Assessment of Aflatoxin contamination in *Arachis hypogaea* L & *Zea mays* L kernels

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Abstract

Introduction: Aflatoxin is the main mycotoxin that harms animal and human health due to its carcinogenic nature and mainly released by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 constitutes the most harmful type of aflatoxin and is a potent hepatocarcinogenic, mutagenic, teratogenic and it suppresses the immune system.

Aim: The present study was aimed at determining the Aflatoxin contamination in the *Zea mays* L. & *Arachis hypogea* L. kernels by using various morphological and analytical techniques.

Materials & Methods: Initially, the cultures were developed using potato dextrose agar medium. The strains were treated with ammonium vapor & lactophenol blue staining to identify the presence of Aspergillus contamination. The Aflatoxins were identified by ELISA by competitive indirect method and HPLC using monolithic column (100 × 4.6 mm) equipped with a fluorescence detector. The mobile phase (water: methanol 55:45, v/v) was pumped at a flow rate of 1.0 ml/min. DNA from mycelia of toxigenic and non-toxigenic *A. flavus, A. parasiticus* were subjected to quadraplex PCR using nor-1, ver-1, omt-A and aflR primers.

Results: Sporulation began after three days from the centre and progressed radially covering the surface of the colony. The conidia produced had yellowish to olive colour. The microscopic features of *A. flavus* under the basic biological light microscope showed that the colonies were biseriate with philiades radiating in all sides from metulae born on subglobose or globose vesicles of variable size. The colony reverse of aflatoxin producing strains of *Aspergillus flavus* and *A. parasiticus* turned pink upon cultures exposed to ammonia vapor. The results obtained from the cultural TLC, ELISA & HPLC showed significant production of AFB1 followed by AFB2 from the isolates of ground nut sample and maize sample. Quadraplex PCR analysis showed the bands of the fragments nor-1, ver-1, omt-A and aflR genes visualized at 400, 537, 797 and 1032 bp, respectively.

Conclusion: There is an emergency to provide insight into the sources of contamination, occurrence, detection techniques and masked aflatoxins, in addition to management strategies to ensure food safety and security.

Key Words: Mycotoxins, Aflatoxin, Aspergillus Sps, *Zea mays* L, *Arachis hypogea* L

Introduction

Fungi are the second largest group of eukaryotes playing a significant role in human health issues. Aspergillus species are the paramount ever present fungi that contaminate various food components and produce biochemical's known as mycotoxins. Aflatoxins are the secondary metabolite compounds produced from filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* contaminating food ingredients causing a serious impact for animal and human health. Aflatoxins are the first known mycotoxins, as a result of turkey "X" disease in the 1960's [1, 2]. More than 18 types of aflatoxins exist naturally, of which AFB1 is the most toxic type. AFB1 and AFB2 produced by *Aspergillus flavus* AFG1 and AFG2 are produced by *Aspergillus parasiticus* that occur in the contaminated feed. AFM1 and AFM2 are present in ruminant milk after the digestion of feed contaminated by AFB1 and AFB2 [3, 4]. These fungal metabolites in high concentrations, can have toxic effects that ranging from acute (liver or kidney deterioration), to chronic (liver cancer), mutagenic, and teratogenic; resulting symptoms range from skin irritation to immunosuppression, neurotoxicity and death. Aflatoxins are chemically difuranocoumarin derivatives with a bifuran group attached to the coumarin nucleus and a pentanone ring (in case of Aflatoxin B) or a lactone ring (in case of Aflatoxin G) [5]. These are insoluble in water, non-polar in nature, stable to heat, physical and chemical treatment that becomes very difficult to remove from the feed contaminated by aflatoxins. Thus, monitoring and prevention of aflatoxins in foods commodities are important issues globally. Due to the development of analytical techniques increasingly faster and more sensitive have come into focus in the last decades, but only a few of them have gained applicability in routine analysis [3]. To protect consumers, many countries have established legislation to regulate the levels of aflatoxins in the feed and food. The United States Food and Drug Administration has set the limit for total Aflatoxins at 20 μg/kg [6], whereas the European Commission established the current limit for AFB1 and total Aflatoxins as 2 μg/kg and 4 μg/kg, respectively, for nuts, dried fruits and cereals.

