Cultural and analytical assays for aflatoxin B production by *Aspergillus flavus* isolates

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ABSTRACT

*Aspergillus flavus* (164 isolates) from wheat flour were subjected to cultural *Aspergillus* differential medium pigmentation and fluorescence (ADM), coconut agar medium pigmentation and fluorescence (CAM), yeast extract sucrose agar supplemented with 0.3% methyl β-cyclodextrin fluorescence (MβCD), Ammonia vapor test on CAM and YES, analytical thin layer chromatography (TLC, AFB₁ & B₂) methods. Positive result percentage for the detection methods 100%, 0%, 9.75%, 12.2%, 0.60%, 9.2%, 27.5%, 9.7% and 4.3%, respectively. Fifteen isolates were analyzed by HPLC for detection of AFB₁ and B₂ production. The HPLC analysis of AFB₁ in isolates extracts were ranged between 1.6 and 4974.3 ppm, AFB₂ were in range 0.1 and 311.8 ppm and total AFBs 1.7-5286.1 ppm.

Key words: *Aspergillus flavus*, cultural methods, TLC, HPLC analysis.

INTRODUCTION

Aflatoxins (AFs) are mycotoxins formed as metabolites by certain *Aspergillus* species (*A. flavus, A. parasiticus, A. nomius* and *A. niger*) in/on foods and feeds [1]. Aflatoxins are group of potent mycotoxins with mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive properties, are of particular importance because of their major occurrence and adverse effects on animal and human health [2]. The Food and Agriculture Organization (FAO) estimated that many basic foods could be contaminated by the mycotoxin producing fungi, which contributes to enormous global losses of food, approximately 1000 million metric tons each year [3]. Among 18 different types of aflatoxins identified, the major members are aflatoxin B₁, B₂, G₁ and G₂ which chemically are coumarin derivatives with a fused dihydrofurofuran moiety [4]. *Aspergillus flavus* produces AFB₁ and AFB₂, whereas, *Aspergillus parasiticus* produces AFB₁, AFB₂, AFG₁ and AFG₂ [5]. The European Union has enacted a very stringent aflatoxin tolerance threshold of 2 µg/kg aflatoxin B₁ and 4 µg/kg total aflatoxins for nut and cereals for human consumption [6].

Numerous publications describe analytical and cultural methods for the detection and quantification of aflatoxins in agricultural commodities and in cultures of fungi isolated from them. These methods vary in accuracy and precision, depending on the end goal of the analysis [7].

Cultural methods include: 1) Lin and Dianese [8] described a coconut agar medium (CAM) for rapid detection of aflatoxins. Blue fluorescence under long-wave UV light was observed on the reverse side of aflatoxin-producing colonies. 2) Bothast and Fennell [9] described *Aspergillus* Differential Medium (ADM) on which *A. flavus* and *A. parasiticus* produced an orange yellow colony reverse. 3) blue fluorescence, particularly in the presence of an enhancer in the medium such as β-methylcyclohexalin [10]; 4) yellow pigmentation (YP), particularly on the underside of colonies [11]; and 5) color change of the yellow pigment to plum-red on exposure of the culture to ammonium hydroxide vapor (AV) [12].
Analytical methods for aflatoxins detection and quantification include 1) thin layer chromatography (TLC) [13]; 2) high-performance liquid chromatography (HPLC) [14]; 3) liquid chromatography/ mass spectroscopy (LC/MS) [15]; 4) enzyme-linked immunosorbent assay (ELISA) [16]; 5) dip-stick kits [17]; and 7) biosensors [18]. The choice of method depends on economic factors, purpose of analysis, and analysts' team, among others [19].

MATERIALS AND METHODS

Isolates of Aspergillus flavus (total 164 isolates) isolated from 75 wheat flour samples by using "dilution-plate method" as described by Johnson and Curl et al. [20] were screened for their potential of aflatoxin B production.

2.1 Cultural detection:
2.1.1 Aspergillus differential medium (ADM):
This was made as described by Beuchat [21]. Colonies showing orange-yellow reverse or fluoresce under UV light (365 nm) were scored as positive.

2.1.2 Coconut based medium test:
Coconut agar medium (CAM) was used according to Zrari [22]. The presence or absence of a fluorescence ring in the agar surrounding the colonies under UV light (365 nm) was noted and the results were scored as positive or negative.

2.1.3 Ammonia vapor test:
Aspergillus isolates were grown on Coconut agar medium (CAM) and (YES) yeast extract sucrose agar according to Saito and Machida [12] and Kumar et al. [23]. After 7 days, a set of plates were inverted over 2 ml of ammonium hydroxide. A change in color of the culture medium was used to determine the toxicity or otherwise of isolates. After ten minutes, the undersides of aflatoxins producing isolates turned into pink to red color. But no color change occurred in the non-toxic isolates.

