DETECTION OF AFLATOXIN PRODUCTION BY FUNGI IN SPICE SAMPLES USING HPLC AND DIRECT VISUAL CULTURAL METHODS

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Abstract

Spice samples detected to be positive for aflatoxin using HPLC were analyzed for their total fungal population and the presence of aflatoxicogenic fungi using direct visual cultural methods. Screening was done using colony fluorescence upon exposure to UV, ammonia vapour test and characteristics in *Aspergillus* differentiation media. The aflatoxicogenic fungi isolated were morphologically identified to be *Aspergillus flavus* with the lone exception of a strain that showed similarity with *Aspergillus parasiticus*. Although, no direct correlation between the numbers of potential aflatoxin producing fungi with the levels of aflatoxin detected in the spice samples could be observed, it is interesting to note that spice samples with low fungal counts were detected to be having more quantity of aflatoxins using HPLC analysis. This leads us to conclude that aflatoxin production by aflatoxicogenic fungi may be considerably reduced or below detectable levels in spice samples having a high population of competing non-producing fungi.

Introduction

Aflatoxin contamination of spices is a major problem from the Indian perspective since, the climatic, agricultural and storage conditions and practices are conducive to fungal growth and toxin production. Aflatoxins are produced mainly by the secondary metabolism of *Aspergillus flavus, Aspergillus parasiticus* and *A. nomius*. Aflatoxin producing *Aspergillus* spp. may proliferate on spice samples if favorable environments for growth are available resulting in production of aflatoxins (Grybauskas *et al.,* 1988). There are many highly specific and sensitive methods for determining aflatoxin concentration in commodities or in culture, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarization assay (Seitz 1975; Trucksess *et al*., 1994; Whitaker *et al.,* 1996; Maragos and

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Thompson, 1999; Stroka and Anklam, 2000; Nasir and Jolley, 2002; Sobolev and Dorner, 2002). Usually these methods are expensive and time-consuming. Chromatographic methods require extraction procedures to remove interfering substances, by using a mixture of water and a polar organic solvent. Commercially available ELISA kits provide a relatively easy assay for quantification of total aflatoxin concentration but do not identify individual aflatoxins present in the sample.

Many areas of the world with a serious aflatoxin problem have difficulty screening potentially toxigenic *A. flavus* cultures because of the expense and expertise required to maintain an analytical laboratory. There has been much interest in developing and using cultural methods for detecting aflatoxins in fungal cultures. Various analytical methods have been compared for the detection of aflatoxins (Seitz 1975; Trucksess *et al.,* 1994; Whitaker *et al.,* 1996; Nilufer and Boyacioglu, 2002).

However, there are only a few studies that compare the cultural methods and analytical methods in detail. The present study therefore attempts to screen, enumerate, isolate and characterize aflatoxicogenic fungi in some spice samples, which are previously detected to be positive for the presence of aflatoxin using standard analytical methods like HPLC with an aim to understand the correlation between the presence of altoxicogenic fungi and levels of aflatoxin detected in the samples.

Materials and Methods

Spice samples used for the study

Five spice samples that were found positive for the presence of aflatoxin using HPLC analysis namely chillies stalkless (sample codes 128982/1 and 12980/1), nutmeg whole and ground (sample codes 127396/1 and 129003/1), and spices mix (masala) (sample code 128994/1) were taken for the present study.

The samples were stored in room temperature in air tight sterile containers.

Enumeration of total fungal population of the spice samples

The cultivable fungal population of the spice samples chillies stalkless (sample codes 128982/1 and 12980/1), nutmeg whole and ground (sample codes 127396/1 and 129003/1), and spices mix (masala) (sample code 128994/1) was enumerated using the standard protocol described in the Bacteriological analytical manual BAM (US FDA) 8th Edition.
(www.fda.gov/Food/ScienceResearch/Laboratory Methods/BacteriologicalAnalyticalManualBAM/ucm071435.htm).
Serial dilutions of the samples using phosphate buffer were made upto 10^{-4} dilution and 1 ml from each dilution was poured to respective petri plates. Potato dextrose agar supplemented with chloramphenicol (4.16 %) was used as the culture media. The PDA plates were allowed to solidify and incubated at $25\ ^{0}C$ for 5 days. After 5 days of incubation the fungal colonies were counted and reported as CFU/g of spice sample tested.

