Aflatoxigenic potential of *Aspergillus* spp. isolated from groundnut seeds, in Burkina Faso, West Africa

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Seven strains of *Aspergillus* spp. have been screened for their ability to produce aflatoxins in coconut broth media. Three were local isolates and four were reference strains obtained from the United States Department of Agriculture (USDA) and Centre for Disease Control and Prevention (CDC) Atlanta and used as positive and negative controls. All strains were previously cultivated in *Aspergillus flavus* and *Aspergillus parasiticus* agar to ascertain if they belong to *A. flavus* or *A. parasiticus* species. The qualitative ability of aflatoxin production was also previously performed by fluorescence emission under ultra violet light at 312 nm after 3 days of incubation at 30°C on coconut agar. Quantitative assessment of aflatoxin production was done by high performance liquid chromatography (HPLC) with post-column derivatisation after immunoaffinity column cleanup. The reference strains (NRRL5862, CDCB5333 and CDCB4571) and one of the local isolates (BfaS1) were aflatoxin producing species in coconut broth as assessed by HPLC. Per isolate aflatoxin B₁ concentration after determination by HPLC was higher than the other types of aflatoxins identified. The strains NRRL5862 and CDCB5333 have, respectively produced the following quantities of aflatoxin B₁: 73.67 and 165.73 ng/ml whereas aflatoxin B2 concentrations were, respectively 0.595 and 1.736 ng/ml.

**Key words:** Aflatoxin B₁, *Aspergillus flavus*, *Aspergillus parasiticus*, high performance liquid chromatography (HPLC).

**INTRODUCTION**

Aflatoxins are secondary metabolites known to be highly toxic and the most carcinogenic of natural toxins (IARC, 2002). They are produced by some strains of section *flavi* (*Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius, Aspergillus bombycis* and *Aspergillus pseudotamarii*) and isolates outside this section such as *Aspergillus ochraceoroseus* in section *circumdati*, and *Emericella astellata* and *Emericella Venezuelesen*s (Criseo et al., 2001; Cary and Ehrlich, 2006; Johnsson et al., 2008; Reddy et al., 2009). This group of fungi has been subjected to detailed investigations. *A. flavus* and *A. parasiticus* are the main aflatoxin producing species. They are frequently found in foodstuffs and animal feeds and are associated with a wide spectrum of stored agricultural commodities. However, not all *Aspergillus* species are able to produce aflatoxins. Different methods are implemented to screen the ability of aflatoxins production of *Aspergillus* species. These methods commonly use the culture of strains in suitable liquid or solid media. Aflatoxins produced are then analysed by chromatographic and ELISA techniques (Lin et al., 1998; Yang et al., 2004). For this purpose many media are used: Yeast extract-sucrose (YES) (Fente et al., 2001), Reddy medium, and natural media with wheat, rice, peanut, malt, date, palm kernel or coconut extracts (Hara et al., 1974; Ahmed and Robinson, 1999; Klich, 2002; Atanda et al., 2006).

To meet the need for more environmentally sound...
methods which may be applicable and available to screen large numbers of strains in a reasonable time, alternative methods were developed. These are based on the use of complex media to detect the natural fluorescence of aflatoxins released by the growing mycelium (Hara et al., 1974; Fente et al., 2001; Maragos et al., 2008) or rely on multiplex Polymerase chain reaction (PCR) and real time Polymerase chain reaction (RT-PCR) detection of genes or their transcripts involved in the aflatoxin biosynthetic pathway (Färber et al., 1997; Criseo et al., 2001; Somashekar et al., 2004; Scherm et al., 2005). New instrumental techniques approaches for aflatoxin determination such as fluorescence polarisation, multiphoton-excited fluorescence, LC separation followed by electrospray ionisation-MS-MS liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC/ESI–MS–MS) detection (Trucksess, 2003) were also developed but all these alternative methods are not always available or affordable to developing countries. In the present study coconut broth has been used as medium to produce aflatoxins which have been analysed by HPLC. A. flavus and A. parasiticus medium have been used to identify isolates belonging to A. flavus or A. parasiticus species. Colonies natural fluorescence under ultraviolet (UV) light on coconut agar medium has also been tested previously. The objective of this is to confirm that Aspergillus species isolated in this study in Burkina Faso are able to produce aflatoxins.

