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Role of mould occurrence in aflatoxin build-up and variability of *Aspergillus flavus* isolates from maize grains across India

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RESEARCH ARTICLE

Abstract

The present study was conducted to study the role of mould/fungal occurrence on aflatoxin build-up in maize grains and correlation between toxigenic and genetic variability present among isolates of *Aspergillus flavus* obtained from maize grain samples. Eighty-six maize samples were collected from farmer's fields from various locations among the four maize growing agro-ecological zones of India. Among the microflora of maize grains studied, *Aspergillus* was the most predominant mould identified. Location Karnal (Haryana) was most sensitive to mould infection; 56.2% of samples), exhibited >20 µg/kg aflatoxin B_1 (AFB_1). On the other hand, Begusarai (Bihar) proves to be a less sensitive area for aflatoxin contamination with 90% of samples exhibiting <20 µg/kg AFB_1. Varied isolates of *A. flavus* from grain samples were established in culture media and studied for toxigenic variability. *In vitro* high level variability (8,116.61-0.21 µg/kg) for aflatoxin production potential was found among these isolates. Random amplified polymorphic DNA (RAPD) analysis using 35 OPERON random primers was used to study the correlation between toxigenic and genetic variability. The study exhibited partial relationship between RAPD dendrogram and geographic origin of these isolates, while no correlation was found between genetic variability and toxin production ability among *A. flavus* isolates studied. Also, three atoxigenic isolates of *A. flavus* were identified, viz. AF-9, AF-36, and AF-39, endemic to India from the Bihar, Haryana, and Delhi regions, respectively. These strains will need further validation.

Keywords: aflatoxin B_1, ELISA, mycotoxin, RAPD, TLC

1. Introduction

Aflatoxins are highly carcinogenic, secondary metabolites mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Christensen, 1983). They contaminate a variety of agricultural commodities, particularly maize and groundnut. Aflatoxins are a group of approximately 20 related fungal metabolites of which the major ones are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 and aflatoxin G_2. AFB_1 is the most potent naturally occurring liver carcinogen (IARC, 1993). Ingestion of aflatoxins in contaminated food or feed results in poisoning (aflatoxicosis), while long-term exposure of moderate to low concentration of aflatoxin causes chronic toxicity. It is one of the most important causes for stunting in children and immune system disorders (Gong *et al.,* 2003; Turner *et al.,* 2003). Hence, their quantity in food and feed is closely monitored and regulated in most countries (Van Egmond, 1995).

In India, human disease outbreaks attributable to the consumption of aflatoxin-contaminated maize have been reported from district of Banswara in Rajasthan and Panchmahal in Gujarat (Krishnamachari *et al.,* 1975). Also, the occurrence of aflatoxins and ochratoxin A has been reported in poultry feeds from India (Thirmula *et al.,* 2002). These observations reinforce the need intervention strategies for aflatoxin exposure within countries. Aflatoxin contributes to significant economic losses in maize which prevents commodities from meeting international standards governing agricultural trade and food safety. Fungal growth and toxin production in maize have been found to depend

on several interacting stress factors (Payne, 1992), including low moisture content of the soil, high daytime maximum temperatures, high nighttime minimum temperatures, and nutrient-deficient soils (Abbas et al., 2002; Miller et al., 1983; Widstrom et al., 1990). The Indian subcontinent has an extreme diversity in soil and climatic conditions. In this varied environment, maize is grown from extreme semi-arid to sub-humid and humid regions. Based on maize cultivation in different agro climatic conditions, India has been demarcated into different zones.

The aim of the present work were: (1) to study of occurrence of mould/fungal species responsible for aflatoxin contamination in maize grain among the four agro-climatic zones of India; (2) to judge the awareness among farmers regarding importance of aflatoxins; (3) to find the toxigenic variability present in isolates of *A. flavus*; and (4) to determine the relationship between toxigenic production potential and the amount of genetic variability present among the isolates.