2.1.4 Detection by fluorescence on YES agar medium:
Five-day-old cultures of A. flavus isolates were placed on yeast extract sucrose agar (YES) containing 0.3% methyl β-cyclodextrin as recorded by Cepeda et al. [24] and Fente et al. [25]. The cultures were examined under UV light (365 nm) for detection of the fluorescence signal. A fluorescent area around each colony of the aflatoxinogenic isolates was observed.

2.2 Analytical detection:
2.2.1 Analysis of Aflatoxin B Production Using TLC and HPLC.
Aspergillus isolates were grown on yeast extract sucrose agar (YES), the culture plates were incubated at 28±2°C for 7 days. Agar plugs were prepared by cutting the fungal colony to a diameter of 5 mm. The plugs were weigh and immersed in 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 28±2°C for 7 days. Agar plugs were prepared by cutting the fungal colony to a diameter of 5 mm. The plugs were weighed and immersed in 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and 2 ml of chloroform for extraction of toxin from fungi. Extracts were purified and were analyzed by HPLC (table, 1). Aflatoxins B1 and B2 were detected in all 15 A. flavus isolates analyzed by HPLC. (table, 1). Aflatoxins B1 and B2 were detected in all 15 A. flavus isolates analyzed by HPLC. Table (2) represents the results of the 15 A. flavus isolates analyzed by HPLC for the detection of AFB1 and B2 production accompanied with their qualitative detection results. The agreement percentage of qualitative detection positives with HPLC results were in range 0.1 and 311.8 ppm and total AFBs 1.7 - 5286.1 ppm. Aspergillus flavus (41-SHG) and A. flavus (66-SHG) isolates were the lowest in AFB1, AFB2 and

RESULTS

Generally, total of 164 A. flavus isolates were recovered from 75 wheat flour samples and showed positive result percentage for the qualitative detection methods (cultural Aspergillus differential medium pigmentation and fluorescence (ADM), coconut agar medium pigmentation and fluorescence (CAM), yeast extract sucrose agar supplemented with 0.3% methyl β-cyclodextrin fluorescence (MβCD), ammonia vapor test on CAM and YES, analytical thin layer chromatography (TLC, AFB1 & B2) 100%, 0%, 9.75%, 12.2%, 0.60%, 9.2%, 27.5%, 9.7% and 4.3%, respectively, and 15 isolates chloroform extracts were analyzed by HPLC (table, 1). Aflatoxins B1 and B2 were detected in all 15 A. flavus isolates analyzed by HPLC. Table (2) represents the results of the 15 A. flavus isolates analyzed by HPLC for the detection of AFB1 and B2 production accompanied with their qualitative detection results. The agreement percentage of qualitative detection positives with HPLC results were 100% for ADM pigmentation, 0% for ADM fluorescence, 100% for CAM pigmentation, 93.33% for CAM fluorescence, 6.66% for YES-MβCD fluorescence, 86.66% for CAM ammonia vapor, 53.33% for YES ammonia vapor, 86.66% for TLC-AFB1 and 46.66% for TLC-AFB2. The HPLC analysis of AFB1 in isolates extracts were ranged between 1.6 and 4974.3 ppm, AFB2 were in range 0.1 and 311.8 ppm and total AFBs 1.7 - 5286.1 ppm. Aspergillus flavus (41-SHG) and A. flavus (66-SHG) isolates were the lowest in AFB1, AFB2 and
total AFBs production; they produced 3.5 ppm AFB$_1$, 0.2 ppm AFB$_2$, 3.7 ppm AFBs and 1.6 ppm AFB$_3$, 0.1 ppm AFB$_4$, 1.7 ppm AFBs, respectively. *Aspergillus flavus* (148-ASW) isolate gained the highest production of 4974.3 ppm AFB$_1$, 311.8 ppm AFB$_2$ and 5286.1 ppm AFBs. *Aspergillus flavus* (16-SHG) and *A. flavus* (106-AST) isolates were also high producers for AFB$_1$, AFB$_2$ and AFBs, their production were 2373.6, 228.7, 2566.3 and 1035.3, 36.7, 1072 ppm, respectively.

Table (1): Detection of AFB$_1$\&A$_2$ of *Aspergillus flavus* isolates from all wheat flour samples using cultural (ADM, CAM, YES-MjCD & Amm. vapor) and analytical (TLC & HPLC) methods.

