



Mycobiota and mycotoxin producing fungi from cocoa beans

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ABSTRACT

The present study reports on the natural mycobiota occurring in cocoa beans, paying special attention to the incidence of fungal species that are potential producers of mycotoxins. The results show that predominant fungi were different species of the genus *Aspergillus* belonging to section *Flavi* and *Nigri*. Of the 214 strains of *Aspergillus* section *Flavi* collected from cocoa beans, 120 were identified as *A. flavus* and 94 as *A. tamarii*. Of *Aspergillus* section *Nigri* 138 strains were isolated, with 132 belonging to *A. niger* aggregate and 6 to *A. carbonarius* species. Potential ability to produce aflatoxins (AFs) B1, B2, G1 and G2, cyclopiazonic acid (CPA) and ochratoxin A (OTA) was studied by isolate culture followed by HPLC analysis of these mycotoxins in the culture extracts. Results indicated that 64.1% and 34.2% of the *A. flavus* strains produced AFs and CPA, respectively. Most of the *A. flavus* strains presented moderate toxigenicity with mean levels of AFs ranging from 100 ng g⁻¹ to 1000 ng g⁻¹. All the CPA-producing strains of *A. flavus* were highly toxigenic producing >30 µg g⁻¹ of CPA. Furthermore, 98% of *A. tamarii* strains produced CPA and over 50% of them were highly CPA toxigenic. With respect to OTA-producing fungi, a high percentage of black aspergilli strains (49.2%) were able to produce OTA. Additionally, most of the OTA-producing isolates were of moderate toxigenicity, producing amounts of OTA from 10 µg g⁻¹ to 100 µg g⁻¹. These results indicate that there is a possible risk factor posed by AFs, CPA and OTA contamination of cocoa beans, and consequently, cocoa products.

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1. Introduction

Cocoa is a very important ingredient in a number of foods, such as cakes, biscuits, child-foods, ice-creams and sweets. Cocoa beans, originating as seeds in fruit pods of the tree *Theobroma cacao*, are source of cocoa powder and come from Africa, and Central and South America. Neither storage nor processing conditions of cocoa are strictly controlled in these tropical countries, thus fungi contamination is possible at many critical points in the cocoa production chain (Magan and Aldred, 2005). The beans are susceptible to fungal spoilage during and after fermentation, the first stage in preparation for cocoa production. Fungal species belonging to the genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* have been observed on mishandled or improperly dried fermented beans (Roelofsen, 1958; Broadent and Oyeniran, 1968). More recently, *Aspergillus* species were the most frequently isolated fungi from samples of ground cocoa-based beverages (Oyetunji, 2006).

Many fungi, especially species from the genera *Aspergillus* and *Penicillium*, produce mycotoxins that can cause acute or chronic

intoxication and damage to humans and animals after ingestion of contaminated food and feed (Marasas and Nelson, 1987; Moss, 1996). Among the mycotoxins, aflatoxins (AFs) and ochratoxin A (OTA) are of special interest, given their high occurrence and toxicity. All AFs are regulated in different products in most countries worldwide (Commission of the European Communities, 2001). Recently, the European Commission has established 2 µg and 1 µg as the maximum level of OTA in raw material for manufacturing cocoa products and consumer products, respectively (Anonymous, 2007).

AFs are hepatotoxic, teratogenic, mutagenic and carcinogenic mycotoxins produced by members of *Aspergillus* section *Flavi* mainly *Aspergillus flavus* and *Aspergillus parasiticus*. The most potent of the four naturally occurring AFs (B1, B2, G1 and G2) is aflatoxin B1 (AFB1), which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1982) because of its demonstrated carcinogenicity to humans (Castegnaro and Wild, 1995). Besides AFs, some *A. flavus* strains together with strains belonging to the species *Aspergillus tamarii*, also included in the section *Flavi*, are reported to produce cyclopiazonic acid (CPA) (Horn, 2007). This mycotoxin is a specific inhibitor of calcium-dependent ATPase, which is toxic to animals and humans (Riley and Goeger, 1992).

Ochratoxin A (OTA) is mainly a mycotoxin with nephrotoxic effects and has been associated with Balkan Endemic Nephropathy (Krogh, 1978; Kuiper-Goodman and Scott, 1989; Abouzied et al., 2002). Recently, black *Aspergillus* species (section *Nigri*), such as *Aspergillus*

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carbonarius and species belonging to the *Aspergillus niger* aggregate, have been described as the main source of OTA contamination in coffee, grapes and other agricultural products (Battilani and Pietri, 2002; Abarca et al., 2003; Pardo et al., 2004; Iamanaka et al., 2005; Magnoli et al., 2006, 2007). OTA occurrence in cocoa, cocoa powder and cocoa marketed products has been reported in different countries (Burdaspal and Legarda, 2003; Serra Bonhevi, 2004; Tafuri et al., 2004; Amezqueta et al., 2005; Brera et al., 2005).