2. Materials and methods

Sample collection

Maize grain samples were collected from five maize producing areas in four agro-climatic zones of India: Dhaulakuan in Himachal Pradesh (zone I), Karnal in Haryana (zone II), Delhi (zone II), Begusarai in Bihar (zone III), and Hyderabad in Andhra Pradesh (zone IV) to evaluate the distribution of aflatoxin producing fungi. Maize kernels were collected randomly from individual cloth bags originating from various farmer's fields with a sample size of 2 kg/bag. 19 samples were collected from Dhaulakuan (30°30' N – 77°20' E) at an altitude of 468.0 m above sea level (m.a.s.l.) with an alluvial and grey brown pod-zolic soil and 840 mm rainfall. 14 samples were collected from Delhi (28°39' N – 77°13' E) at an altitude of 228 m.a.s.l., with a loamy to sandy loam soil type and an average annual rainfall of 714 mm. 16 samples originated from Karnal (29°41' N – 76°59' E) at an altitude of 257 m.a.s.l., a loamy soil type and an average rainfall of 573 mm. Begusarai (25°54' N – 83°36' E) at altitude of 51.8 m.a.s.l. has a *rabi* (winter) season as the main growing season of maize. It has a sandy loam soil type and an average rainfall of 1,053 mm. 10 samples were drawn from this location. Finally, 27 samples were taken from Hyderabad (17°23' N – 78°29' E) which has a topography suitable for growing maize year-round. It lies at an altitude of 530 m.a.s.l. and has a black clay loamy soil and an average rainfall of 783 mm. During sample collection, farmers were assayed for awareness regarding the importance of mycotoxin/aflatoxin contamination in maize.

Isolation and identification of fungi

From each sample, thirty kernels were taken at random and surface sterilised for 1 min in 2.5% NaOCl, washed in three changes of sterile distilled water and aseptically plated on Potato Dextrose Agar (PDA; HiMedia Laboratories, Mumbai, India). Three replications for each sample with 10 kernels per replication were placed on each culture plate and incubated at 28±2 °C for three days. Fungal colonies on maize kernels were counted and identified up to genus level using a stereo-binocular microscope (Olympus BH 2, Tokyo, Japan). *Fusarium* was identified using the key by Nelson *et al.* (1983) and *Aspergillus*, *Penicillium* and other fungi by Pitt and Hocking (1997). From each sample, only fungi belonging to the *Aspergillus* genus were transferred to new PDA plates, and further identified up to the species level using the taxonomic key and species descriptions of Singh *et al.* (1991). Single colonies from *A. flavus* cultures were transferred to PDA, incubated for a week (28±2 °C) and sub-cultured to obtain pure strains for testing of aflatoxin production. The *A. flavus* strains were enumerated AF-1 to AF-50. A further 100 kernels from each sample were taken to study the frequency and occurrence of fungal microflora.

Aflatoxin B_1 production

From grain samples

Estimation of AFB_1 was done one month after the date of sample collection, in order to follow uniformity. Maize kernels from each sample were grounded in a grinder, and 20 g of the maize powder was mixed thoroughly with 70% methanol containing 0.5% KCl in a blender for 30 min at 300 rpm. The mixture was filtrated through Whatman filter paper No. 41 (Maidstone, UK), and the obtained extract ('A') was used for aflatoxin determination.

From Aspergillus flavus *culture*

In total 50 isolates of *A. flavus* were selected randomly from maize grain samples from each location. Isolates were maintained on two 50 ml flasks of potato dextrose broth (HiMedia Laboratories) inoculated with 2 mm plugs of a six days old fungal culture and incubated at 27±2 °C. After 10 days, the culture broth was filtered through Whatman filter paper No. 41 and extracted with chloroform (25:25, v/v) by a separating funnel. The bottom aqueous layer of chloroform was passed through an anhydrous sodium sulphate column to dry the remaining chloroform layer, which contained the dissolved aflatoxins. The eluted solution/chloroform was evaporated at 60 °C in a vacuum evaporator, after which the residue was dissolved in 500 µl acetonitrile. Confirmation of AFB_1 and AFB_2 was done by thin layer chromatography (TLC), using a slightly modified AOAC technique (Kumar *et al.*, 2007). Aliquots of the acetonitrile extract (20 µl) were spotted in duplicate on a TLC silica gel plate (Merck 60