<table>
<thead>
<tr>
<th>No. of Isolate</th>
<th>Test positive &amp; negative</th>
<th>ADM</th>
<th>CAM</th>
<th>YES-MjCD Fluorescence</th>
<th>Ammonia Vapor</th>
<th>TLC</th>
<th>HPLC</th>
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<td></td>
<td>Pigment</td>
<td>Fluorescence</td>
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<td>164</td>
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<td>0</td>
<td>15</td>
<td>15</td>
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<td>15</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>164</td>
<td>0</td>
<td>9.75</td>
<td>12.2</td>
<td>0.60</td>
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<td>$q_{15}$</td>
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<td>90.25</td>
<td>87.8</td>
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<td>90.8</td>
<td>72.5</td>
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<tr>
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</table>

Table (2): Detection of AFB$_1$\&B$_2$ of *Aspergillus flavus* isolates from wheat flour samples using cultural (ADM, CAM, YES-MjCD & Amm. vapor) and analytical (TLC & HPLC) methods.

<table>
<thead>
<tr>
<th>Isolates codes</th>
<th>ADM</th>
<th>CAM</th>
<th>YES-MjCD Fluorescence</th>
<th>Ammonia vapor</th>
<th>TLC</th>
<th>HPLC (ppb)</th>
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*+ve* = Positive & *-ve* = Negative

DISCUSSION

*Aspergillus flavus* isolates (164) and their chloroform extracts were subjected to cultural *Aspergillus* differential medium pigmentation and fluorescence (ADM), coconut agar medium pigmentation and fluorescence (CAM), yeast extract sucrose agar supplemented with 0.3% methyl β-cyclodextrin fluorescence (MJCD), Ammonia vapor test on CAM and YES, analytical thin layer chromatography (TLC, AFB$_1$ & B$_2$) methods. Positive results percentages for the detection methods were 100%, 0%, 9.75%, 12.2%, 0.60%, 9.2%, 27.5%, 9.7% and 4.3%, respectively.

Not all strains of *Aspergillus* are able to produce mycotoxins [32], therefore, there is a need for screening for their toxin production abilities [33]. The development of a yellowish orange color in the reverse of the colonies grown on *Aspergillus* differentiation agar was a result of the reaction of ferric ions from ferric citrate with aspergillic acid molecules. This result was taken as a positive reaction for the production of aflatoxin [34].

Davis et al. [35] described a method using coconut agar medium (CAM) for detection of aflatoxin. Yazdani et al. [33] also reported that production of aflatoxins was detected on coconut milk agar (CMA) media by the presence of a fluorescence ring around the colonies. Abbas et al. [36] while comparing the cultural and analytical methods for determination of aflatoxin production by *Aspergillus* isolates reported that cultures capable of producing greater than 61 ng/g of aflatoxin in fungal structures were fluorescent on PDA. However, visual detection of aflatoxins using coconut agar medium can be conducted multiple ways, using either ammonium vapour or fluorescence detection [37]. Coconut agar medium is very popular in developing countries due to its cheap and ready available ingredients. However, its reliability is considered controversial [33, 38].

Aflatoxicogenic strains produced a pink color against ammonium vapour and green-blue fluorescence under UV light on the reverse side of colonies [33]. The colony reverse of aflatoxin producing strains of *A. flavus* and *A. parasiticus*.
parasiticus turned pink to red when their cultures were in contact with ammonia vapour. This colour change occurred immediately after Aspergillus colonies were exposed to ammonia vapour [12]. No colour change was observed for non aflatoxin-producing strain of A. oryzae. Versicolorin, a precursor of AFB1, produced by A. flavus and A. parasiticus, is the compound that produces purple-red colour in alkaline conditions [12, 39, 40]. The explanation for colour change is that anthraquinone compounds behave as pH indicator dyes which will change colour [41]. Shier et al. [42] showed that there are some other compounds that can be responsible for this colour change. It has been identified that these compounds are anthraquinone derivatives associated with aflatoxin biosynthetic pathway in Aspergillus species, as the biosynthetic intermediates (norsolorinic acid, averantin, averufin, versicolorin A) for AFB1. Of these compounds, averufin was produced by a non-aflatoxigenic mutant of A. parasiticus [43].

Yazdani et al. [33] concluded that the ammonium hydroxide test was an unreliable method for detection of aflatoxins as false negatives and positives were produced when compared with TLC and HPLC analysis results of tested Eurotium spp. and A. flavus isolates. It was reported by Saito and Machida [12] that the ammonium hydroxide vapour test are 11% false positive and 6% false negative results for aflatoxicogenicity. Kumar et al. [23] reported a 92% efficacy for the ammonium vapour test having 8% false negatives. Abbas et al. [36] reported on using a yellow pigmentation combined with the ammonium hydroxide vapour test and reduced false negatives to 7%. Yazdani et al. [33] findings showed that the methyl-β-cyclodextrin test did not have enough sensitivity for detection of aflatoxins.