Taking all this information into account, it would seem relevant to determine the mycoflora of cocoa and the potential ability of the isolated fungi to produce mycotoxins.

2. Materials and methods

2.1. Samples and reference strains

Fungi were isolated from nine samples (0.5 kg) of fermented and sun-dried cocoa beans from Sierra Leona (Forastero variety), Equatorial Guinea (Amazon Forastero variety) and Ecuador (Amazon–Trinitario–Canelo Amazon hybrid). Beans were provided by a Spanish import factory. Ten beans were picked from each sample and ground into smaller pieces. In order to avoid skin contamination from the beans, their surface was first decontaminated using a 5% chlorine solution for 1 min followed by two rinses with sterile-distilled water. Ten small pieces were taken randomly from each bean and directly plated onto plates of Dichloran Rose Bengal Chloramphenicol medium (DRBC) (Pitt and Hocking, 1997). Plates were incubated at 25 °C for 7 days. All fungi considered to represent different species were isolated and transferred to Malt Extract Agar (MEA) plates for identification (Pitt and Hocking, 1997). Isolates were identified through macroscopic and microscopic observation, with the aid of published guidelines (Klich, 2002; Samson et al., 2004a,b). The identity of the different isolates was confirmed by 5.8-ITS sequencing.

2.2. DNA preparation

All strains were grown on MEA medium for 6–8 days. Mycelium was collected from the plates, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg of powder and the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

2.3. PCR reactions and sequencing

The 5.8S-ITS region was amplified by PCR using universal primers its5 and its4 (White et al., 1990). PCR reactions were performed in 100 µl of final volume, containing 100–200 ng of DNA, 50 mM KCl, 10 mM Tris–HCl, 80 µM of each dNTP, 1 µM of each primer, 2 mM MgCl₂ and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, N.E.E.D, SL, Valencia, Spain). The reaction mixtures were incubated in a thermalcycler (Techne TC-512) for 35 cycles consisting of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72 °C.

PCR products were cleaned with the GeneClean II Purification Kit (Bio 101, La Jolla, CA, USA) and directly sequenced using the Taq DyeDeoxy terminator cycle sequencing Kit (Applied Biosystems, Falmer, Brighton, UK), according to the manufacturer's instructions in an Applied Biosystems automatic DNA sequencer (model 373A). The primers its5 and its4 were also used to obtain the sequence of both strands (White et al., 1990).

2.4. Extraction and detection of mycotoxins from culture

Mycotoxins were extracted using a variation of a simple method described previously (Bragulat et al., 2001). Briefly, three agar plugs (diameter: 6 mm) were obtained from the inner, middle and outer areas of each colony of potential mycotoxin-producers and were

injected in a vial containing 900 µl of methanol. After 60 min, the extracts were shaken and filtered (Millex® SLHV 013NK, 0.45 µm. Millipore, Bedford, MA, USA) into another vial and stored at 4 °C until chromatographic analysis.

For the determination of AFs and CPA, fungal isolates were grown on yeast extract sucrose agar (YES) and Czapeck Agar (CZ), respectively and incubated at 25 °C, 14 days (Pitt and Hocking, 1997). For the determination of OTA, fungal isolates were grown on Czapeck Yeast Extract Agar (CYA) and incubated at 25 °C for 7 days (Pitt and Hocking, 1997). Separation, detection and quantification were performed by injecting 20 µl of extract from each vial into an HPLC system consisting of a Dionex model P680A pump (Sunnyvale, CA, USA) connected to a Dionex model RF-2000 programmable fluorescence detector and to a Dionex PDA-100 photodiode array detector.