Calculation

N=∑C/(1×n₁)+(0.1×n₂)×d

$$N=\sum C/(1 \times n_1)+(0.1 \times n_2) \times d$$

where

N - number of colonies/ml or g

∑C - sum of all colonies in all plate counted

n_1 - number of plates in first dilution counted*

n_2 - number of plates in second dilution counted*

  d - dilution factor

  *count range - 15-150 colonies

Screening of Aflatoxicogenic fungi in the spice samples

The spice samples used in the present study were subsequently screened for the presence of aflatoxin producing fungi using potato dextrose agar (PDA) (Himedia Laboratories, Mumbai, India), coconut milk PDA (Davis *et al.*, 1987) and *Aspergillus* differentiation agar (Pitt *et al.*, 1983) (Himedia Laboratories, Mumbai, India). Coconut milk PDA media composed of 30 g of potato dextrose agar (PDA) x 100 mL coconut milk (Dabur, India) per 1000mL of final media prepared. Fungal colonies growing on PDA and coconut milk PDA media were subjected to the ammonia vapour test (Saito and Machida, 1999) and UV radiation at 365 nm

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for detection of resultant fluorescence upon aflatoxin production (Davis *et al.*, 1987). In case of *Aspergillus* differentiation agar, development of a yellowish orange colour in the reverse of the colonies, due to the reaction of ferric ions from ferric citrate with aspergillic acid molecules (Assante *et al.,* 1981) was taken as a positive reaction for the production of aflatoxin. Fungal colonies detected to be producing aflatoxin using the three different media were enumerated in relation to the total fungal population of the spice samples.

Isolation and Characterization of aflatoxin producing fungi

Fungal colonies showing fluorescence upon exposure to UV radiation and pink reverse upon exposure to ammonia vapours were isolated and pure cultured from PDA and coconut milk PDA media respectively. Colonies giving a positive reaction in *Aspergillus* differentiation agar were picked up and subsequently pure cultured onto PDA plates and slants. Microscopic observation of the fungal morphology in case of each of the isolates was done using Leica DME binocular Microscope.(Leica Microsystems, Germany) The morphology and cultural characteristics of each of the aflatoxin producing fungal isolates were compared to known descriptions of fungi in different fungal identification manuals (Domsch and Gams, 1972; McClenny, 2005 and Dugan, 2006) and identified.

Detection and quantification of aflatoxin production by the fungal isolates using HPLC

The aflatoxin producing fungal isolates were cultured in liquid potato dextrose media at 25 ºC for 10 days. The fungal culture broth of each of the aflatoxicogenic fungal isolate was centrifuged at 4 ºC in 10000 rpm using e in Hermle Z 383 ultracentrifuge (Germany).

The resultant supernatant was filtered using a microfiber filter of 110mm size (VICAM, Waters USA) to make the filtrate free from any fungal mycelia. The aflatoxin producing fungal isolates were analyzed for aflatoxin by High Performance Liquid Chromatography (HPLC) using Shimadzu DGU 20A5 (Japan) HPLC unit as per the standard analytical method of the American Spice Trade Association (ASTA, analytical manual method no 24.2).

Sample preparation: 12.5 ml of fungal culture broth was mixed with 50 ml extraction buffer containing methanol and water in 9:1 ratio.

The solution was shaken in a Cap blender jar for 15 minutes and filtered through micro fiber filter paper 10 ml of the filtrate was taken and volume made up to 50 ml using HPLC grade water.

Sample clean up: During the clean up stage, the total aflatoxin present in the sample is separated by an immunoaffinity column. In the present study the immunoaffinity column (Aflatest column, VICAM, Waters, USA) was used. The column was rinsed with HPLC grade methanol and 10 ml of the sample was passed through it. The aflatoxins present in the sample based on the highly specific antibody antigen reaction form a conjugate with the antibody and the remaining impurities are separated out. Finally aflatoxins bound in the column are cleaved from their respective antibodies using methanol. The separated aflatoxin methanol mixture was collected in 2 ml HPLC vials (Waters business, made in USA). A volume of 50 µl of the sample was subsequently injected into the HPLC column for analysis. Detection of aflatoxin in the samples was done by comparing the retention time and peaks generated with that of standards.

Calculation of the quantity of aflatoxin was done using the following formulae:

$$\text{Aflatoxin (ug/kg)} = \frac{\text{Pg (conc)} \times 1000ul \times 50ml \times 100ml}{50ul \times 10ml \times 10ml \times 25 \times 10^3 \, mg}$$

HPLC conditions used:

Mobile Phase: 55% HPLC water, 45% HPLC methanol. Flow: 1.2 ml/min, Isocratic flow. Column oven temperature: 40^0C: column CTO-10AS vp. Detector: fluorescence, wave length Ex-365-EM-464, UV/Visible detector (SPD-20A). Post column derivation: Photochemical reactor for enhanced detection (PHRED) Aura industries, USA. Standards used: G2= 37.500 Pg, G1=125.000 pG, B2= 37.500 Pg, B1=125.000 pG (Supleco analytical, made in USA).

