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ORIGINAL PAPER

ITS-RFLP characterization of black *Aspergillus* isolates responsible for ochratoxin A contamination in cocoa beans

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Abstract In the present study, ochratoxigenic mycobiota in cocoa beans was identified at species level by digestion of the ITS products using the endonucleases *Hha*I, *Nla*III and *Rsa*I. Of the 132 isolates of *Aspergillus* section *Nigri* collected from cocoa beans, 89 were identified as *A. tubingensis*, 27 as *A. niger*, 10 as *A. tubingensis*-like and 6 as *A. carbonarius*. No variation was observed between RFLP patterns (C, N, T1 and T2) described previously for grape isolates and those of the cocoa isolates analysed. With respect to OTA-producing fungi, a high percentage of black aspergilli (50.7%) was 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}. Percentages of OTA-producing isolates in the *A. niger* aggregate were higher than in other substrates, ranging from 30% to 51.7%. Furthermore, the detected levels of OTA production in the *A. niger* aggregate, particularly in *A. tubingensis* species was higher than in *A. carbonarius*, ranging from 0.7 μg g^{-1} to 120 μg g^{-1} (mean 24.55 μg g^{-1}). Due to the high occurrence, percentage of ocratoxigenic isolates and their ability to produce OTA, isolates belonging to the *A. niger* aggregate could be considered as the main cause of OTA contamination in cocoa beans used for manufacturing cocoa products.

Keywords Black aspergilli · Cocoa beans · Cocoa products · Food safety · Identification · Ochratoxin A · RFLP analysis

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Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic effects and has been associated with Balkan Endemic Nephropathy [1–4]. OTA occurrence in cocoa, cocoa powder and cocoa marketed products has been reported in different countries [5–9] The European Union has set maximum permitted levels of OTA in certain products (cereals, coffee, wine, etc) and is considering its extension to other food commodities such as cocoa and its derivatives [10].

In recent years, black *Aspergillus* species (section *Nigri*) have been described as the main source of OTA contamination in coffee, grapes and other agricultural products [11–18]. Recently, the presence of abundant black *Aspergillus* filamentous fungi during cocoa processing has been reported [19]. More recently, black *Aspergillus* species, mainly members of the *A. niger* aggregate and *A. carbonarius*, have been reported as the prevalent fungal species occurring in cocoa beans [20]. It is important to identify black aspergilli commonly found on cocoa beans at species level in order to establish the relationship between species and OTA production correctly. *A. carbonarius* can easily be recognised by following morphological criteria. In contrast, species included in the *A. niger* aggregate have always been extremely difficult to distinguish from each other morphologically. Molecular methods have led to

important progress in deciphering the relationships among these black aspergilli [21–24]. In our laboratory, a method that differentiates the species of *Aspergillus* section *Nigri* from grapes into four ITS-RFLP patterns (type N, type T1, type T2 and type C), corresponding to the species of *A. niger*, *A. tubingensis*, *A. tubingensis*-like and *A. carbonarius*, respectively, was described [25].

A previous study on mycobiota from cocoa beans showed that the predominant OTA-producing fungi belonged to the *Aspergillus* section *Nigri*, but these were not identified at the species level [20]. It is important to accurately identify black aspergilli occurring on cocoa beans because the toxin profiles of black *Aspergillus* species vary and the fungi present in the beans represent and define potential toxicological risks. In this study, *Aspergillus* strains belonging to the section *Nigri,* which had been previously isolated from cocoa beans and tested for OTA production, were identified at species level from their ITS-RFLP patterns. The potential of black *Aspergillus* species for producing OTA is also discussed.

Material and methods

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 (Amazón-Trinitario-Canelo Amazón hybrid). Beans were provided by a Spanish import factory. *Aspergillus* strains are held in the Institute of Agrochemistry and Food Technology of the National Spanish Research Council (IATA-CSIC). They were previously identified by morphological criteria and tested for OTA production [20]. Reference strains were provided by Centralbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and the Spanish Type Culture Collection (CECT, Valencia, Spain). Strains of *Aspergillus aculeatus* CECT 2968, *A. carbonarius* CBS 111.26, *A. niger* CECT 2090 and *A. tubingensis* CBS 115.29 were included as reference species. The grape isolate W04-17 identified as *A. tubingensis*-like by Martínez-Culebras and Ramón [25] was also included as a reference.

