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