Our results indicated that the agreement percentage of qualitative detection positives with HPLC results were 100% foradm pigmentation, 0% for ADM fluorescence, 100% for CAM pigmentation, 93.33% for CAM fluorescence, 66.66% for YES-MβCD fluorescence, 86.66% for CAM ammonia vapor, 53.33% for YES ammonia vapor, 86.66% for TLC-AFB1; and 46.66% for TLC-AFB2.

Fani et al. [37] obtained 524 A. flavus isolates from 681 pistachio nuts and orchard soils and analyzed them by cultural and analytical methods for aflatoxins production. The accuracy of cultural methods declined in the order YES-AV, PDA-AV, CAM-FD and YES-MβC-FD, respectively. The frequency of false negatives ranged from 2.6% (AV using YES) to 15% (FD using YES-MβC). Assays HPLC and TLC found 63 atoxigenic isolates, all of which were correctly identified by ammonium vapour assays using CAM. Coconut agar medium was very precise and accurate using ammonium vapour (0% false negatives) but not with fluorescence detection (13% false negatives) [37]. Of 43 isolates of A. flavus, 9 (20.93%) produced some AFs on YES medium when analyzed by HPLC; only 2 (4.6%) isolates were determined to be aflatoxicogenic by presence of blue fluorescence under UV light on APA and CAM. From these AF producer strains, 8 (18.7%) isolates produced only AFB1; and one strain produced both AFB1 and AFB2 [44]. Aflatoxin production capability of all Aspergillus spp. was analysed with two chromatographic techniques (HPLC and TLC) and the results were in agreement with each other [33]. Aflatoxins also generated by A. ostianus as reported by Saber et al. [45], the color response to ammonia vapor was in 100% agreement with CAM and TLC detection of the toxicity of A. ostianus isolate.

Atanda et al. [46] reported that aflatoxicigenic isolates reverse exhibit a characteristic blue or blue green fluorescence under long wave UV that was confirmed by TLC. However, some non-aflatoxicigenic isolates also fluorescence under UV light [47]. Strains of A. flavus and A. oryzae produce several compounds other than aflatoxins such as asperoperterin A or B [48], flavacol and deoxy-hydroxy-aspergillic acid [49] give a blue fluorescence under UV light. In addition to aflatoxins B1 and B2, A. flavus also produces many other mycotoxins such as cyclopiazonic acid, kojic acid, beta-nitropipionic acid, aflatropin, aflatrem and aspergillic acid [50].

Fifteen isolates were analyzed by HPLC for detection of AFB1 and B2 production. The obtained data by HPLC analysis of AFB1 in isolates extracts were ranged between 1.6 and 4974.3 ppm, AFB2 were in range 0.1 and 311.8 ppm and total AFBs 1.7-5286.1 ppm. Aswan-September A. flavus (148-ASW) isolate gained the highest production of 4974.3 ppm AFB1, 311.8 ppm AFB2 and 5286.1 ppm AFBs. Sohag-February A. flavus (41-SHG) and Sohag-March A. flavus (66-SHG) isolates were the lowest in AFB1, AFB2 and total AFBs production; they produced 3.5 ppm AFB1, 0.2 ppm AFB2, 3.7 ppm AFBs and 1.6 ppm AFB1, 0.1 ppm AFB2, 1.7 ppm AFBs, respectively.

Among the 150 A. flavus strains tested, 60 produced amounts of AFB1 ranging from 12.1 to 234.6 µg/g [51]. The production of aflatoxins by different strains of A. flavus varies widely. The A. flavus strains tested by [52] produced AFB1 in the range from 175 to 124101 µg kg-1 and AFB2 from not detected to 10328 µg kg-1. Abbas et al. [53] observed greater variations in aflatoxins production by A. flavus. Different aflatoxin production capabilities of the A. flavus strains would be influenced by different sources of the strains and environmental conditions [52].
In particular, analytical methods are proven accurate and reliable, but require expensive laboratory equipment and supplies [54]. Availability of these methods may be limited especially in developing countries. Here, qualitative cultural methods provide a cheaper alternative to screen large number of fungal isolates for aflatoxin production. Among the many cultural methods, visual indicators are thought to be highly predictive for aflatoxin development [35, 55]. These cultural methods have been developed many decades ago and their precision and reliability are widely criticized. Moreover, these methods have low degree of sensitivity and do not allow the specification of mycotoxigenic species [56]. High performance liquid chromatography is ideal and more useful than the other methods in terms of specificity and sensitivity of this process [57]. As an analytical tool, HPLC offers the advantage of a high resolution, limit of detection, with the possibility to be coupled to multiple detection automated systems. Although accurate and specific, most of the chromatographic assays are expensive, time-consuming and require expensive equipment and clean-up procedures [58].

REFERENCES