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 power and the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

PCR reactions and DNA digestions

The 5.8S-ITS region was amplified by PCR using universal primers its5 and its4 [26]. PCR reactions were performed in 100 μL as the final volume, containing 100–200 ng of DNA, 50 mM KCl, 10 mM Tris–HCl, 80 μM (each) dNTP, 1 μM of each primer, 2 mM $MgCl_2$ and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, NEED, SL, 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 digested with the restriction enzymes *Hha*I, *Nla*III and *Rsa*I (MBI Fermentans, Lithuania). PCR products and their restriction fragments were separated on 1 and 3% agarose gels, respectively, with 0.5× TBE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 mg mL^{-1}), and the DNA bands were visualised under UV light. Sizes were estimated by comparison with a DNA standard length (GeneRuler ™ 100 bp DNA ladder, MBI Fermentans, Lithuania).

Sequencing analysis

PCR products were cleaned with UltraClean PCR Clean-up DNA Purification Kit (Mo Bio, 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.

The 5.8S-ITS sequences from the cocoa isolates *A. carbonarius* Co-140, *A. niger* Co-27, *A. tubingensis* Co-26 and *A. tubingensis*-like Co-131 were obtained for phylogenetic analysis. 5.8S-ITS sequences obtained from grape isolates belonging to these fungal species and reference species were included in the analysis. The 5.8S-ITS region sequences were aligned and analysed using the computer program MEGA version 4.1 [27]. The genetic distances were calculated using the Jukes-Cantor model and the phylogenetic inference was obtained by the neighbour-joining (NJ) method. *Aspergillus flavus* NRRL 4818 was designated as outgroup for the sequence analyses.

Results

Molecular characterization of the black *Aspergillus* isolates

The ITS-5.8S rDNA of the 132 isolates of black *Aspergillus* analysed in this study was amplified using its5 and its4 primers. The size of all amplified PCR products was estimated to be 650 bp. PCR products were digested by the

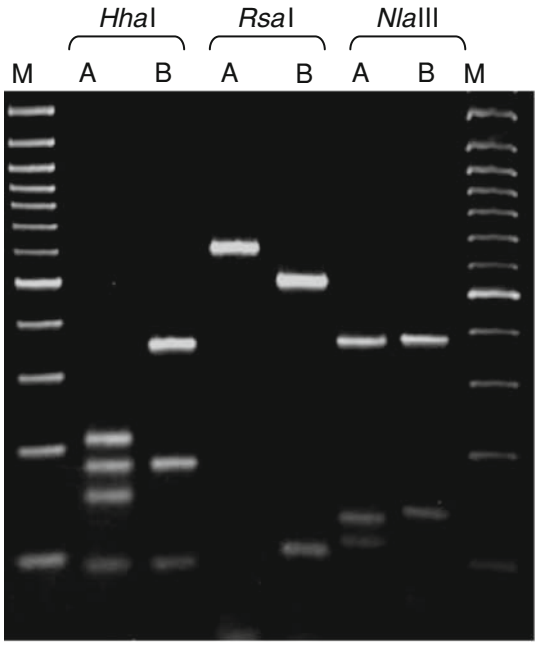

Fig. 1 Ribosomal DNA restriction patterns exhibited by black *Aspergillus* isolates from cocoa beans after digestion with the restriction endonucleases *Hha*I, *Rsa*I and *Nla*III. Lanes M correspond to the 100 bp molecular weight marker