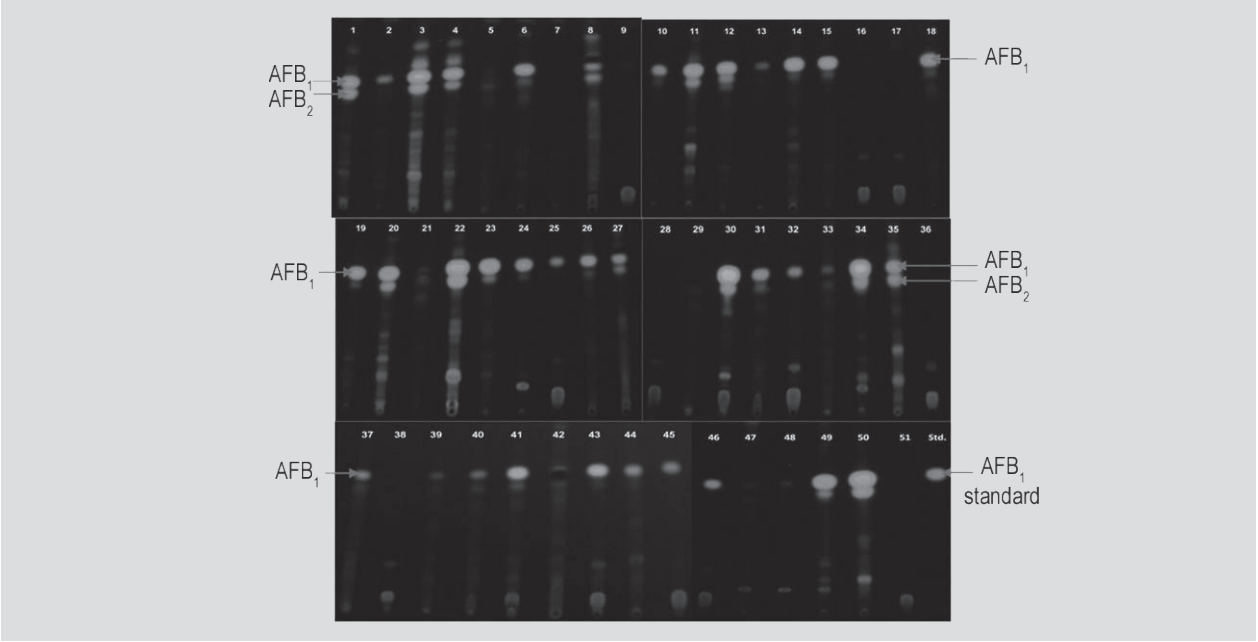

Figure 1. Aflatoxin B_1 produced by different *Aspergillus flavus* isolates on TLC plates.

F_{254}; Merck, Kenilworth, NJ, USA) that were pre-activated at 100 °C for 25-30 min. The TLC plates were developed in a chloroform acetone mixture (9:1, v/v) and analysed under UV light for fluorescence (Figure 1). Individual spots of AFB_1 were scraped of TLC plates and dissolved in 1 ml acetonitrile to quantify toxin production from separately cultures of *A. flavus* (extract 'B').

Quantitative analysis of aflatoxin B_1

The quantitative analysis of AFB_1 in extracts 'A' and 'B' was performed by indirect competitive ELISA using the method of Kumar *et al.* (2009). Microtitre plates (Nunc-Immuno™ MicroWell™ 96-well; Sigma-Aldrich, St. Louis, MO, USA) were coated with AFB_1-bovine serum albumin (BSA) in carbonate buffer (pH 9.6), left overnight at 4 °C, and washed thrice with phosphate buffered saline containing Tween (PBST). After addition of 0.2% BSA, microtitre plates were incubated for 1 h at 37 °C. Extract 'A' and antiserum diluted in PBST-BSA (1:6,000, v/v) were added to each well and incubated for 1 h at 37 °C. Goat-antirabbit immunoglobulin conjugate to alkaline phosphatase was added at a 1:4,000 dilution and incubated for 54-60 min at 37 °C, followed by thrice washing. Absorbance was recorded at 405 nm with an ELISA plate reader (Thermo Multiskan EX; Thermo Fisher Scientific, Waltham, MA, USA) after incubation. A similar method was followed for extract 'B'.