This study sought to identify aflatoxigenic Aspergillus species from groundnuts & maize kernels using an integration of morphological and molecular approaches.

Methodology

Sample Collection, Preparation / Extraction

Groundnut & Maize kernel samples were obtained from sundry shops in Tirupati, Andhra Pradesh, India. Raw kernel samples was obtained and ground from which twenty grams of the ground samples were weighed into a clean conical flask (250 mL) with a glass lid tightly sealed. Solvent solution (methanol: distilled water; 70 : 30 v/v ratio) was prepared and 100 mL was added to samples in the conical flasks and the sealed tightly. The samples were extracted in a ratio of 1 : 5 of sample to extraction solution respectively and then stirred thoroughly at room temperature by using a mechanical wrist shaker for 30 minutes. Finally, the samples were allowed to settle and filtered

using the Whatman # 1 filter paper and the filtrates were collected for the analysis [7].

Identification of Aspergillus species

The kernels of ground nut & maize were surface sterilized for 1 min in 2.5% NaOCl, washed with sterile distilled water, in accordance with the method described by Samson [8]. Seeds from the sundry shops were thoroughly mixed after which approximately 100 seeds were randomly selected and washed in 350 ml of 0.5% ethanol solution and rinsed with distilled water twice. The kernels were randomly obtained and plated using sterile forceps onto potato dextrose agar (PDA) (20 g dextrose, 4 g Potato extract, and 15 g Agar) growth media and incubated at 28°C for 07 days. Any visible Aspergillus sps like mycelial growth or spores characterized by greenish coloration was considered.

Morphological Characterization of Aspergillus species

Macroscopic features of the fungi including colony growth, colour, texture, conidia were observed after 07 days of inoculation. For microscopic assessment, the slide cultures were prepared in accordance with description of Diba [9]. The 18 × 18 mm cover slip was placed gently at an angle of 45° on inoculated culture media. Upon fungus sporulation, the cover slip was gently removed and placed on the microscope slide and a drop of lactofuchsine was added and covered with a small cover slip. Another drop of lactofuchsine was placed on top of the small cover slip before completing the assembly with a 22 × 22 mm cover slip. The microscopic features such as conidiophores, vesicles, metulae, philiades, conidia shape, and texture were observed under a basic biological light microscope using the immersion oil (100x) objective.

Lactophenol cotton blue treatment

A quick method is simply to push an 18 × 18 mm cover slip at a 45 degree angle into a sporulation media, such as potato flake agar. When the mould sporulates, the cover slip is carefully withdrawn from the agar and mounted in a drop of lacto-phenol blue or lacto-fuchsin on a microscope slide. Another drop is placed on top of the small cover slip before completing the assembly with a 22 × 22 mm cover slip [10, 11].

Thin Layer chromatography

Thin layer chromatograph differentiation of fungi was done by growing the A. flavus isolates on PDA medium for 7 days at 28 ± 2^0 C. A 9 mm diameter plug of medium of each isolate was transferred to an eppendorf tube and extracted with 500 µL chloroform and evaporated to dryness. The residue was redissolved in 50µL chloroform and applied onto precoated silica gel TLC plate with 20 x 20cm, 0.25mm thickness. 5µL of aflatoxin extract along with aflatoxin standard were spotted on precoated TLC plates. The plate was first eluted with anhydrous ethyl ether [32], dried up in a fume hood for 5 minutes, and developed in mobile phase chloroform: acetone (9:1 v/v) in the same direction. TLC Plates were dried and observed under ultraviolet light (366 nm) light. The blue (AFB1 and AFB2) and green (AFG1 and AFG2) florescent spots on TLC plates were identified by comparing them with the standard aflatoxin [12].