Results and Discussion

Total fungal population of the spice samples

The total fungal count obtained in case of each of the spice samples are given in Table 1. The fungal population was enumerated using potato dextrose agar and a modified media i.e. coconut milk potato dextrose agar. The total fungal population of the samples ranged from 4×10^1 CFU/g to 1.21×10^5 CFU/g (Table 1). Sample 128980/1 showed a high fungal count in both the test media used. In PDA media the count for this sample was 1.05×10^5 CFU/g while in coconut milk media the count was 1.21×10^5 CFU/g. Sample 129003/1 showed low fungal counts 4.0×10^1 CFU/g and 4.5×10^1 CFU/g for potato dextrose agar and coconut milk potato dextrose agar respectively (Table 1).

Table 1. *Enumeration of fungal count in PDA and Coconut PDA plates*

No	Spice sample	Total fungal count in PDA plate (CFU/g of spice sample)	Total fungal count in coconut PDA plate (CFU/g of spice sample)
1	Nutmeg whole (127396/1)	8.2×10^2	9.8×10^2
2	Nutmeg ground (129003/1)	4×10^1	4.5×10^1
3	Chillies stalk less (128982/1)	5.768×10^4	5.83×10^4
4	Chillies stalk less (128980/1)	1.05×10^5	1.21×10^5
5	Mixed spices (128994/1)	7.75×10^3	8.28×10^3

*values are mean of three replicates

The total fungal population of the spice samples enumerated using two culture media i.e. potato dextrose agar and coconut milk potato dextrose agar showed similar patterns for each of the samples (Table 1). The results for the total number of fungi using the coconut milk media were slightly higher than that of the PDA media (Table 1). Domir and his co-workers, while studying the effect of elm selection, explant source and medium composition on growth of the fungus *Ophiostoma ulmi* on callus cultures, also reported that coconut milk, being a rich source for growth hormones, stimulated fungal growth in the absence of callus (Domir *et al.*, 1992). Coconut milk added culture media has been reported to be excellent for screening of aflatoxin producing fungi (Lin and Dianese, 1976; Yazdani *et al.*, 2010). Our results reveal that the total fungal counts obtained in the two media were in the same range although higher counts were recorded for the coconut milk supplemented PDA media in comparison to PDA alone (Table 1).

Aflatoxicogenic fungi in the spice samples

Detection of aflatoxin producers based on colony fluorescence

The screening for aflatoxin producing fungi in the spice samples was carried out based on the fluorescence of positive colonies upon exposure to UV radiation at 365 nm. The total number of fungal colonies showing fluorescence (Fig. 1) after 7 days of growth in potato dextrose agar and coconut milk potato dextrose agar is given in table 2. The highest number of colonies showing fluorescence was recorded for the sample 128980/1 followed by sample 128980/1, 128994/1, 127396/1 and 129003/1 (Table 2).

The methodology for detection of aflatoxin by fluorescence of agar medium under ultraviolet light provides a simple and reliable means of eliminating non-producing strains and also avoids difficulties encountered in extracting aflatoxin from complex natural substrates (Hara *et al.*, 1974).

The highest number of colonies showing fluorescence was recorded for the sample 128980/1 followed by sample 128980/1, 128994/1, 127396/1 and 129003/1 (Table 2). In the present study detection of aflatoxin producing fungi using UV fluorescence in the two culture media showed similar patterns in terms of the total numbers of colonies showing fluorescence upon exposure to UV radiation.

The blue fluorescence of aflatoxins has been used for developing qualitative cultural methods for detecting aflatoxin production by *Aspergillus* species grown on suitable media (Abbas *et al.*, 2004).

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Table 2. Enumeration of colonies showing fluorescence in PDA and coconut PDA plates

No	Spice sample	Total fungal count in PDA plate (CFU/g of spice sample)	Total fungal count in coconut PDA plate (CFU/g of spice sample)
1	Nutmeg whole (127396/1)	3.5×10^1	4×10^1
2	Nutmeg ground (129003/1)	1.2×10^1	1.5×10^1
3	Chillies stalk less (128982/1)	1.0×10^4	1.205×10^4
4	Chillies stalk less (128980/1)	1.25×10^3	2.5×10^3
5	Mixed spices (128994/1)	4.8×10^2	5.25×10^2