Standard AFB_1 was prepared in concentrations ranging from 0.9 to 1000 ng/ml. Standard curves were obtained by plotting log10 values of AFB_1 dilutions at 405 nm. Concentration of AFB_1 (ng/ml) in a sample was determined from the standard curves by putting aflatoxin concentration on the 'X' axis and optical density values on the 'Y' axis of the desired sample. In order to test the recovery of AFB_1, 20 g healthy maize grain extract was mixed with pure AFB_1 (Sigma-Aldrich) to provide a concentration range of 0.9, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0 and 1000.0 ng/ml. Quantification of unknown samples A and B was done. The limit of detection of AFB_1 using this method was 0.02 ng/ml.

Fungal DNA extraction

Plugs (2 mm) of seven days old culture of *A. flavus* were inoculated into 100 ml potato dextrose broth and incubated at 25 °C for 48 h without shaking (Lee *et al.,* 2004). Mycelial mats were filtered and air dried. The mycelial mat was grounded to a fine powder by addition of liquid nitrogen. Approx. 20 mg mycelial powder was mixed with 500 µl extraction buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 2% sodium dodecyl sulphate), and incubated at 65 °C for 60 min in a water bath. To this, 150 µl of potassium acetate (pH 4.8; 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water) was added, vortexed briefly and centrifuged at 10,000 rpm for 1 min. The supernatant was transferred to another tube and again centrifuged for 1 min at 10,000 rpm. The solution was mixed with an equal volume of isopropanol, followed by gentle mixing and incubated at -20 °C for 30 min. DNA was then pelleted by centrifugation at 10,000 rpm for 2 min, washed with 70% ethanol, pelleted again and dried at room temperature. Finally, the DNA pellet was dissolved in 50 µl of 1× TE buffer and stored at -20 °C until use.

Random amplified polymorphic DNA analysis

To identify the best primers for the random amplified polymorphic DNA (RAPD) profiling, 35 OPERON random primers (Invitrogen, Carlsbad, CA, USA) were tested. Ten primers – OPA8, OPA11, OPA15, OPA19, OPB4, OPB5, OPB8, OPB11, OPS 12 and OPB 2 – gave good reproducibility and were selected. PCR amplifications were performed in microvial containing a 25 µl reaction mixture of $MgCl_2$-free reaction buffer, 3 mM $MgCl_2$, 2.5 U of Taq polymerase, 200 µM of each dNTP, and 0.25 µM of each primer and 10 ng template. Amplification consisted of an initial denaturation step at 94 °C for 5 min followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min and amplification at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplification products of RAPD were separated on a 1.5% agarose gel by electrophoresis and photographed under UV light. The RAPD data were analysed using NTSYS-PC computer package 18 (Exeter Software, Setauket, NY, USA). The Jaccard's coefficients between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA).

Statistical analysis

Data on fungal incidence of microflora and aflatoxin level were analysed using SAS (version 9.1; SAS Institute, Cary, NC, USA). Prior to analysis, AFB_1 concentration were transformed to log (x+1) to normalise residuals. The means were separated using Fisher's protected least significant difference test to determine whether there were significant differences between the samples obtained from the different locations under the agro-ecological zones.

3. Results

Distribution of fungi/moulds on grain samples collected from different agro ecological zones

During the collection of samples, the awareness among farmers regarding importance of aflatoxin contamination in maize was also assessed. It was found that in Dhaulakuan (zone I) and Begusarai (zone III), respectively 90 and 92% of the farmers rated mycotoxins as an important problem. However, 75% of the farmers in Hyderabad did not consider it a problem, while 25% were unaware of it. In Karnal (zone II), 80% farmers were not aware of mycotoxin/aflatoxin problem.

The assessment of the microflora present on maize grains revealed the presence of five different fungal genera: *Aspergillus*, *Fusarium*, *Penicillium, Trichoderma* and *Rhizopus,* in varying frequencies (Figure 2). *Aspergillus* was the most predominant fungal genus identified and present in all samples across the agro ecological zones. Within locations, the occurrence of *Aspergillus* species was highest in Karnal (98.2% of samples; zone II) and lowest in Dhaulakuan (89%; zone I). Maximum load of *Fusarium* was observed in Karnal (97.2%), of *Penicillium* in Begusarai (zone III) (90%), of *Trichoderma* in Hyderabad (Andhra Pradesh, zone IV) (55.5%) and of *Rhizopus* in Dhaulakuan with 28.5% abundance. Among the *Aspergillus* species, *A. flavus* and *Aspergillus niger* were present in samples of all four agro ecological zones (Figure 3). The frequency of *A. flavus* was more than 95% in all sampled locations, whereas occurrence of *A. niger* varied from 100% in Begusarai to 41.1% in Karnal.