For the determination of AFs and CPA, a C18 reversed-phase column (250×4.6 mm i.d., 5 µm particle size; Supelcosil LC-18 (Supelco, Bellefonte, PA, USA)) connected to a precolumn Security Guard (20×4.6 mm i.d., 5 µm particle sizes, LC-18 Supelguard, Supelco) were used. For chromatographic separation of AFs the mobile phase was water:acetonitrile:methanol (3:1:1, v/v/v) under isocratic elution during 15 min, at a flow rate of 1 ml min⁻¹. AFs were determined by fluorescence detection at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Standard of AFs (B1, B2, G1 and G2) was obtained from Supelco (aflatoxin mix kit). The chromatographic separation of CPA was based on Urano et al. (1992) and Hayashi and Yoshizawa (2005) with some modifications. The mobile phase consisted of 85% v/v methanol in water (eluant A), 85% v/v methanol in water and 4 mM ZnSO₄·7H₂O (eluant B) and 0.1% v/v acetic acid in water (eluant C) with a multi-step gradient elution at a flow rate of 1 ml min⁻¹. The elution program was as follows: 100% A to 100% B (0–10 min), 100% B (10–12 min), 100% B to 100% C (12–15 min), 100% C (15–25 min), 100% C to 100% A (25–27 min) and 100% A (27–32 min). The UV-visible spectra were recorded from 200 to 400 nm. The elution time and spectra on the samples were compared with pure CPA standard (Sigma-Aldrich, Steinheim, Germany). For the determination of OTA, a C18 reversed-phase column (150×4.6 mm i.d., 5 µm particle size Kromasil C18 (Análisis Vínicos S.L., Tomelloso, Spain)), connected to a precolumn Kromasil C18 (10×4.6 mm i.d., 5 µm particle sizes, Análisis Vínicos S.L.) was used. For chromatographic separation of OTA, the mobile phase was acetonitrile:water:acetic acid, (57:41:2 v/v/v) under isocratic elution during 10 min, at a flow rate of 1 ml min⁻¹. OTA was determined by fluorescence detection at an excitation wavelength of 330 nm and an emission wavelength of 460 nm. The ochratoxin standard was obtained from *A. ochraceus* (Sigma-Aldrich).

3. Results

3.1. Fungal contamination of cocoa beans

Fig. 1 summarises the fungi isolated from cocoa beans. Predominant mycobiota belonged to the genera *Aspergillus* section *Flavi* (214 strains, 51% of the total strains) and section *Nigri* (138 strains, 32.8% of the total strains), constituting 83.8% of the total 420 fungal strains. The incidence of other species belonging to the genera *Aspergillus* (4.4%) and *Penicillium* (6.6%) was very low compared to *Aspergillus* section *Flavi* and *Nigri*. All *Aspergillus* spp. and *Penicillium* spp. considered to represent different species were isolated and identified by ITS sequencing. Within the genera *Aspergillus*, we identified strains from *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus ochraceus*, *Aspergillus terreus* and *Aspergillus versicolor* species. From the genera *Penicillium*, strains belonging to eight different species were identified. They included *Penicillium citrinum*, *Penicillium commune*, *Penicillium chrysogenum*, *Penicillium glabrum*, *Penicillium griseoroseum*, *Penicillium olsonii*, *Eupenicillium cinnamopurpur* and *Eupenicillium tropicum*. Finally, other fungal species were also isolated and identified

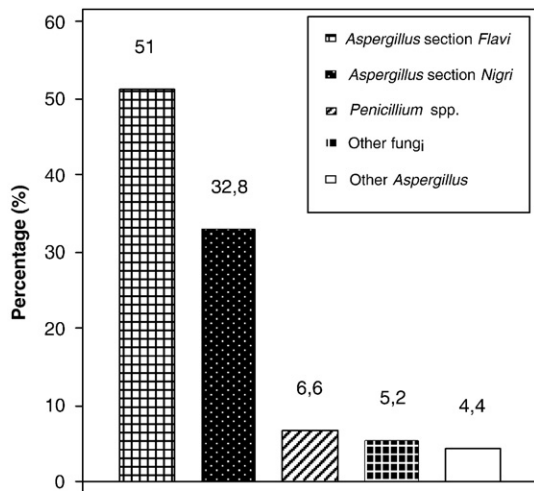


Fig. 1. Percentage of fungi isolated in cocoa beans.

by ITS sequencing, although at very low frequencies (5.2%). They included strains from *Chaetomium globosum*, *Cladosporium oxisporum*, *Emericella rugolosa*, *Eurotium amstelodami*, *Eurotium chevalieri*, *Nectria haematococca*, *Mucor racemosus*, *Phoma glomerata*, *Phoma medicaginis* and *Rhizopus oryzae* species.

3.2. Identification of strains and mycotoxigenic capacity

One of main goals of this study was to identify *Aspergillus* strains belonging to sections *Flavi* and *Nigri*, given the high frequency of isolation and potential for producing mycotoxins, such as AFs, CPA and OTA (Abarca et al., 2004, Horn, 2007). All *Aspergillus* strains belonging to the aforementioned sections were identified at species level, based on morphological characteristics such as colony morphology, morphology of the conidial head and conidial size and shape, as well as published reference guidelines (Klich, 2002; Samson et al., 2004a,b). Identities of representative strains were confirmed by 5.8-ITS sequencing.