**MATERIALS AND METHODS**

**Chemicals**

Aflatoxins B₁, B₂, G₁, and G₂ standards were purchased from Sigma Aldrich Chemical Company. Standard solutions were prepared in methanol and stored at –20°C for up to three months. Immunoaffinity columns were purchased from Romer Labs Diagnostic GmbH (Germany) and stored at 4°C until expiry date.

**Microorganisms**

Reference strains of Aspergillus spp. were graciously offered by the USDA-Research, Education and Economics Agricultural Research Service and the CDC Atlanta (USA). They have the following characteristics: Strains NRRL 5862 (A. parasiticus, highly aflatoxinogenic) and NRRL 484 (A. flavus, non- aflatoxinogenic) was from USDA whereas strains B4571 (A. parasiticus) and B5333 (A. flavus) were from CDC and their ability to produce aflatoxins had not been studied yet by CDC by the time they were offered.

**Local Aspergillus spp. isolates**

Local Aspergillus species were isolated from groundnut seeds. The seeds were wet in glassware and left at the ambient temperature (27 to 34°C) until proliferation of mould, from the consortium of moulds grown, Aspergillus strains were isolated and purified on bean broth agar by multiple exhausted seeding. Czapek Yeast extract Agar (CYA) slant was used for further purification and identification using the systematic classification of the Aspergillus strains based on morphological characters described by Christensen (1981), Hocking (1982), Cotty (1993) and Klich and Samson (2009). Isolates were thereafter grown on A. flavus and A. parasiticus to ascertain if they belong to A. flavus or A. parasiticus species. Finally three local isolates of Aspergillus spp. were coded BfaS0, BfaS1 and BfaS5 and assessed for aflatoxin production.

**Aflatoxin production**

Natural Coconut Broth medium (CB) and CYA were used in this study (Cotty, 1994; Nguyen, 2007).

**Preparation of spore suspension**

Strains were grown on CYA slant for 10 days at 30 ± 1°C. Spores were harvested in sterilized water containing 0.01% (v/v) tween 80 and centrifuged at 4500 g for 20 min. They were re-diluted in sterilized water, then centrifuged at 2000 g for 20 min. The operation was repeated three times. The number of conidia was estimated by count under microscope using a Nageotte cell.

**Culture conditions for aflatoxin production**

CB was used as a basal medium for aflatoxin production in stationary cultures, in 250 ml Erlenmeyer flasks. Appropriate quantity (about 10⁵) of spores was added to 100 ml of sterilised coconut broth pH 7.06 so that the final concentration was 10⁵ spores/ml. The cultures were incubated at 30 ± 1°C for 13 days. At the end of the incubation, methanolic extracts of these cultures were analysed by HPLC as described below to determine the content of aflatoxins produced.

**Assessment of aflatoxins produced by HPLC**

**Extraction, cleanup and determination of aflatoxins by HPLC**

The presence of aflatoxins in culture media was confirmed by HPLC method with immunoaffinity column cleanup and post-column derivatisation according to European standards ISO 16050 CEN/TC-34, (2006). The cultures were filtered through Whatman No. 4 filter paper. The paper containing the mycelium was washed twice with 5 ml of pure methanol HPLC grade. Lipids were extracted from the filtrate with hexane (filtrate-hexane/100:50; v/v). Fifteen (15) ml of the final filtrate were diluted with 15 ml of phosphate buffered saline (PBS) (for 1 liter of PBS pH 7.4: (KCl, 200 mg; KH₂PO₄, 200 mg; NaH₂PO₄·H₂O, 116 mg; NaCl, 8.0 g, make up to volume with distilled water) and applied to the immunoaffinity column “Afacest” manufactured by Romer Labs Diagnostic GmbH. The immunoaffinity column was washed twice with 10 ml of distilled water and aflatoxins were eluted with pure methanol. Aflatoxins were separated and quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post-column derivatisation (PCD) involving bromination followed by fluorescence detection. The post-column derivatisation was achieved using a Cobra Cell to obtain electrochemically generated bromine (ISO 16050 CEN/TC-34, 2006); Romer Labs, Fact Sheet, 2004).