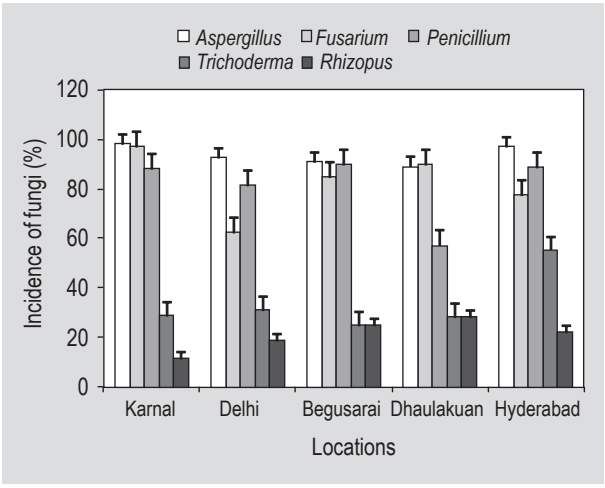

Figure 2. Occurrence of moulds/fungal species isolated from maize kernels collected from different maize growing regions of India. Zone I to IV indicate the different agro-ecological zones. Values shown are based on a sample of 100 kernels. For each vertical bar, vertical lines represent the standard errors of the mean.

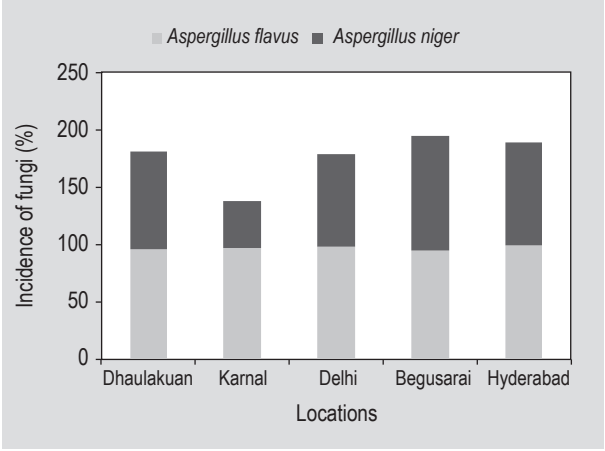

Figure 3. Incidence of species within the *Aspergillus* genus isolated from maize kernels from different maize growing regions of India. Zone I to IV indicate the different agro-ecological zones.

Aflatoxin contamination in maize samples collected from farmer's field

The level of aflatoxin contamination in maize grains, collected from farmer's fields varied widely between the locations (Table 1). The highest concentration of AFB_1 (91.4 µg/kg) was detected in a sample from Karnal, whereas lowest (0.14 µg/kg) was from Begusarai. Average aflatoxin contamination was highest in Karnal with 56.2% of total samples having a concentration above 20 µg/kg AFB_1 – the maximum concentration permitted by the FDA. Mean aflatoxin content was almost the same for Dhaulakuan and Delhi locations, but at Dhaulakuan one grain sample contained 55.7 µg/kg, while 90% of samples exhibited AFB_1 concentrations <20 µg/kg. In Hyderabad, 48.1% samples exhibited aflatoxin concentrations higher than the permitted by the FDA guidelines. In Begusarai, 90% of the maize samples had a concentration <20 µg/kg AFB_1.

In vitro aflatoxin B_1 production by *Aspergillus flavus* isolates

Estimation of AFB_1 production by *A. flavus* isolates was performed *in vitro*. Highest toxigenic isolate, producing 8,116.61 µg/kg AFB_1, originated from Hyderabad, whereas the least toxigenic isolate (0.21 µg/kg) originated from Begusarai. The highest minimum AFB_1 production level was found in an isolate from Delhi. A high level of variability in aflatoxin production among isolates was found in agro-ecological zone II (0.42-4,387.2 µg/kg for Karnal and 1.03-3,072.50 µg/kg for Delhi), zone III (0.21-6,878.59 µg/kg) and zone IV (0.53-8,116.61 µg/kg), while isolates from Dhaulakuan in zone I exhibited a minimum variability (0.27-0.91 µg/kg) in aflatoxin production (Table 2).