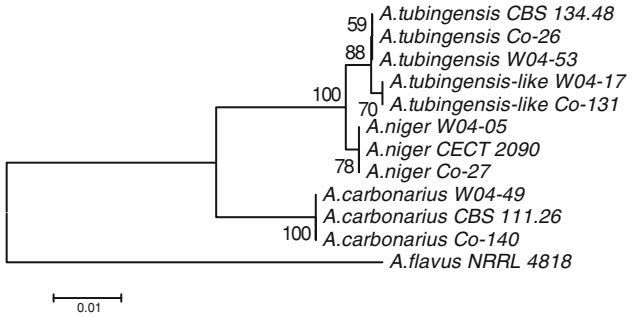

Fig. 2 Neighbour-Joining tree based on nucleotide divergences, estimated according to Jukes-Cantor model, from the 5.8S-ITS sequences. The numbers on the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1,000 runs. The phylogenetic tree shows the relationships among black aspergilli isolates from cocoa beans, grapes and reference strains belonging to *A. carbonarius*, *A. niger, A. tubingensis* and *A. tubingensis*-like

endonucleases *Hha*I, *Nla III* and *Rsa*I in order to classify the isolates using the RFLP technique previously described by Martínez-Culebras and Ramón [25] Their typical restriction patterns are shown in Fig. 1. These individual profiles, designated with the letters A–B, were combined into composite restriction patterns or RFLP types (Table 1). Each one of the 132 isolates analysed was then assigned to its RFLP type. The restriction patterns obtained for the different isolates were compared with those obtained from the reference strains of black *Aspergillus* (see "Material and methods").

For the 132 black *Aspergillus* isolates analysed, four RFLP types were observed (Table 1). The most common was type T1, represented by 89 isolates (67.4%). This RFLP type corresponded to *A. tubingensis* species. Type N included 27 isolates (20.5 %) and corresponded to *A. niger* species. Type T2 included 10 isolates (7.6 %) and corresponded to a new group of ochratoxigenic black aspergilli described recently in grapes and named as *A. tubingensis*-like [25] Finally, type C included six isolates (4.52%) corresponding to *A. carbonarius* species. Sequences of the ITS region of one representative isolate of *A. carbonarius*, *A. niger*, *A. tubingensis* and *A. tubingensis*-like respectively were examined. The ITS-5.8S sequences from these representative species were identical to representative isolates of these fungal species isolated from grapes and reference species. The phylogenetic relationship among cocoa isolates, grape isolates and reference species from *A. carbonarius*, *A. niger*, *A. tubingensis* are illustrated in a cluster analysis (Fig. 2).

Ochratoxigenic ability of black *Aspergillus* isolates

Black *Aspergillus* strains included in this study were tested for OTA production by Sanchez-Hervás et al. (2008) and OTA production data were assigned to each strain identified by ITS-RFLP in the present study (Table 1). Twelve out of the 27 *A. niger* isolates produced OTA (44.4%). The concentrations of OTA detected for these isolates varied from 0.4 μg g^{-1} to 90 μg g^{-1} (mean 16.58 μg g^{-1}). Forty-six isolates of *A. tubingensis* (51.7% of the 89 tested) and

Table 1 Ribosomal restriction patterns and composite patterns or types exhibited by the black *Aspergillus* isolates analysed in the present study

Species	Type	*Hha*I	*Nla*III	*Rsa*I	Number of isolates (%)	Number of OTA+ isolates (%)	OTA produced (μg/g)	
							Range	Mean
A. niger	N	A	A	A	27 (20.5)	12 (44.4)	0.4–90	16.58
A. tubingensis	T1	A	A	B	89 (67.4)	46 (51.7)	0.7–120	24.55
A. tubingensis-like	T2	A	B	B	10 (7.6)	3 (30)	0.2–36	7.25
A. carbonarius coccodes	C	B	A	A	6 (4.5)	6 (100)	0.2–8	2.15

Occurrence and ochratoxin-producing ability analysed by Sánchez-Hervás et al. [20] assigned to the identified black *Aspergillus* isolates

three isolates of *A. tubingensis*-like (30% of the 10 tested) were OTA producers on CYA medium. *A. tubingensis* isolates were able to produce higher levels of OTA than *A. niger* isolates, with OTA levels ranging from 0.7 μg g^{-1} to 120 μg g^{-1} (mean 24.55 μg g^{-1}). The levels of OTA production by *tubingensis*-like isolates were lower and ranged from 0.2 μg g^{-1} to 36 μg g^{-1} (mean 7.25 μg g^{-1}). Finally, six isolates of *A. carbonarius* (100% of the six tested) were able to produce OTA levels that ranged from 0.2 μg g^{-1} to 8 μg g^{-1} (mean 2.15 μg g^{-1}).