Aspergillus section *Flavi* were the most frequently found fungi, isolated in 80% of the cocoa beans analysed. Of the 214 strains of *Aspergillus* section *Flavi* collected from cocoa beans, 120 were identified as *A. flavus* and 94 as *A. tamarii*. *A. flavus* strains contained typical morphological features of *A. flavus* with a green-coloured colony. Three representative strains from each geographical origin had an identical nucleotide sequence in the ITS regions. Based on the BLAST searches (National Center for Biotechnology Information, USA), these ITS sequences had 99% identity with the ITS sequence of *A. flavus* (AY373848). Additionally, the colonies of *A. tamarii* strains were brown in colour. When ITS sequences for representative strains from each geographical origin were compared with those available in the database, they were nearly identical (99% identity) to ITS sequence of *A. tamarii* (AY373870). No strain isolated in this study was identified as *A. parasiticus*.

Black aspergilli were also very frequent and were isolated in 60% of the cocoa beans analysed. Of the 138 strains of *Aspergillus* section *Nigri* collected from cocoa beans, 132 were identified as *A. niger*

Table 1
Occurrence and aflatoxin-producing ability of *A. flavus* strains isolated from cocoa beans

Species	Total strains (%)	AF positive strains (AF range in ng/g)				
		Total (%)	B1	B2	G1	G2
<i>A. flavus</i>	120 (29)	77 (64.1)	35 (20–3500)	65 (0.5–250)	15 (50–2500)	8 (2–12)

Table 2
Occurrence and cyclopiazonic acid producing ability of *Aspergillus* section *Flavi* isolated from cocoa beans

Species	Total strains (%)	CPA positive strains (%)	CPA produced ($\mu\text{g/g}$)	
			Range	Mean
<i>A. flavus</i>	120 (29)	41 (34.2)	33.3–240.7	121.7
<i>A. tamarii</i>	94 (22)	92 (98)	28.6–253.3	96.5

aggregate and 6 as *A. carbonarius*. The two relevant species, *A. niger* aggregate and *A. carbonarius*, can easily be differentiated by conidia dimensions (3–5 μm for *A. niger* aggregate and 7–10 μm for *A. carbonarius*). No black aspergilli isolated in this study was identified as uniseriate (*Aspergillus aculeatus* and *Aspergillus japonicus*) species.

3.2.1. Production of AFs

A total of 214 *Aspergillus* section *Flavi* were tested for their ability to produce aflatoxins B1, B2, G1 and G2 in YES medium (Table 1). Seventy-seven isolates (64.1%) identified as *A. flavus* were aflatoxigenic as demonstrated by HPLC analysis. Of the 77 *A. flavus* isolates positive for AFs, 35 and 65 isolates produced aflatoxins B1 and B2, respectively. Aflatoxins G1 and G2 were detected in 15 and 8 isolates, respectively. These strains also produced aflatoxins B1 and B2, indicating that a total of 8 strains were able to produce all aflatoxins (B1, B2, G1 and G2). The mean levels of AFs ranged from 100 ng g^{-1} to 1000 ng g^{-1} of medium; however, 9 (7.5%) strains were able to produce $>1000 \text{ ng g}^{-1}$. Table 1 shows the potential for AFs production by *A. flavus* strains isolated from cocoa beans.

3.2.2. Production of CPA

The 214 *Aspergillus* strains belonging to section *Flavi* were also tested for their ability to produce CPA in CZ medium (Table 2). Ninety-two strains of *A. tamarii* (98% of the 94 tested) and 41 strains of *A. flavus* (34.2% of the 120 tested) were CPA producers on CZ medium. Most of the *A. tamarii* strains produced high levels of CPA ranging from 28.62 to 253.3 $\mu\text{g g}^{-1}$ of medium. The CPA production ability by strains of *A. flavus* was similar that in the case of *A. tamarii* strains with mean levels of CPA ranging from 33.3 $\mu\text{g g}^{-1}$ to 240.7 $\mu\text{g g}^{-1}$. Table 2 shows the potential for CPA production by *Aspergillus* spp. strains isolated from cocoa beans.