Ammonium Vapor test

The fungal isolates were inoculated on PDA medium as single colonies by 5mm diameter in the center of plate and incubated at 280 C for 7 days. The petri plate was inverted and 2 drops of concentrated ammonium hydroxide solution placed on the inside of the lid of petri dish. The petri dish was inverted over the lid containing the ammonium hydroxide [13].

Enzyme Linked Immunosorbent Assay

A competitive Enzyme Linked Immunosorbent Assay (ELISA) was performed using the Aflatoxin ELISA test kit. Five grams of feed samples were weighed into five 50 mL tubes. As described in HPLC procedure, the first tube was designated as blank (0 ppb), the other tubes were spiked to get 5, 10, 20 and 30 ppb concentrations of Aflatoxin B1 & B2 and 25 mL of 70% methanol was added in each tube and shaken vigorously for 5 min using shaker. The extract was filtered through Whatman No. 1 filter paper and 1 mL of the filtrate was diluted with 1 mL of deionized distilled water and 50µL of the diluted filtrate was transferred to each well of the microtiter plate for analysis. In separate wells, 50 µL of standards were added and 50 µL of enzyme was added to each well. The wells of microtiter plates were coated overnight at 4^0 C with 100µL of AFB1-OVA conjugate in carbonate buffer (100mM), at pH 9.6, and then washed thrice with PBS containing 0.05% Tween 20 (PBS-T). AFB1 standards (Sigma) in 10% (v/v) methanol-PBS (50µL), or different dilutions of samples (50 µL), were added to the walls. After 15 min incubation at 37^0 C, 100 µL of anti-AFB1antibody (Sigma) (1:10000) in PBS were added and incubated at 37^0C for 30 min. The plates were washed with PBST 4 to 5 times. Subsequently, 100 µL- of secondary antibody conjugated with horseradish peroxidase (HRP) (1:10000) in PBS was added and incubated at 37^0 C for 30 min. At the end of the incubation period, the plates were washed 4 to 5 times with PBST, 100 µL of substrate (TMB-H202) were added and incubated at room temperature for 10 to 15 min. The reaction was stopped by adding 50 µL of stop solution (2M H_2S0_4) and the colour developed was read at 492 nm by ELISA Reader [14, 15].

High Performance Liquid Chromatography

The chromatographic method used for Aflatoxins extraction from the samples and the conditions were completely based on the AOAC official method with some minor modifications [16]. Fifty grams of samples with 5 g of sodium chloride and 300 mL of methanol: H_2O (80:20 v/v) was shaken for 30 min. After filtration, 20 mL of the filtrate was diluted with 130 mL of deionized water and filtered through the whatman filter, and 75 mL of the filtrate was used for further clean up on a Afla test IAC column. The Afla test column was preconditioned with 10 mL of phosphate buffered saline (2-3 mL/min) and then 75 mL of the diluted sample extract was passed through the column (2-3 mL/min). Finally, the column was washed with 15 mL water. For AFB1 elution from the column, a portion of 0.5 mL HPLC grade methanol was passed through the column followed by an additional portion of 0.75 mL of the same solvent one minute afterward. HPLC grade water was

added to the eluent to the volume of 3 mL and 100 µL of the final solution was injected into HPLC. For all samples, separation was performed on a monolithic column (100 × 4.6 mm) using a HPLC system equipped with a fluorescence detector. Mobile phase was water: methanol (55:45, v/v) with a flow rate of 1.0 mL/min. The fluorescence detector was operated at excitation wavelength of 365 nm and emission wavelength of 435 nm. Post-column derivatization was carried out with pyridinium hydrobromide perbromide (PBPB) at flow rate of 1 mL/min.