*values are mean of three replicates

In majority of the studies using UV fluorescence, potato dextrose agar and coconut agar were used (Davis *et al.*, 1987; Gupta and Gopal, 2002; Lemke *et al.,* 1988, 1989; Lin and Dianese, 1976; Saito and Machida, 1999; Wei *et al.*, 1981). Yabe *et al.* (1987) used GY-agar medium as a simple method for screening aflatoxin-producing species by UV photography. It was possible to detect production of aflatoxins even by small colonies that were only 36 hr old, and many colonies could be examined

on a single plate (Yabe *et al., 1987*). Ultraviolet photography can therefore also be used as a rapid method for the identification of aflatoxin-producing isolates. The aflatoxin-producing isolates appeared as gray or black colonies in the UV photographs, whereas non producing isolates appeared as white colonies. Aflatoxins B1 and G1 were primarily responsible for the absorbance of UV light (Abbas *et al.*, 2004). In the present study coconut milk PDA media showed an increase in the number of fungal colonies exhibiting blue fluorescence zones in comparison to the PDA media (Table 2). This can be explained by the enhancement of aflatoxin production and detection using UV light in the case of coconut supplemented agar media (Lin and Dianese, 1976; Davis *et al.*, 1987; Lemke *et al.,* 1988). Yazdani *et al.* (2010) while evaluating the various detection techniques for toxigenic *Aspergillus* isolates 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.* (2004) while comparing the cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates reported that cultures capable of producing greater than 61 ng/g of aflatoxin in fungal structures were fluorescent on PDA.
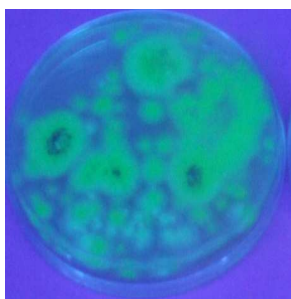

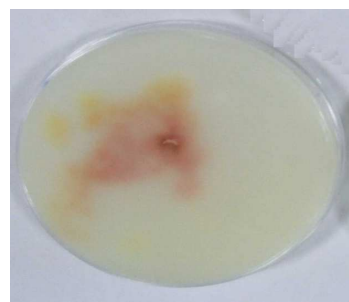*Figure 1. PDA plate with aflatoxin producing fungal colonies showing fluorescence on UV exposure*

Figure 2. Aflatoxigenic fungal isolate SNRB 48 producing pink color when exposed to ammonium vapour

Detection of aflatoxin producers based on the Ammonia vapour test

Fungal colonies that exhibited the phenomenon of development of pink reverse (Fig 2) after exposure to vapours of ammonia were considered positive for aflatoxin.

The number of colonies showing the positive reaction in both the test culture media i.e. potato dextrose agar and coconut milk potato dextrose agar are given in table 3.

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Table 3: Screening of aflatoxigenic fungi using the Ammonium vapour test

No	Spice sample	Number of colonies showing pink color in the back side of PDA plate (CFU/g of spice sample)	Number of colonies showing pink color in the back side of coconut PDA plate (CFU/g of spice sample)
1	Nutmeg whole (127396/1)	4×10^1	4×10^1
2	Nutmeg ground (129003/1)	1.2×10^1	1.5×10^1
3	Chillies stalk less (128982/1)	1.1×10^4	1.0×10^4
4	Chillies stalk less (128980/1)	2.68×10^3	2.55×10^3
5	Mixed spices (128994/1)	3.57×10^2	5.12×10^2

*values are mean of three replicates

The number of positive colonies detected using this methodology also followed a similar pattern as exhibited by the results obtained from colony fluorescence (Table 2). The number of positive colonies detected using this methodology also followed a similar pattern as exhibited by the results obtained from colony fluorescence (Table 2). The highest number for colonies exhibiting the pink colouration was observed in case of sample 128982/1 followed by samples 128980/1, 128994/1, 127396/1 and 129003/1 respectively. Saito and Machida, 1999, using the same technique reported that the colony reverse of aflatoxin (AF)-producing strains of *Aspergillus flavus* and *A. parasiticus* turned pink when their cultures were exposed to ammonia vapour.

Further, they also observed that the colour change was visible for colonies grown on media suitable for aflatoxin production such as potato dextrose, coconut, and yeast extract sucrose agar after 2 days of incubation at 25ºC. They also reported that out of the 120 strains of *A. flavus, A. parasiticus,* and two related species in *A. flavus* group: *A. oryzae* and *A. sojae* tested in the study, only the aflatoxin producing strains of *A. flavus* and *A. parasiticus* showed the pink pigmentation. The colour change occurred immediately after the colony was contacted with ammonia vapour.

The aflatoxin production for the strains showing pink reverse ranged from 1.9 ng/g to 40,200 ng/g for aflatoxin B1. Although the color change was visible after 2 days, 3-4 days of incubation was sufficient for detection of even very weak AF production. Incubation at 20 ºC slightly reduced the intensity of fluorescence, whereas no difference was observed at 25 ºC, 30 ºC, or 35 ºC (Saito and Machida, 1999).