Discussion

Although OTA has been reported to occur in cocoa products in several countries [5–9], little research has been done on fungal identification and ability to produce OTA. In previous studies, *Mucor*, *Penicillium, Rhyzopus* and especially *Aspergillus* were the most frequently isolated fungi from cocoa beans [28–30] Recently, high percentages of black *Aspergillus* species have been isolated from cocoa beans [19, 20] with black aspergilli being identified according to morphological criteria; however, no molecular back-up methods were used.

The present study employed the ITS-RFLP method developed by Martínez-Culebras and Ramón [25] to identify black *Aspergillus* species from cocoa beans. By comparison with reference species, 132 black aspergilli from cocoa beans were assigned to *A. carbonarius, A. niger, A. tubingensis* and *A. tubingensis*-like. No variation was observed between RFLP patterns (C, N, T1 and T2) described for grape isolates in the aforementioned study and any of the cocoa isolates analysed in the present study. Additionally, the 5.8S-ITS sequences for representative cocoa isolates from *A. carbonarius, A. niger, A. tubingensis* and *A. tubingensis*-like were identical to sequences of these fungal species from grape and reference species. Phylogenetic tree supports the sequence data indicating a close relationship between black aspergilli from cocoa and grape. The most common species were *A. tubingensis* (67.4%) followed by *A. niger* (20.5%), *A. tubingensis*-like (7.6%) and *A. carbonarius* (4.5%). However, in the current study no black aspergilli was identified belonging to the unisseriate species *A. aculeatus* and *A. japonicus*, which are commonly found on grapes. Data on occurrence and frequency agree with the reported percentages of black aspergilli in grapes, where isolates of the *A. niger* aggregate appear to be the dominant black *Aspergillus* [23]. Furthermore, these results are also in agreement with those of Mounjouenpou et al [19] who determined that isolates belonging to the *A. niger* aggregate were predominant (90–100%) within the total of black aspergilli found on cooca beans from Cameron. Moreover, *A. carbonarius* was mostly found on unfermented beans. The low incidence of *A. carbonarius* species in the present study could be explained by the fact that all the samples analysed here were fermented and sun-dried cocoa beans. It is also interesting to note that *A. tubingensis*-like, a new group of ocratoxigenic isolates found on grapes closely related to *A. tubingensis*, was also present in this study though its occurrence (7.6%) in cocoa beans was lower than in grapes (14.2%) [25].

Regarding the ability of black *Aspergillus* isolates to produce OTA, results of the present study indicate that the highest percentage of OTA-producing isolates corresponded to *A. carbonarius* (100%) followed by *A. tubingensis* (51.7%), *A. niger* (44.4%) and *A. tubingensis*-like (30%). Here it is interesting to note the unusual result concerning the high percentages of OTA producers within the *A. niger* aggregate, considering that in other substrates the reported percentages of OTA-producing isolates in the *A. niger* aggregate are usually lower, ranging from 0.2% to 30% [15, 16, 31]. However, these results agree with those of Mounjouenpou et al. [19] where 70% of the isolates belonging to the *A. niger* aggregate were toxigenic. Additionally, most of the OTA-producing isolates belonging to the *A. niger* aggregate 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} [25, 31]. Among the species belonging to the *A. niger* aggregate identified, *A. tubingensis* isolates displayed the highest detected level of OTA production with a mean level of 24.55 μg g^{-1}. On the other hand, although all the *A. carbonarius* isolates were able to produce this mycotoxin, only six (4.5%) strains out of the total isolated black aspergilli were identified as *A. carbonarius*. Furthermore, the detected levels of OTA production were lower than in the *A. niger* aggregate, ranging from 0.2 μg g^{-1} to 8 μg g^{-1}.

Conclusions

This is the first study to provide significant information on the key black *Aspergillus* species responsible for OTA contamination of cocoa beans used for cocoa-product manufacture. Although different OTA-producing species might participate in OTA contamination of cocoa beans, and consequently of cocoa products, our results provide strong evidence of the important role played by isolates belonging the *A. niger* aggregate, particularly *A. tubingensis*. This is supported not only by its high frequency of isolation in cocoa beans, but also by its ample ability to produce OTA.

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