Mycoflora and mycotoxin producing fungi from cocoa beans

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ABSTRACT

The present study reports on the natural mycoflora 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.

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 Openiran, 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 Aspergillius 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...
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% chloroform 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 Ltd, Ireland). 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, Foster City, CA, USA) and directly sequenced using the Taq DyeDeoxy terminator cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) 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-producing fungi.
by ITS sequencing, although at very low frequencies (5.2%). They included strains from Chaetomium globosum, Cladosporium oxysporum, Emericella rugulosa, Eurotium amstelodami, Eurotium chevalieri, Nectria haematococa, Mucor racemosus, Phoma glomerata, Phoma medicaginis and Rhyzopus 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 conidal 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 5.8S-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 uniseriate (Aspergillus aculeatus and Aspergillus japonicus) species.

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 aggregate and 6 as A. carbonarius. The two relevant species, A. niger aggregate and A. carbonarius, can easily be differentiated by conidial 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 YESS medium (Table 1). Seventy-seven isolates (64.1%) were 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 9 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 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 µg 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 µg g⁻¹ to 240.7 µg 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

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**Table 1**

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Total strains (%)</th>
<th>AF positive strains (AF range in ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (%)</td>
<td>B1</td>
</tr>
</tbody>
</table>

**Table 2**

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Total strains (%)</th>
<th>CPA positive strains (%)</th>
<th>CPA produced (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>120 (29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. tamarii</td>
<td>94 (22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3**

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

<table>
<thead>
<tr>
<th>Chemotype</th>
<th>Mycotoxins</th>
<th>No. of strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB</td>
<td>AFG</td>
<td>CPA</td>
</tr>
<tr>
<td>I (B1&gt;B2)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>II (B1&gt;B2)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>IV</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>VI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
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 µg g\(^{-1}\) to 90 µg g\(^{-1}\) and 0.2 µg g\(^{-1}\) to 8 µg 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.

### Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>Total strains (%)</th>
<th>Ochratoxigenic strains (%)</th>
<th>OTA produced (µg/g)</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger aggregate</td>
<td>132 (98)</td>
<td>50 (44.7)</td>
<td>0.5–90</td>
<td>11.52</td>
<td></td>
</tr>
<tr>
<td>A. carbonarius</td>
<td>6 (4.5)</td>
<td>6 (100)</td>
<td>0.2–8</td>
<td>2.15</td>
<td></td>
</tr>
</tbody>
</table>

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 (Roelofsen, 1958; Broadent 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 Nigrri. 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 (Emericella 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 Circumdata, 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 µg 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. isolated, 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 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 µg 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-toxigenic 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. tamauri 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. tamauri; however, a very high percentage (98%) of them was CPA-producing strains. Additionally, more than 50% of the strains of A. tamauri were highly toxigenic producing > 30 µg g\(^{-1}\) of CPA. Incidence of Aspergillus section Flavi belonging to A. flavus and A. tamauri 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; Belli et al., 2004; Iamanaka et al., 2005; Magnoli et al., 2007; Martínez-Culebras and Ramón, 2007). Nevertheless, results indicated a high percentage of ocratoxigenic 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% (Belli 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 µg g\(^{-1}\) to 100 µg g\(^{-1}\), whereas in other substrates, such as grapes and coffee, lower amounts of OTA have been recorded, ranging from 1 µg g\(^{-1}\) to 10 µg g\(^{-1}\) (Belli 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 µg g\(^{-1}\) to 8 µg 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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