The chromatographic system consisted of an automatic Agilent HEWLETT PACKARD brand n°1100, is managed by computer with the chemstation software. It is equipped with an auto-sampler (100 µl, injector loop), a Zorbax column with a Reverse-Phase C18 (4.6 x 250 mm, 5 µm) and a fluorescence detector. The detector was set at λex = 360 nm, λem = 440 nm. The mobile phase was isocratic and composed of water – acetonitrile - methanol (6/2/3, v/v/v) with 120 mg of potassium bromide and 350 µl of 4 M nitric acid per litre of mobile phase. The flow rate was set at 1 ml/min. Each experiment
was conducted in duplicate and aflatoxins contents were determined according to their corresponding standard curves. Calibration curves for each aflatoxin were determined, using a series of standard solutions prepared in methanol. Linear calibration graphs were obtained by plotting the peak area against the aflatoxin amount injected. Quantification of aflatoxins was performed by comparing the peaks areas with the calibration curves. With regard to the limits of determination, limits of detection (LOD) and limits of quantification (LOQ) were calculated for each type of aflatoxin as follows:

$$\text{LOD} = \frac{b_0 + 3 \times S(b_0)}{b_1}$$  
$$\text{LOQ} = \frac{b_0 + 10 \times S(b_0)}{b_1}$$

Where $b_0$ is the intercept of the calibration curve, $S(b_0)$ is the standard deviation of the blank and $b_1$ is sensitivity (calibration curve slope).

Statistical analysis

All experiments were conducted in duplicates and the data were presented as Mean ± SD. Statistical significance was ascertained by using the single linear method. Statistical relation between variables was analyzed by correlation analysis which is highly significant.

RESULTS AND DISCUSSION

Aflatoxins production and their determination by fluorescence HPLC

Aflatoxin production abilities tested previously by fluorescence (Figures 1 and 2) under UV light of strains by cultivating them on coconut agar were in concordance with those obtained by HPLC determination. We have found that all strains showing fluorescence under UV light produced aflatoxins in coconut broth medium. No aflatoxin was detected in the methanolic extract of the isolates BfaS0, BfaS5 and the strain NRRL 484 (Table 1). This table compared the response of strains on *A. flavus* and *A. parasiticus*, CA and their aflatoxin producing ability assessed by HPLC. These findings are similar to those observed by Jaimez et al. (2003) where all the blue fluorescence observed around an isolate was associated with the presence of aflatoxins, detected by the HPLC.

Extraction of CB-culture filtrates with methanol followed by subsequent analysis by HPLC allowed the detection and the quantification of the four types of aflatoxins (AFB1, AFB2, AFG1 and AFG2) according to the species (*A. flavus* and *A. parasiticus*) (Table 2).

The culture with local isolates BfaS1 showed detectable contents of AFB1 and AFB2. This isolate have produced the same types of aflatoxins as the reference strain *A. flavus* CDCB5333. However, there is a big difference in the aflatoxins quantities produced. The concentrations of AFB1 and AFB2 produced by isolate BfaS1 after 13 days of incubation in CB medium were 0.59 and 0.011 ng/ml of culture filtrate, respectively, whereas the strain *A. flavus* CDCB5333 has produced 165.73 and 1.74 ng/ml of AFB1 and AFB2, respectively. A comparison has showed that *A. flavus* CDCB5333 has produced 281.8 and 156.4 fold higher quantity of AFB1,
Figure 2. Fluorescence under UV light at 312 nm in coconut agar medium on the fourth (4th) day of incubation at 30°C. A, colony of the aflatoxigenic strain NRRL 5862 showing a blue fluorescence around; B, colony of the aflatoxigenic strain BfaS1 showing a slight blue fluorescence around; C, colony of the non-aflatoxigenic strain BfaS5 without fluorescence.