Amplification of Internal Transcribed Spacer Region

The primers nor1 enclosing a fragment of 400 bp from nucleotide 501-900 of the *A.flavus* nor1 gene; ver1 enclosing a fragment of 537 bp from nucleotide 623-1160 of the *A.flavus* ver-1 gene; omt1, enclosing a fragment of 797 bp from nucleotide 301-1098 of the *A.flavus* omt-A gene; aflR1, enclosing a fragment of 1032 bp from nucleotide 450-1482 of the *A.flavus* aflR gene designed on the basis of sequence alignments [24] were used to amplify the partial sequence of ITS region of the rDNA. The PCR reaction was done in 25 µl PCR tubes containing 2.5 µl of the DNA template, 1.5 µl of each primer set, 2.5 µl of reaction buffers, 1 µl of MgCl2, 0.25 µl of dNTPs, and 0.2 µl of Taq DNA polymerase. The PCR amplification parameters were set as follows: initial denaturation cycle of 5 minutes at 95°C, 30 cycles of 30 seconds each at 95°C for the subsequent denaturation, 30 cycles of 30 seconds each at 58°C for annealing, 30 cycles of 45 seconds at 72°C for extension, and final cycle of 5 minutes at 72°C for final extension. The PCR products were then held at 4°C indefinitely and visualized in 1.2% agarose gels in TE buffer and compared with 100 bp DNA molecular ladder. Electrophoresis was conducted at 80 V for 1 hour and the gel was observed under UV light [17].

Results & Discussion

Aflatoxins are toxic substances formed by the fungi that have potential to contaminate feed, crops and pose a serious health risk to humans and livestock and are also assumed to be responsible for the annual loss of 25% or more of the world's food crops that has significant economic implications. Various procedures for the detection and analysis of Aflatoxins are available in feed and food, as they are highly specific, practical and useful [18, 19]. The prevention of mycotoxicity is one of the most perplexing toxicology issues of recent times. Mycotoxins are toxic chemical compounds with low molecular weight (MW < 1000) and due to their diverse chemical structure; there exists no single standard technique for their analysis or detection [20].

Seed infection by *Aspergillus flavus* contamination:

Seed samples with varied levels of storage mould and field fungal incidence showed different symptoms and abnormal growth depending on the extent of infection.

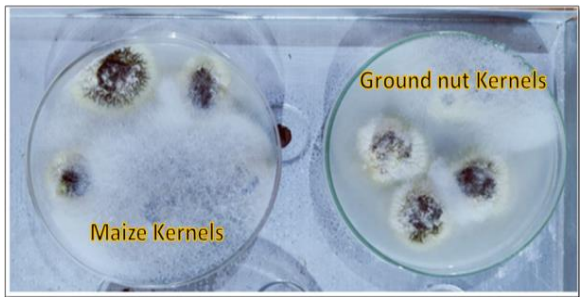

Figure 1: Fungal growth using PDA culture medium

Macroscopic Morphological Features

Microbial growth was observed on the PDA media. Sporulation began after three days from the centre and progressed radially covering the surface of the colony. The conidia produced had yellowish to olive colour. As the sporulation spread outwards, it gave a characteristic white border encircling the sporulating mycelia. The white border was eventually covered as the entire mycelia continued to sporulate and to produce more conidia by day 07. These colonies had clear exudates and cream colour on the reverse. The isolates representative of *A. flavus* had a greenish colony that spread radially from the point of inoculation. As the colony progressively grew, raised as mycelia piled and the centre becomes floccose and rough.

Figure 2: Identification of *Aspergillus flavus* by macroscopical examinations

Microscopic Morphological Features

Morphological characterization is the commonly adopted method for fungal isolation and characterization. It employs the use of culture media to support & facilitate the growth and development of the fungus for observation. Growth culture media such as malt extract agar (MEA), sabouraud dextrose agar (SDA), rose bengal chloramphenicol agar (RBCA), czapek dox agar (CZA) [21] and potato dextrose agar (PDA) [9, 21] have been used previously. These media can provide adequate requirements for fungus colony establishment that allows the development of the macroscopic and microscopic features suitable for assessment [9, 21]. This study employed the use of PDA medium provided adequate growth and sporulation of the fungus allowing satisfactory evaluation.