Molecular characterisation

50 toxigenic and non-toxigenic isolates of *A. flavus* were subjected to RAPD analysis. Initially, 35 OPERON random primers were tested for their polymorphism, reproducibility and capacity to differentiate the isolates. Out of 35, only 10 primers gave distinct amplification products (Figure 4). These 10 primers generated a total of 83 reproducible RAPD bands, averaging to 7.5 bands per primer. Dendrogram analysis grouped the isolates into seven clusters with a genetic similarity of 52% based on Jaccard similarity coefficients (Figure 5). The first cluster included the isolates AF-1, AF-2, AF-38 and AF-18 with a genetic similarity of 52%. Cluster 2 comprised of 11 strains, with a maximum similarity of 68% which could be further subdivided into three sub-clusters. Sub-cluster 2.1 (3 strains) included the highly toxic strains AF-03 and AF-12. Sub-cluster 2.2 comprised of highly toxic strains AF-22, AF-23 and AF-24. Moderately to low toxic isolates with less than 1 µg/kg aflatoxin production, i.e. strains AF-39, AF-40, AF-42, AF-45 and AF-48, were found in sub-cluster 2.3. Cluster 3 contained 17 strains with a genetic similarity of 68%, with further division into three sub-clusters. Cluster 4 contained 6 strains varying from least toxic (AF-16 and AF-17) to highly toxic (AF-19 and AF-20), while cluster 5 included 6 low to moderately toxic strains. Cluster 6 comprised of 2 strains from Delhi, AF-14 and AF-21, indicating that the primers were somewhat able to differentiate between the different locations/regions. Cluster 7 comprised of only one highly toxic strain from Karnal (AF-15). With reference to this data, there was partial relationship between clustering in the RAPD dendrogram and geographic origin of the isolates tested.

Table 2. *In vitro* toxigenic potential of *Aspergillus flavus* strains isolated from different locations in India.

Location	No. of isolates	Range of aflatoxin production (µg/kg)
Dhaulakuan (zone I)	2	0.27-0.91
Karnal (zone II)	13	0.42-4,387.2
Delhi (zone II)	13	1.03-3,072.50
Begusarai (zone III)	20	0.21-6,878.59
Hyderabad (zone IV)	2	0.53-8,116.61
Total	50	

Table 1. Aflatoxin B_1 content in maize grain samples collected from farmer's fields.

Location	Sample size (n)	Mean ± SD	Range (µg/kg)	Percent of sample with >20 µg/kg aflatoxin
Dhaulakuan (zone I)	19	7.5±17.0	0.6-55.7	10.5
Karnal (zone II)	16	30.3±26.2	1.4-91.4	56.2
Delhi (zone II)	14	6.5±12.9	0.3-50.4	7.10
Begusarai (zone III)	10	13.1±24.3	0.14-81.6	10.0
Hyderabad (zone IV)	27	16.7±18.0	0.19-67.4	48.1

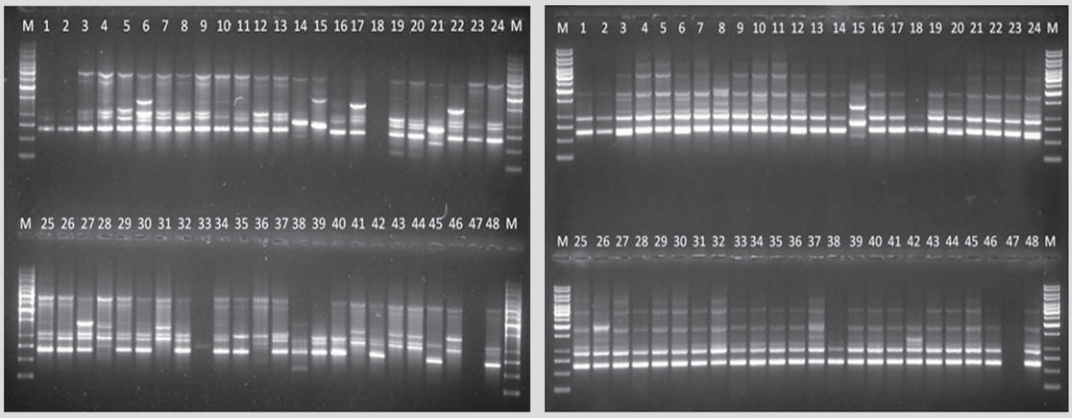

Figure 4. RAPD profiles of some *Aspergillus flavus* isolates generated by random primer (A) OPS-12 and (B) OPP-2. M = 1 Kb marker, lane 1-48: isolate AF-1 to AF-48; lane 47 = water control.