Table 1. Comparison of aflatoxigenic and non-aflatoxigenic strains responses for three methods of identification (response on AFPA, fluorescence under UV light on coconut agar medium and HPLC response after culture in coconut broth).

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Response on AFPA</th>
<th>Fluorescence under UV light on CA</th>
<th>Aflatoxins production in CB with HPLC response</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. parasiticus NRRL5862</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>A. flavus NRRL48</td>
<td>+</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>A. flavus CDCB5333</td>
<td>+</td>
<td>++</td>
<td>nd</td>
</tr>
<tr>
<td>A. parasiticus CDCB4571</td>
<td>-</td>
<td>± (slight)</td>
<td>+</td>
</tr>
<tr>
<td>BfaS0</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>BfaS1</td>
<td>+</td>
<td>± (slight)</td>
<td>+</td>
</tr>
<tr>
<td>BfaS5</td>
<td>+</td>
<td>-</td>
<td>nd</td>
</tr>
</tbody>
</table>

(+) negative; (±) slightly positive; (+) positive; (++) definitely positive; (nd) not detected.

respectively AFB2 than the local isolate BfaS1. The Aspergillus parasiticus strain NRRL5862 has produced 73.67 ng/ml of AFB1; 0.60 ng/ml of AFB2; 11.64 ng/ml of AFG1 and 0.05 ng/ml of AFG2. As it can be seen from the chromatograms at Figure 3, all peaks are well separated and it is easy to notice the difference of AFB1 quantity produced by the local isolate BfaS1 and also the strain of A. parasiticus CDCB4571. Hence, isolate BfaS1 has produced about 24.5 fold higher quantity of AFB1 than A. parasiticus CDCB4571. The fit parameters of the linear regression performed for each aflatoxin are presented in Table 3 and all the coefficients of determination R² of the linear regression curves of the calibration of the aflatoxins standards aliquots were higher than 0.9994 which is a very good value. It is generally requested to obtain R² ≥ 0.99. The Residual Standard Deviation (RSD) was 0.303% < 1% and the recovery rate was 78.8%. However, we have classified the strain BfaS5 between some A. flavus species as non-aflatoxins-producing, in agreement with strains NRRL 484 of the USDA-Research. Furthermore, it is important to mention that in some particular cases such as:

(i) Sometime, in mutant strains, aflatoxins are not produced due to the oxidative stress, though aflatoxins biosynthesis genes are present and all the precursors are accumulated (Chang et al., 1999; Yu et al., 2004; Bhatnagar et al., 2006; Narasaiah et al., 2006).

(ii) Ancestral trait of some strains where aflatoxins biosynthesis pathway genes are missing.

Cary and Ehrlich (2006) had demonstrated that for conversion of the last aflatoxin precursor; (sterigmatocystin) to aflatoxin, the closest Aspergillus nidulans required genes aflU (CypA), aflP (OmtA) and aflQ (OrdA). The strain BfaS5 could be in these above
Table 2. Different types and quantity (ng/ml) of aflatoxins produced by Aspergillus strains in coconut broth after 13 days of incubation at 30°C.