3.2.3. Identification of chemotypes in *A. flavus* strains

The strains were classified into seven chemotypes based on AFs and CPA production patterns (Table 3). This classification was done similarly to previous studies conducted in Iran (Razzaghi-Abyaneh et al., 2006) and Italy (Giorni et al., 2007). Chemotypes I and II represented strains able to produce both AFs B and CPA including 7 and 8 strains, respectively (12.5%). The chemotype III, represented by 32 strains (26.6%), corresponded to strains able to produce only AFs B. Chemotype IV included 17 strains (14.2) that corresponded to strains able to produce only CPA. Around 30% of total strains were of the

Table 3
Chemotype patterns of *Aspergillus* section *Flavi* strains based on aflatoxins and cyclopiazonic acid producing ability

Chemotype	Mycotoxins			No. of strains (%)
	AFB	AFG	CPA	
I (B1 > B2)	+	–	+	7 (5.8)
II (B1 < B2)	+	–	+	8 (6.7)
III	+	–	–	32 (26.6)
IV	–	–	+	17 (14.2)
V	–	–	–	36 (30)
VI	+	+	+	9 (7.5)
VII	+	+	–	11 (9.2)

Table 4
Occurrence and ochratoxin producing ability of black *Aspergillus* isolated from cocoa beans

Species	Total strains (%)	Ochratoxigenic strains (%)	OTA produced ($\mu\text{g/g}$)	
			Range	Mean
<i>A. niger</i> aggregate	132	59 (44.7)	0.5–90	11.52
<i>A. carbonarius</i>	6	6 (100)	0.2–8	2.15

chemotype V, representing the isolates unable to produce any mycotoxin. The chemotype VI included 9 strains (7.5%) that corresponded to strains able to produce all mycotoxins (AFB, AFG and CPA). Finally, chemotype VII included 11 strains (9.2%) that corresponded to strains able to produce both aflatoxins, AFB and AFG.

3.2.4. Production of OTA

A total of 138 black aspergilli strains were tested for their ability to produce OTA in CYA medium. Sixty-five (47.1% of the 138 tested) isolates were shown to produce OTA. Fifty-nine strains positive for OTA production belonged to *A. niger* aggregate (44.7% of the 132 tested), while the remaining 6 strains were classified as *A. carbonarius* (100% of the 6 tested). The mean levels of OTA ranged from $0.5 \mu\text{g g}^{-1}$ to $90 \mu\text{g g}^{-1}$ and $0.2 \mu\text{g g}^{-1}$ to $8 \mu\text{g g}^{-1}$ in *A. niger* aggregate and *A. carbonarius*, respectively. Table 4 shows the potential for OTA production by black aspergilli isolated from cocoa beans.

4. Discussion

Despite the importance of cocoa-based products, not much is known about the mycobiota present on the raw material destined to chocolate manufacture, i.e. cocoa beans. In previous studies, *Mucor*, *Penicillium*, *Rhizopus* and especially *Aspergillus* were the fungi most frequently isolated from cocoa beans (Roelofs, 1958; Broadbent and Oyeniran, 1968; Oyetunji, 2006). However, little research has been done on the occurrence of mycotoxigenic fungi, species identification and mycotoxin evaluation. The results obtained in this study have provided, for the first time, information about the presence and distribution of mycotoxigenic fungi in cocoa beans and their ability to produce different mycotoxins. The main fungi isolated from cocoa beans were *Aspergillus* strains belonging to sections *Flavi* and *Nigri*. It is well known that some species of these two *Aspergillus* sections are considered the most significant toxigenic fungi (Moss, 1996). In addition, other fungal species belonging mainly to genera *Aspergillus* and *Penicillium*, were isolated and identified. Although some of the *Aspergillus* spp. isolated have a well-known potential for producing mycotoxins, such as sterigmatocystin (*Emerella* spp.) (Pitt and Hocking, 1997), given their low incidence they are an unlikely source of mycotoxins in this substrate. The incidence of species belonging to *Aspergillus* section *Circumdati*, which are traditionally considered ochratoxigenic, was very low. Only one out of the two isolated strains of *A. ochraceus* produced OTA at a level of $12.7 \mu\text{g g}^{-1}$ (data not shown). These data suggest that *A. ochraceus* is probably a relatively unimportant source of OTA in cocoa products. The major *Penicillium* species responsible for ochratoxin production, *P. verrucosum* and *P. nordicum* (Pitt and Hocking, 1997), have not been isolated from cocoa beans. Nevertheless, among the *Penicillium* spp. identified, there were some important mycotoxin-producers, such as *P. citrinum* (citrinin) and *P. chrysogenum* (roquefortine C) (Pitt and Hocking, 1997). Given the low frequency of isolation, their potential for mycotoxin production is not a cause of concern. Other fungi isolated such as *Mucor* and *Rhizopus* have not been reported as toxigenic, but can produce enzymes resulting in reduced cocoa quality, in particular by increasing its acidity.