Detection of aflatoxin producers based on cultural characteristics in Aspergillus differentiation media

Aflatoxin is primarily produced by the fungal species of the genus *Aspergillus*. As such in the present study efforts were made to screen the aflatoxin producing species from the non producers using a differentiation media i.e. *Aspergillus* differentiation media. The total number of colonies in CFU/g and the number of colonies showing a yellowish orange colour in the reverse of the colonies (Fig 3), due to the reaction of ferric ions from ferric citrate present in the media with the aspergillic acid molecules are shown in Table 4.

The highest number of positive fungal colonies was recorded for the sample 128982/1 whereas the sample 129003/1 showed the least count i.e. 1.0×10^1 CFU/g (Table 4). Aflatoxin is primarily produced by the fungal species of the genus *Aspergillus*. The two most important species producing aflatoxin are *Aspergillus parsiticus* and *Aspergillus flavus.* In the present study the numbers of positive colonies showing characteristics of these two species in relation to the total number of fungi in *Aspergillus* differentiation media are given in Table 4. Both *Aspergillus parsiticus* and *Aspergillus flavus* develop an intense yellow orange colour at the base of the colonies which is a differential characteristic for these in comparison with other related species.

Assante *et al.* (1981) showed that the orange yellow colouration was due to reaction of ferric ions from ferric citrate with aspergillic acid molecules forming a coloured complex. In a similar study for comparing four media for the isolation of *Aspergillus flavus* group of fungi, it was found among a mix of about four hundred

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related isolates only isolates of *A. flavus* and *A. parasiticus* produced the colour reaction. Further, it was reported that the *Aspergillus* differentiation media failed to detect strains of *A. nomius* (Cotty, 1994). However, it is crucial to note that this may not be a problem for many researchers as *A. nomius* occurs far less frequently than either *A. flavus* or *A. parasiticus* in most locations/ commodities.

Table 4. *Screening of aflatoxigenic fungi using Aspergillus differentiation medium*

No	Spice sample	Total Number of colonies in *Aspergillus* differentiation medium (CFU/g of spice sample)	Number of colored colonies in *Aspergillus* differentiation medium (CFU/g of spice sample)
1	Nutmeg whole (127396/1)	5.6×10^2	2.0×10^1
2	Nutmeg ground (129003/1)	3.0×10^1	1.0×10^1
3	Chillies stalk less (128982/1)	1.4×10^4	1.27×10^4
4	Chillies stalk less (128980/1)	5.2×10^3	2.7×10^3
5	Mixed spices (128994/1)	3.4×10^3	5.0×10^2

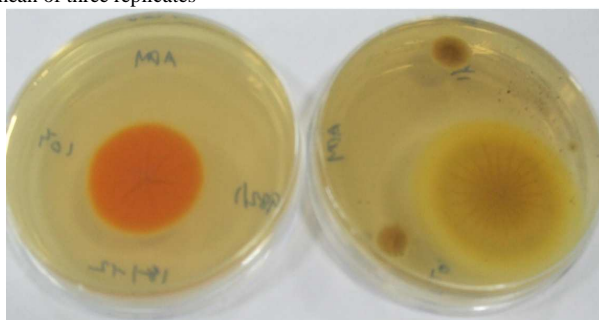*values are mean of three replicates

Figure 3: *Aspergillus flavus isolate SNRB23 producing orange red color in Aspergillus differentiation medium. Non producing colonies did not produce the colour change*

Table 5. *Summary of results obtained from the three different screening methods*

No	Spice sample	Total Aflatoxin detected in the spices sample using HPLC (µg/kg)	Average of Total fungal counts from PDA and Coconut PDA media (CFU/g of spice sample)	Average number of aflatoxigenic fungi detected using the three screening methods (CFU/g of spice sample)
1	Nutmeg whole (127396/1)	**1070.2** B1=1026.3 B2=43.4	9.0×10^2	3.5×10^1
2	Nutmeg ground (129003/1)	**41.8** B1=38.1 B2=2.8 G1=0.9	4.3×10^1	1.3×10^1
3	Chillies stalk less (128982/1)	**23.7** B1=23.2 B2=0.5	5.799×10^4	1.115×10^4
4	Chillies stalk less (128980/1)	**12.2** B1=12.2	1.13×10^5	2.336×10^3
5	Mixed spices (128994/1)	**3.7** B1=3.7	8.015×10^3	4.75×10^2

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Table 5 summarizes the results obtained from the three different screening strategies for the total number of aflatoxin producing fungal population using three different media i.e. potato dextrose agar, coconut milk potato dextrose agar and *Aspergillus* differentiation media. The levels of aflatoxin detected in each sample, prior to the enumeration of the fungal population using HPLC are also given (Table 5).