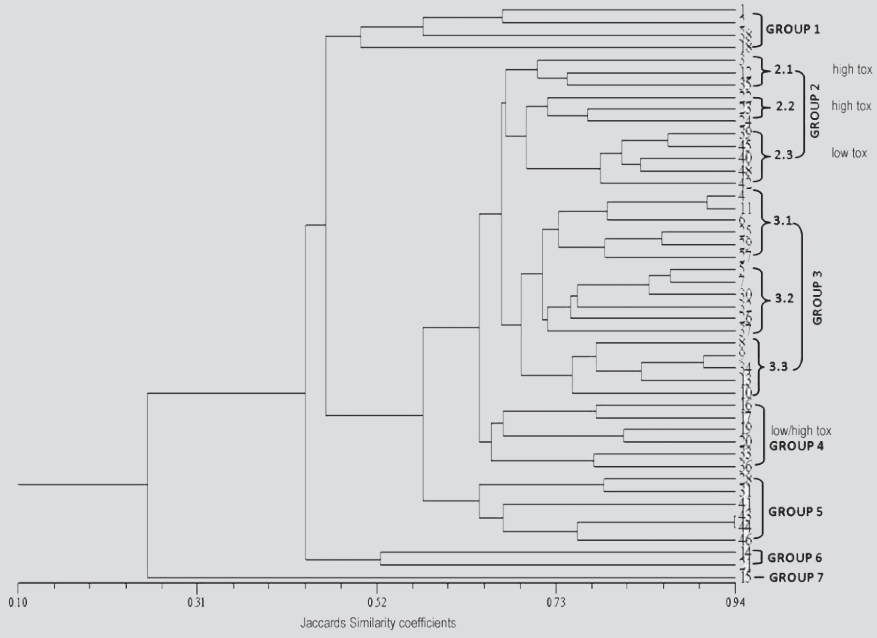

Figure 5. Dendrogram of 50 *Aspergillus flavus* isolates constructed by UPGMA based on a similarity matrix produced with simple matching coefficients.

4. Discussion

Maize is a high risk agricultural commodity for aflatoxin contamination, particularly in the USA and South East Asian countries (Jelinek *et al.,* 1989). In present study, 35.48% of the samples collected from farmer's fields were contaminated with >20 µg/kg AFB_1, the tolerance limit for humans according to the FDA guidelines. Aflatoxin contamination is prevalent in both warm humid climates and irrigated hot deserts. The variable concentration of aflatoxin observed in different agro-ecological zones may be due to the prevailing climatic conditions in that area. *A. flavus* is the most widely isolated *Aspergillus* species across the maize zones of India. The highest load of AFB_1 was recorded on maize grain from Karnal (91.4 µg/kg). At this location the occurrence of other fungi, i.e. *Trichoderma* sp. and *A. niger*, was low, which may have influenced the high amount of aflatoxin found. *Trichoderma* spp. and *A. niger* have been considered as biocontrol agents by some workers (Dhavan and Choudhary, 1995; Thimmappa *et al.,* 2014; Vinale *et al.*, 2014). The low frequency of fungi other than *A. flavus* at Karnal may not have be sufficient to suppress the growth of toxigenic *A. flavus,* and subsequent aflatoxin build-up.

In Karnal and Delhi (zone II), with an average annual rainfall 714 mm, maize is a main summer (*kharif*) crop. At the time of maturity of the crop, high humidity combined with high temperature prevails, which is a predisposing factor for fungal growth and aflatoxin contamination.