The fungal species most frequently isolated from cocoa beans was *A. flavus* (29%). The other major *Aspergillus* species belonging to section *Flavi* responsible for AFs production, *A. parasiticus* (Horn, 2007), was not isolated from cocoa beans. Furthermore, a high percentage of the

A. flavus strains (64.1%) were positive for aflatoxin production. These data are similar to those found in other substrates such as bee pollen (Gonzalez et al., 2005), dusts generated by agricultural processing facilities (Sales and Yoshizawa, 2006) and maize (Giorni et al., 2007). Aflatoxin-producing strains were mostly *A. flavus* producing only B aflatoxins (35/77 AFB1 and 65/77 AFB2). None of the strains produced only G aflatoxins; however, 18 strains out of 77 produced both B and G aflatoxins. Although it is generally accepted that *A. flavus* produces only B aflatoxins, production of G aflatoxins has also been reported in the literature (Gabal et al., 1994; Giorni et al., 2007). Most of the strains were of moderate toxigenicity with mean levels of AFs ranging from 100 ng g^{-1} to 1000 ng g^{-1} . Nevertheless, the presence of 9 (7.5%) highly aflatoxigenic strains ($>1000 \text{ ng g}^{-1}$) was corroborated. Besides AFs production, 34.2% of the *A. flavus* strains were also able to produce CPA, in fact all of them proved to be high CPA producers ($>30 \mu\text{g g}^{-1}$). Regarding the results obtained from the chemotypes found in this study, they are in agreement with those found in field soils in Iran by Razzaghi-Abyaneh et al. (2006) and differ from those found in maize in Italy (Giorni et al., 2007). The non-toxic group was the most common chemotype found in this work. Furthermore, strains able to produce either more AFB than AFG or more AFG than AFB were also found in the present study. Finally, although *A. flavus* appears to be the dominant species within the section *Flavi* invading cocoa beans, *A. tamarii* was also a frequently isolated species (22%), and its presence can also contribute to mycotoxin contamination in cocoa products. Aflatoxins were not produced by any of the 94 strains of *A. tamarii*; however, a very high percentage (98%) of them was CPA-producing strains. Additionally, more than 50% of the strains of *A. tamarii* were highly toxigenic producing $>30 \mu\text{g g}^{-1}$ of CPA. Incidence of *Aspergillus* section *Flavi* belonging to *A. flavus* and *A. tamarii* species, together with the high percentage of toxigenic isolates, is considered to pose a potential risk of AFs and CPA contamination in cocoa products. Aflatoxins and CPA commonly co-occur in contaminated agricultural commodities, such as maize and peanuts (Fernandez-Pinto et al., 2001; Giorni et al., 2007). In order to establish the potential risk posed by human exposure to these mycotoxins in different cocoa products, it is essential to compile data concerning their content in such products.

Presence of black aspergilli was also found to be very important (33%) in cocoa beans, though lower than in other substrates such as grapes and coffee (80–90%) (Pardo et al., 2004; Bellí et al., 2004; Iamanaka et al., 2005; Magnoli et al., 2007; Martínez-Culebras and Ramón, 2007). Nevertheless, results indicated a high percentage of ochratoxigenic strains (47.1%), which is mainly due to the high percentage (44.7%) of ochratoxigenic strains identified as *A. niger* aggregate. In the literature, the reported percentages of OTA production by *A. niger* aggregate from different substrates are usually lower, ranging from 0.2 to 30% (Bellí et al., 2006; Iamanaka et al., 2005; Magnoli et al., 2006). Additionally, most of the OTA-producing strains studied in the present work produced amounts of OTA ranging from $10 \mu\text{g g}^{-1}$ to $100 \mu\text{g g}^{-1}$, whereas in other substrates, such as grapes and coffee, lower amounts of OTA have been recorded, ranging from $1 \mu\text{g g}^{-1}$ to $10 \mu\text{g g}^{-1}$ (Bellí et al., 2006; Martínez-Culebras and Ramón, 2007). Regarding the ability of *A. carbonarius* species to produce OTA, all the strains were able to produce this mycotoxin. Nevertheless, only 6 (4.5%) strains out of the total isolated black aspergilli were identified as *A. carbonarius*, moreover, the detected levels of OTA production was lower than in the *A. niger* aggregate, ranging from $0.2 \mu\text{g g}^{-1}$ to $8 \mu\text{g g}^{-1}$. Although different OTA-producing species might participate in OTA contamination of cocoa beans, and consequently cocoa products, our results provide strong evidence of the role played by black aspergilli, particularly the *A. niger* aggregate. This is supported not only by its important role in the mycobiota of cocoa beans, but also by its strong ability to produce OTA and the scarce or null contribution of the typical OTA-producing species, *A. ochraceus* and *P. verrucosum*.