Isolation and characterization of aflatoxin producing fungi from the spice samples

Fungal colonies that were positive for aflatoxin production were pure cultured by repeated transfers to PDA plates and slants. Isolates showing different colony characteristics were designated as different isolates. Morphological characterization and identification of each of the isolates is presented in Table 6. Photomicrographs of the different isolates as observed under the light microscope are shown in Figs 4 to 6. The aflatoxin producers detected by the screening methodologies were subsequently isolated and pure cultured onto potato dextrose agar. All the isolates based on their cultural and morphological characteristics were identified to be species of *A. parasiticus or A. flavus* (Table 6) (Figs 3 to 5).

Figure 4: Fungal isolate SNRB23 under 40x magnification

Figure 5: Fungal isolate SNRB23 under 40x magnification

Figure 6: Fungal isolate SNRB23 under 40x magnification

However, it is interesting to note that isolates of *A.flavus* was isolated from all the five spice samples whereas *A. parasiticus* was isolated only from the sample 129003/1. If we compare the levels of aflatoxins detected in the spice samples prior to the initiation of the present study, using the standard HPLC protocol (Table 5), it can be observed that apart from other aflatoxins, aflatoxin G1 has been detected only in the sample 129003/1.

This leads us to conclude, that the production of aflatoxin G1 in the ground nutmeg sample may be due to the presence of *Aspergillus parasiticus* in addition to *A.flavus*. This is supported by the experimental finding previously made by Gourama and Bullerman in 1995 in which they noted that aflatoxin G1 and G2 are produced exclusively by *A.parasiticus*.

Table 6. *Aflatoxin producing fungal isolates obtained from the spice samples*

Fungal isolate number	Sample from which isolated	Colony colour	Morphological identity
SNRB4	Nutmeg whole(127396/1)	Green	*Aspergillus flavus*
SNRB23	Nutmeg ground(129003/1)	Green	*Aspergillus flavus*
SNRB48	Nutmeg ground(129003/1)	Dark Green	*Aspergillus parasiticus*
SNRB56	Chillies stalkless(128982/1)	Green	*Aspergillus flavus*
SNRB78	Chillies stalkless(128980/1)	Green	*Aspergillus flavus*
SNRB89	Mixed spices (128994/1)	Green	*Aspergillus flavus*

No direct correlation between the numbers of potential aflatoxin producing fungi as detected by the screening methods could be seen with the levels of aflatoxin detected in the spice samples (Table 5). However, if we look at the results from a total fungal population perspective, one interesting

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phenomenon emerges. Samples having high fungal population in general also showed a comparatively high number of possible aflatoxin producing fungi whereas the samples having comparatively low total fungal counts had even lower number of possible aflatoxin producers as detected by our screening strategy. However, these samples with low fungal counts i.e. 127396/1 and 129003/1 were detected to be having more quantity of aflatoxins i.e. 1070.2 and 41.8 μg/kg respectively (Table 6).

This phenomenon can be possibly due to the increased competition of aflatoxin producing fungi with non-producers or probable antagonistic fungi in spice samples where the fungal population is comparatively high. In samples where, the total fungal population is less the aflatoxin producing fungi has to compete with a lesser number of fungal species for occupying its preferred niche in search of nutrients for growth and eventual production of aflatoxin.

Similar observations were made by Horn and Dorner in 2002 while studying the effect of competition and adverse culture conditions on aflatoxin production by *Aspergillus flavus* through successive generations. They reported that the aflatoxin-producing ability was considerably reduced or absent in *A. flavus* stains grown in co-culture with other fungi (Horn and Dorner, 2002).

Detection and quantification of aflatoxin production by the fungal isolates in liquid media by HPLC

The levels of aflatoxin detected in liquid potato dextrose agar media for each of the aflatoxin producing fungal isolates is shown in Table 7. Only fungal isolates having green colonies showed presence of aflatoxin in the culture broth after 10 days of incubation at 25ºC (Table 7). The HPLC chromatogram for the aflatoxicogenic fungal isolate SNRB23 is given in Figure 7.

The Chromatogram generated from the standards of Aflatoxin in the HPLC system is shown in Figure 8. Only the isolates that were identified to be *Aspergillus flavus* with light green colonies were detected to be producing aflatoxin using the HPLC analysis (Table 7).The chromatogram of one representative isolate of *A. flavus* is shown in

Figure 6. *Aspergillus parasiticus* isolates were not detected to produce aflatoxin after 10 days of incubation in liquid culture media by the HPLC analysis. This may be due to the fact that the *Aspergillus parasiticus* isolates in the present study were slow producers of aflatoxin in liquid culture conditions in comparison to solid media.

Moreover, the ability of the isolates may have been lost during the pure culturing stage owing to susceptibility of the isolates to such interventions.