<table>
<thead>
<tr>
<th>Type and quantity of aflatoxins produced (ng/ml)</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
</tr>
<tr>
<td>AFB₁</td>
<td>nd</td>
</tr>
<tr>
<td>AFB₂</td>
<td>nd</td>
</tr>
<tr>
<td>AFG₁</td>
<td>nd</td>
</tr>
<tr>
<td>AFG₂</td>
<td>nd</td>
</tr>
</tbody>
</table>

**Ratio CDCB5333 AFB₁**
Other strains AFB₁*  
2.3  
1  
6901.3  
281.8

**Ratio CDCB5333 AFB₂**
Other strains AFB₂*  
2.9  
1  
156.4

nd = not detected. * AFB₁ and AFB₂ productivity ratio of the more producing strain CDCB5333 and the other strains; local isolate and reference. AFB₁ and AFB₂ productivity ratio of the more producing strain CDCB5333 and the other strains; local isolate and reference.

In our results in Table 2, the amount of aflatoxin B₁ is higher than the others. Similar data were reported by Bokhari (2002). The ratio of the quantity of AFB₁ and AFB₂ produced by the reference isolate CDCB5333 on the other strains of AFB₁ and AFB₂ production (shown in the last two rows of Table 2) display that strain CDCB5333 produced AFB₁ two to three folds more than the strain NRRL5862 and 281.8 fold more than the isolate BfaS1. Our results also show that the species of A. flavus produced only aflatoxins B₁ and B₂ (reference isolate CDCB5333), on the other hand, A. parasiticus produced the four types of aflatoxins (reference isolate A. parasiticus NRRL5862 and CDCB4571). Cary and Ehrlich in (2006) explained this metabolic behaviour of strains by the trough of the molecular genetics, phylogenetic relationships and evolutionary implications. They have shown that within section flavi isolates, the ability to produce both B and G aflatoxins is the ancestral trait and that A. flavus diverged from A. parasiticus by loss of a portion of the gene, aflU (cypA), involved in G type aflatoxin production. A. flavus and A. parasiticus group forms a polyphyletic assemblage containing isolates of different morphotypes and having the ability to produce aflatoxins. Thus in the present findings, the isolate CDCB4571 did not produce detectable quantity of aflatoxins B₂ and G₂. This is certainly due to the slight capability of its aflatoxins productivity (0.024 ng/ml of aflatoxin B₁ and 0.018 ng/ml of AFG₁).

**CONCLUSION AND PERSPECTIVES**

This study has demonstrated the ability to produce aflatoxins by the local Aspergillus spp. BfaS1 in the natural coconut broth and agar media. Whereas the local isolates BfaS0 and BfaS5 have shown no aflatoxicogenic potential. The study has also shown that the aflatoxin producing isolate (BfaS1) produced relatively feeble quantities of aflatoxins B₁ and B₂ comparing to the level of aflatoxins produced by the reference strain CDC5333. Having isolated two local A. flavus species; one non aflatoxicogenic BfaS5 and the second producing aflatoxins BfaS1 is a way to focus aflatoxins control in order to reduce the exposure of the population to the aflatoxins toxic effect. In perspective, it is very interesting to use the two isolates BfaS1 and BfaS5 in bio-control tests by cultivating them in competitive growth to check if the isolate BfaS5 could reduce the growth of isolate BfaS1 as well as the aflatoxins production of BfaS1 by bio-competitive exclusion. Another way to develop these findings is to enhance the research on the strain present in groundnut in Burkina Faso by studying the kinetic production of aflatoxins by our Aspergillus isolate. Then, inhibition of Aspergillus species growth and aflatoxins production by plants extracts will be tested for the reduction of illness due to aflatoxins consumption in feed and foodstuffs.

**ACKNOWLEDGEMENTS**

The authors thank Dr. S.W. Peterson from the Research, Education and Economics...
Figure 3. HPLC chromatograms of aflatoxins standards (AFB₁, AFB₂, AFG₁, and AFG₂): A, AFB₁ and AFB₂ produced by local isolate BfaS1; B, AFB₁ and AFG₁ produced by the strain CDC4571; C.
Agricultural Research Service of USDA and Mary E. Brandt and Dr. Beth Skaggs from CDC Atlanta for providing graciously cultures.

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