In conclusion, this study has provided the first significant body of relevant information on the key fungal species responsible for

mycotoxin (AFs, CPA and OTA) contamination of cocoa beans used for manufacturing cocoa products.

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References

- Abarca, M.L., Accensi, F., Bragulat, M.R., Castellá, G., Cabañes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection* 66, 504–506.
- Abarca, M.L., Accensi, F., Bragulat, M.R., Castellá, G., Cabañes, F.J., 2004. Taxonomy and significance of black aspergilli. *Antonie van Leeuwenhoek* 86, 33–49.
- Abouzie, M.M., Horvat, A.D., Podlesny, P.M., Regina, N.P., Metodiev, V.D., Kamenova-Tozeva, R.M., Niagolova, N.D., Stein, A.D., Petropoulos, E.A., Ganev, V.S., 2002. Ochratoxin A concentrations in food and feed from a region with Balkan endemic nephropathy. *Food Additives and Contaminants* 19, 755–764.
- Amezqueta, S., Gonzalez-Peñas, E., Murillo, M., López de Cerain, A., 2005. Occurrence of ochratoxin A in cocoa beans: effect of shelling. *Food Additives and Contaminants* 22, 590–596.
- Anonymous, 2007. Discussion paper on ochratoxin A in cocoa. Codex Alimentarius Commission, Codex Committee on Food Additives and Contaminants, First session. Beijing, China (16–20 April 2007, CX/CF 07/1/19).
- Battilani, P., Pietri, A., 2002. Ochratoxin A in grapes and wine. *European Journal of Plant Pathology* 108, 639–643.
- Bellí, N., Pardo, E., Marín, S., Farré, G., Ramos, A.J., Sanchis, V., 2004. Occurrence of ochratoxin A and toxicogenic potential of fungal isolates from Spanish grapes. *Journal of Science of Food and Agriculture* 84, 541–546.
- Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2001. An easy screening method for fungi producing ochratoxin A in pure culture. *International Journal of Food Microbiology* 71, 139–144.
- Brera, C., Grossi, S., Miraglia, M., 2005. Interlaboratory study for ochratoxin A determination in cocoa powder samples. *Journal of Liquid Chromatography and Related Technologies* 28, 35–61.
- Broadent, J.A., Oyeniran, J.O., 1968. A new look at mouldy cocoa. *Proceeding 1st International Biodeterioration Symposium*, pp. 693–702.
- Burdaspal, P.A., Legarda, T.M., 2003. Occurrence of ochratoxin A in samples of different types of chocolate and cacao powder, marketed in Spain and fifteen foreign countries. *Alimentaria* October, pp. 143–153.
- Castegnaro, M., Wild, C.P., 1995. IARC activities in mycotoxin research. *Natural Toxins* 3, 327–331.
- Commission of the European Communities, 2001. EC regulation 466/01. *Official Journal of the European Union* L 77/1 (16.03.2001).
- Fernandez-Pinto, V., Patriarca, A., Locani, O., Vaamonde, G., 2001. Natural co-occurrence of aflatoxin and cyclopiazonic acid in peanuts grown in Argentina. *Food Additives and Contaminants* 18, 1017–1020.
- Gonzalez, G., Hinojo, M.J., Mateo, R., Medina, A., Jiménez, M., 2005. Occurrence of mycotoxin producing fungi in bee pollen. *International Journal of Food Microbiology* 105, 1–9.
- Gabal, M.A., Hegazi, S.A., Hassanin, N., 1994. Aflatoxin production by *Aspergillus flavus* field isolates. *Veterinary and Human Toxicology* 36, 519–521.
- Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., Battilani, P., 2007. Studies on *Aspergillus* Section *Flavi* Isolated from Maize in Northern Italy, vol. 113, pp. 330–338.
- IARC, 1982. The evaluation of the carcinogenic risk of chemical to humans. IARC Monograph Supplement, vol. 4. International Agency for Research on Cancer, Lyon, France.
- Hayashi, Y., Yoshizawa, T., 2005. Analysis of cyclopiazonic acid in corn and rice by a newly developed method. *Food Chemistry*, 93, 215–221.
- Horn, B.W., 2007. Biodiversity of *Aspergillus* section *Flavi* in the United States: a review. *Food Additives and Contaminants* 24, 1088–1101.