In a similar observation Drummond and Pinnock (1990), while studying the aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus in* liquid culture reported that aflatoxin B_1 production was greatest between 7 and 10 days and continued even upto 20 days for some isolates.

Table 7. *Determination of aflatoxins in the liquid culture media of the fungal isolates after 10 days of inoculation using HPLC*

Fungal isolate number	Colony colour	Morphological identity	Total amount of aflatoxin detected by HPLC (μg/kg)
SNRB4	Green	*Aspergillus flavus*	23.3
SNRB23	Green	*Aspergillus flavus*	25.6
SNRB48	Dark Green	*Aspergillus parasiticus*	N.D
SNRB56	Green	*Aspergillus flavus*	22.2
SNRB78	Green	*Aspergillus flavus*	20.1
SNRB89	Green	*Aspergillus flavus*	22.4

N.D: Not detected

Significantly higher quantities were produced by *A. flavus* in comparison to *A. parasiticus*. Further they observed that aflatoxin production was not a stable trait in one *A. parasiticus* isolate passaged 50 times on agar. In addition to loss of aflatoxin production, an associated loss in sporulation ability was also observed in this passaged isolate, although it did maintain pathogenicity against its host mealybug *Saccharicoccus sacchari* (Drummond and Pinnock, 1990).
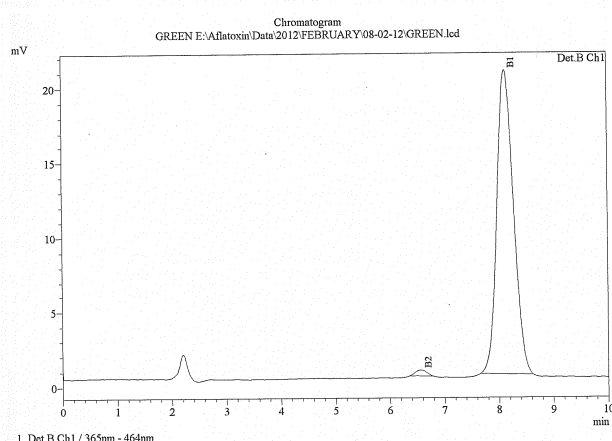
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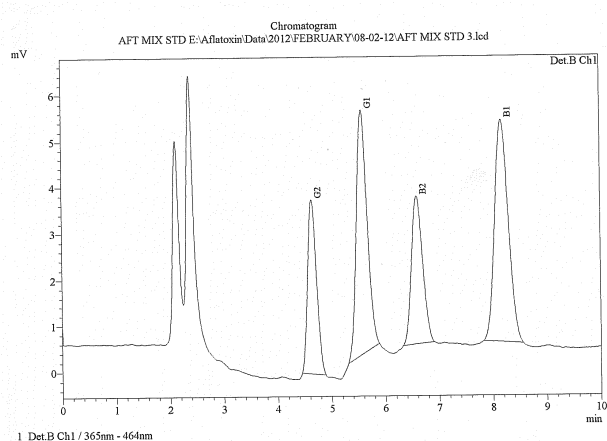*Figure 7. HPLC Chromatogram for Aspergillus flavus (SNRB23) showing detection of aflatoxin in the liquid culture media*

Figure 8. HPLC Chromatogram for aflatoxin standards

Conclusion

The present study successfully attempted in trying to establish a link between the enumeration and detection of aflatoxin producing fungi to the levels of aflatoxin detected in spice samples using the standard HPLC analytical procedures. However, more work needs to be done with larger number and types of spice samples in relation to their geographical origins. Molecular characterization of the fungal species involved followed by the development of a database of aflatoxin producing fungi from spice and spice products may help us to accurately predict the possible levels of aflatoxin in the spice samples prior to using expensive analytical tools for elucidating the same.

References

Abbas H.K., Shier W.T., Horn B.W., Weaver M.A. (2004). Cultural methods for aflatoxin detection. *Journal of Toxicology*, 23,295-315.

Assante G., Camrada L., Locci R.R., Merlini L., Nasini G., Popadopoulos E. (1981).Isolation and structure of red pigments from *Aspergillus flavus* and related species, grown on a differential medium. *The Journal of Agricultural and Food Chemistry*, 29,785-787.

Cotty P.J. (1994). Comparison of four media for the isolation of *Aspergillus flavus* group fungi. *Mycopathologia*, 125, 157-162.

Davis N.D., Iyer S.K., Diener U.L. (1987). Improved method of screening for aflatoxin with a coconut agar medium. *Applied and Environmental Microbiology*, 53, 1593-1595.

Domir S.C., Schreiber L.R., Ichida J.M., Eshita S.M. (1992). Effect of elm selection, explant source and medium composition on growth of *Ophiostoma ulmi* on callus cultures. *Journal of Environmental Horticulture*, 10, 59-62.