The microscopic features of *A. flavus* under the basic biological light microscope showed that the colonies were biseriate with philiades radiating in all sides from metulae born on subglobose or globose vesicles of variable size. The conidia had a globose shape ranging between 250 µm and 450 µm in diameter with thin walls and rough texture. The conidiophores had a rough texture and thick walls were nonpigmented and unbranched conidiophore which is nonseptate, rough and hyaline.

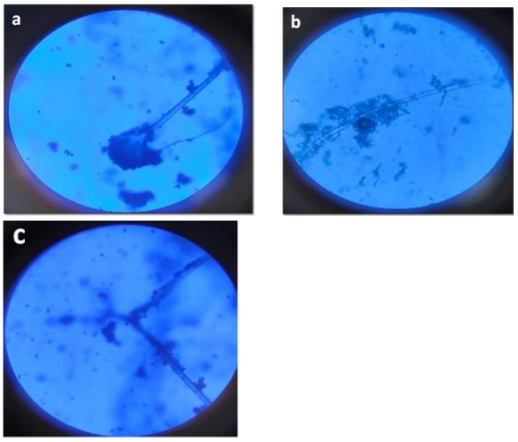

Figure 3: Identification of Aspergillus Sps by microscopical examinations

(3a, 3b, 3c) Aspergillus Sps showing conidia having a globose shape ranging between 250 µm and 450 µm in diameter with thin walls and rough texture. The conidiophores had a rough texture and thick walls were nonpigmented and unbranched

Aspergillus Sps on lactophenol cotton blue staining, showed septate hyphae and swollen vesicle giving rise to phialides from which chains of conidia arise.

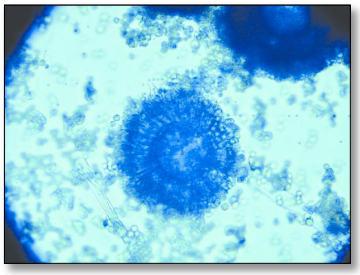

Figure 4: Identification of Aspergillus Sps. by lactophenol cotton blue staining

Ultra violet and ammonia vapor tests showed variable results with aflatoxigenic strains upon repeating the experiments. The colony reverse of aflatoxin producing strains of *Aspergillus flavus* and *A. parasiticus* turned pink upon cultures exposed to ammonia vapor. This color change was visible for colonies grown on media suitable for aflatoxin production after 3 days of incubation at 25°C. The color change occurred immediately after the colonies were contacted with ammonia vapor [22].

Table 1: Preliminary Identification of Aspergillus sps by conventional methods

Isolate	Conventional Methods		Aflatoxin Production
	UV test	AV Test	
Isolate form *Arachis hypogea*	+	+	**Positive**
Isolate from *Zea Mays*	+	+	**Positive**

Differentiation of Aflatoxigenic and Non-Aflatoxigenic *A. Flavus* isolates

The results obtained from the cultural TLC, ELISA were presented in Table 2. TLC analysis showed a clear demarcation between aflatoxin producing and non producing strains of *Aspergillus flavus*. ELISA method is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific aflatoxin. The competitive ELISA method is commonly used for aflatoxin analysis [23]. A conventional microtiter plate ELISA requires equilibrium of the antibody antigen reaction. ELISA results showed that, two *Aspergillus flavus* isolates (AFG19 and AFG23) were found positive for aflatoxin production but was reported as non-aflatoxin producer on TLC analysis which is due to higher sensitivity of ELISA. Among 21 aflatoxigenic isolates identified, AFB1 was significantly produced followed by AFB2 from the isolates of ground nut sample, and few strains produced only AFB1 from the isolates of maize sample.