- Iamanaka, T.B., Taniwaki, M.H., Menezes, C.H., Vicente, E., Fungaro, M.H.P., 2005. Incidence of toxigenic fungi and ochratoxin A in dried fruits sold in Brazil. *Food Additives and Contaminants* 22, 1258–1263.
- Klich, M.A., 2002. Identification of common *Aspergillus* species. *Centraalbureau Voorschimmelculturs*. Utrecht, The Netherlands.
- Krogh, P., 1978. Causal associations of mycotoxic nephropathy. *Acta Pathol Microbiol Scand Sect A* 269, 1–28 (Suppl).
- Kuiper-Goodman, T., Scott, P.M., 1989. Risk assessment of the mycotoxin ochratoxin A. *Biomedical Environmental Science* 2, 179–248.
- Magan, N., Aldred, D., 2005. Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Additives and Contaminants* 22, 10–16.
- Magnoli, C., Astoreca, A., Ponsone, L., Fernandez-Juri, M.G., Chiacchiera, S., Dalcerio, A., 2006. Ochratoxin A and the occurrence of ochratoxin A-producing black aspergilli in stored seeds from Cordoba, Argentina. *Journal of the Science of Food and Agriculture* 86, 2369–2373.
- Magnoli, C., Astoreca, A.L., Chiacchiera, S.M., Dalcerio, A., 2007. Occurrence of ochratoxin A and ochratoxigenic mycoflora in corn and corn based foods and feeds in some south American countries. *Mycopathologia*, 163, 249–260.
- Marasas, W.F.O., Nelson, P.E., 1987. *Mycotoxicology*. Pennsylvania State University, University Park, PA.
- Martínez-Culebras, P.V., Ramón, D., 2007. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. *International Journal of Food Microbiology* 113, 147–153.
- Moss, M.O., 1996. Mycotoxins. Centenary review. *Mycological Research* 100, 513–523.
- Oyetunji, T.O., 2006. Mycological evaluation of a ground cocoa-based beverage. *African Journal of Biotechnology* 5, 2073–2076.
- Pardo, E., Marín, S., Ramos, A.J., Sanchis, V., 2004. Occurrence of ochratoxigenic fungi and ochratoxin A in green coffee from different origins. *Food Science and Technology International* 10, 45–49.
- Pitt, J.I., Hocking, A.D., 1997. *Fungi and Food Spoilage*. Blackie Academic and Professional, London.
- Razzaghi-Abyaneh, M., Shams-Ghahfarokhi, M., Allameh, A., Kazeroon-Shiri, A., Ranjbar-Bahadori, S., Mirzahoseini, H., Rezaee, M., 2006. A survey on distribution of *Aspergillus* section *Flavi* in corn field soils in Iran: populations patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. *Mycopathologia* 161, 183–192.
- Riley, R.T., Goeger, D.E., 1992. Cyclopiazonic acid: speculations on its function in fungi. In: Bhatnagar, D., Lillehoj, E.B., Aroa, D.K. (Eds.), *Handbook of Applied Mycology. Mycotoxins in Ecological Systems*, vol. 5. Marcel Dekker, New York, NY, p. 385–342.
- Roelofs, P.A., 1958. Fermentation, drying and storage of cocoa beans. *Advances in Food Research* 8, 225–229.
- Sales, A.C., Yoshizawa, T., 2006. *Aspergillus* section *Flavi* and aflatoxins in dusts generated by agricultural processing facilities in the Philippines. *Journal of the Science of Food and Agriculture* 86, 2534–2542.
- Samson, R.A., E.S., Frisvad, J.C., 2004a. *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. *Studies in Mycology*, vol. 49. Centraalbureau Voorschimmelculturs, Utrecht, The Netherlands.
- Samson, R.A., Hoekstra, E.S., Frisvad, J.C., 2004b. *Introduction to Food- and Airborne Fungi*, 7th edn. Centraalbureau Voorschimmelculturs, Utrecht, The Netherlands.
- Serra Bonhevi, J., 2004. Occurrence of ochratoxin A in cocoa products and chocolate. *Journal of Agricultural and Food Chemistry* 52, 6347–6352.
- Urano, T., Trusckess, M.W., Matusik, J., Dorner, J.W., 1992. Liquid chromatographic determination of cyclopiazonic acid in corn and peanuts. *Journal of AOAC International* 75, 319–322.
- Tafari, A., Ferracane, R., Ritieni, A., 2004. Ochratoxin A in Italian marketed cocoa products. *Food Chemistry* 88, 487–494.
- White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), *PCR protocols. A Guide to Methods and Applications*. Academic Press, San Diego, CA, pp. 315–322.