Domsch K.H., Gams W. (1972). Fungi in agricultural soils. London: Longman.

Drummond J., Pinnock D.E. (1990). Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus*. *Journal of Invertebrate Pathology*, 55,332–336.

Dugan F.M. (2006). The identification of fungi: An illustrated introduction with keys, glossary, and guide to literature. pp 176, APS Press, St Paul, MN.

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Innovative Romanian Food Biotechnology (2014) 14, 1-12

Gourama H., Bullerman L.B. (1995).Antimycotic and anti aflatoxigenic effect of lactic acid bacteria: A review. *Journal of Food Protection*, 58, 1275-1280.

Grybauskas A.P., Thomison P.R., Cassel E.K. (1988). Aflatoxins. Cooperative Extension Service, University of Maryland, USA. Retrieved May 15, 2009, fromhttp://dairy.vitaplus.com/pdf/ Maryland%20Cooperative%20Extension%3B%20 Aflotoxins.pdf

Gupta A., Gopal M. (2002). Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. *World Journal of Microbiology and Biotechnology*, 18,325-331.

Hara S., Fennell I.D.I., Hesseltine C.W. (1974). Aflatoxin-producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. *Applied Microbiology,* 27,1118-1124.

Horn B.W., Dorner J.W. (2002). Effect of competition and adverse culture conditions on aflatoxin production by *Aspergillus flavus* through successive generations. *Mycologia*, 94,741 -751.

Lemke P. A., Davis N.D., Creech G.W. (1989). Direct visual detection of aflatoxin synthesis by minicolonies of *Aspergillus* species. *Applied and Environmental Microbiology,* 55, 1808 - 18 10.

Lemke P.A., Davis N.D., Lyer S.K., Creech G.W. , Diener U.L. (1988). Fluorometric analysis of iodinated aflatoxin in mini cultures of *Aspergillus flavus* and *Aspergillus parasiticus*. *Journal of Industrial Microbiology*, 3, 119- 125.

Lin M.T., Dianese J.C. (1976). A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology*, *66*, 1466-1499.

Maragos C.M., Thompson V.S. (1999). Fiber-optic immunosensor for mycotoxins. *Natural Toxins*, 7, 371–376.

McClenny N. (2005). Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: The traditional approach. *Medical Mycology*, 43,125-128.

Nasir M.S., Jolley M.E. (2002). Development of a fluorescence polarization assay for the determination of aflatoxins in grains. *The Journal of Agricultural and Food Chemistry*, 50, 3116–3121.

Nilufer D., Boyacioglu D. (2002). Comparative study of three different methods for the determination of aflatoxins in tahini. *The Journal of Agricultural and Food Chemistry,* 50, 3375–3379.

Pitt J.D., Hocking D., Glenn R. (1983). An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. *Journal of Applied Bacteriology*, 54, 109–114.

Saito M., Machida S. (1999). A rapid identification method for aflatoxin producing strains of *A. flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience*, 40,205 -211.

Seitz L.M. (1975). Comparison of methods of aflatoxin analysis by high-pressure liquid chromatography. *Journal of Chromatography*, 104, 81–91.

Sobolev V.S., Dorner J.W. (2002). Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *Journal of AOAC International*, 85, 642–645

Stroka J., Anklam E. (2000). Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. *Journal of Chromatography*, 904, 263–268.

Trucksess M., Stack M.E., Nesheim S., Albert R.H., Romer T R. (1994). Multifunctional column coupled with liquid chromatography for determination of aflatoxins B1, B2, G1 and G2 in corn, almonds, brazil nuts, peanuts and pistachio nuts: collaborative study. *Journal of AOAC International*, 77, 1512–1521.

Wei D.L., Chen W.L., Wei R.D., Jong S.C. (1981). Identity and aflatoxins producing ability of *Aspergillus* reference cultures. pp. 87-97 *In:* Kurata H., Ueno Y. eds. *Toxigenic Fungi-Their Toxins and Health Hazard.* Amsterdam: Elsevier

Whitaker T., Giesbrecht F., Wu J. (1996). Suitability of several statistical models to simulate

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Innovative Romanian Food Biotechnology (2014) 14, 1-12

observed distribution of sample test results in inspections of aflatoxin-contaminated peanut lots. *Journal of AOAC International,* 79, 981-988.

Yabe K., Ando Y., Ito M., Terakado N. (1987). Simple method for screening aflatoxin-producing molds by UV photography. *Applied and Environmental Microbiology*, 53, 230-234.

Yazdani D., Zainal Abidin M.A., Tan Y.H., Kamaruzaman S. (2010). Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. *African Journal of Biotechnology*, 9, 7654-7659.