Table 2: Detection of Aflatoxin producing ability of *Aspergillus flavus* isolates from TLC & ELISA

Isolate	TLC	ELISA	Aflatoxin Production
Isolate form *Arachis hypogea*	**B1, B2**	+	**Positive**
Isolate from Zea Mays	**B1**	+	**Positive**

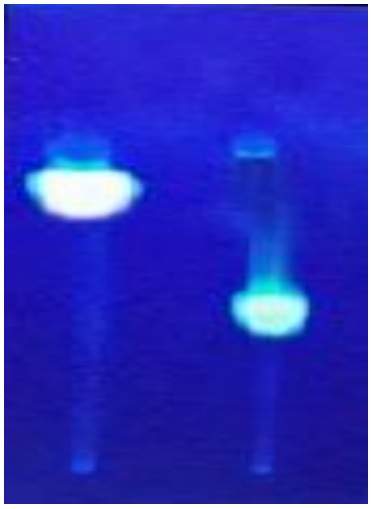

Figure 5: Thin Layer Chromatography of Aflatoxin identification

Quadraplex PCR analysis:

Bands of the fragments nor-1, ver-1, omt-A and aflR genes can be visualized at 400, 537, 797 and 1032 bp, respectively.

Table 3: Detection of Aflatoxins by Quadraplex PCR

Isolate	Quadraplex PCR				Aflatoxin Production
	aflR	Omt-A	Ver-1	Nor-1	
Isolate form *Arachis hypogea*	+	+	+	+	**Positive**
Isolate from *Zea Mays*	+	+	+	+	**Positive**

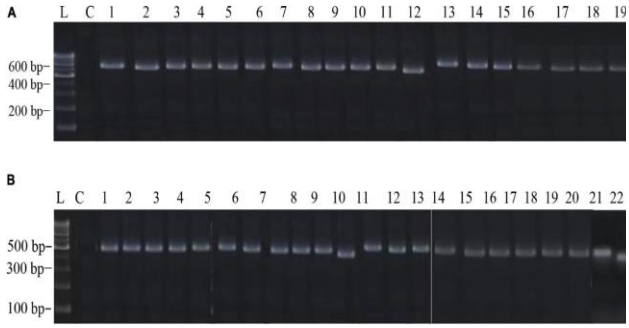

Figure 6: Quadraplex PCR analysis

Identification of Aflatoxins by HPLC method

Linearity was assessed for AFB1 over a range of 0.4-3.6 ng/g and reasonable correlation coefficients (r2 > 0.995) were obtained which indicated a good linearity of the analytical response over the specified concentration range. The estimated LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 9) of AFB1 were 0.01 ng/g and 0.03 ng/g, respectively, which indicated that applying monolithic column improved sensitivity of the method compared to the previously reported methods. The recovery percentage ranged from 86 to 96%, with mean and relative standard deviation (RSD) values of 92.42 and 5.97%, respectively for HPLC.

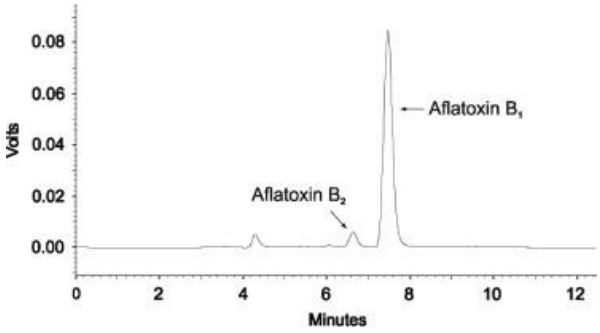

Figure 7: Chromatogram of Aflatoxin B1 & B2

Conclusion

The identification and quantification of Aflatoxins in food and feed is a major challenge to guarantee food safety globally. Therefore, developing feasible, sensitive and robust analytical techniques for the identification and quantification of Aflatoxins present in low concentrations in food and feed. Various analytical methods have the potential to improve the efficiency of Aflatoxins decontamination as well as to overcome the limitations of any specific technology. However, it is vital to understand the mechanisms of Aflatoxins detoxification so that no residues are left when applied in food and feed samples analysis. Furthermore, as there is less information on the masked Aflatoxins present in food and feed, it requires in depth research and understanding with regards to identification, detection and control strategies.

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