Begusarai in Bihar and Hyderabad in Andhra Pradesh are the main hub for maize seed production. In Begusarai, the maximum concentration of aflatoxin in grains was 81.6 µg/kg, and only 10% of the total samples exhibited values >20 µg/kg AFB_1. *Rabi* (winter) crop for maize cultivation is common in Bihar. The occurrence of *A. flavus* was found to be 100%, which is similar to Karnal and Delhi, however the frequency of *A. niger* was much higher compared to Karnal and Delhi. Moreover, the prevailing environmental temperature is low during cropping season (winter), which is unfavourable for fungal growth. Both factors may explain why aflatoxin contamination was low. Our findings correlate with that of Shearer *et al.* (1992), who indicated that *A. flavus* competes poorly under cool conditions (20-25 °C) and that the prevalence of *A. flavus* is higher in warmer environments (above 25 °C). Another study by Sinha (1990) also confirmed that *kharif* crop (summer) had a greater incidence of aflatoxin than winter crop.

The climatic conditions at Hyderabad are most favourable for maize and maize is grown throughout the year. Hence, the fungal load increases easily such an environment, even in presence of *A. niger,* leading to increased aflatoxin production. This is reflected in present study by 48.1% of the samples exhibiting values >20 µg/kg AFB_1. Therefore, special care must be taken at the time of harvesting with respect to grain moisture as well as mechanical damage.

Dhaulakuan is a temperate region in zone I, where low temperature prevails during summer (main) cropping season. Only 10.5% of the grain samples showed AFB_1 values above 20 µg/kg; one sample exhibited 55.7 µg/kg AFB_1, the highest from this area, which could be linked to a farmers handling error/mechanical damage at the time of harvest.

A total of 50 strains of *A. flavus* were isolated from maize samples of these locations on PDA and tested in *in vitro* for their aflatoxin producing potential. A high level of toxigenic variability was exhibited by the isolates, ranged from 0.214 to 8,116.61 µg/kg AFB_1. Our result corroborate with the study by Hussein and Brasel (2001) which showed variability in aflatoxin production by *A. flavus* under similar conditions. Also recent work done in rice by Xianwen *et al.* (2014) showed a high level of variability in AFB_1 production potential, ranging from 175 to 24,101 µg/kg.

To determine the relationship between toxigenic and genetic variability, we characterised the isolates molecularly. Cluster analysis showed that both high and low toxic strains fell in the same group, revealing that no relationship exists between genetic variability of *A. flavus* isolates based on RAPD and their toxic production ability. Environmental conditions and handling error are the predominant factors for aflatoxin production. Fluctuations in climate also influence predisposition of hosts to contamination by altering crop development and by affecting insects that create wounds on which aflatoxin-producers proliferate (Cotty *et al.,* 2008).

Biological control of aflatoxin contamination with atoxigenic isolates of *A. flavus* has been practiced for over a decade in commercial agriculture in several regions of the USA (Cotty, 2006; Cotty *et al.,* 2008; Dorner, 2004; Probst *et al.*, 2012). Atoxigenic isolate-based biological control competitively exclude aflatoxin producing isolates from the environment thereby reducing the aflatoxin-producing potential of the resident fungal community. In order to achieve such benefits, atoxigenic isolates must be adapted to both target crops rotations and the target environments. According to Abbas *et al.* (2004), isolates that produce less than 5 ng/g of aflatoxin by ELISA are identified as 'atoxigenic' isolates.

The current study provides the first attempt to identify locally adapted endemic atoxigenic isolates, that could have potential value in aflatoxin mitigation in various agro-ecological zones of India where maize is an important crop. One of the major outcomes of the current study is the identification of three atoxigenic isolates of *A. flavus,* i.e. AF-9, AF-36 and AF-39 (from Bihar, Haryana and Delhi, respectively), that produce less than 0.28 µg/kg aflatoxin – far below the FDA regulatory limits. These isolates need to be further evaluated as potential biocontrol agents for aflatoxin control of maize in India.

5. Conclusions

Aflatoxin contamination in both food and feed, leads to significant nutritional and economic losses, posing a major obstacle to agriculture producer, consumer and stakeholder. It prevents them from meeting international, national and standard governing agriculture trade and food safety policy. It can be mitigated mainly by making farmers and stakeholder aware about aflatoxin contamination and post-harvest handling practices. The present study was done in India in major maize growing areas, which is one of the few works done till now. During the collection of maize grain samples, some interesting observations were noticed: nearly 90% of farmers showed their awareness for mycotoxin contamination in Himachal Pradesh (zone I) and Bihar (zone III), whereas in Haryana nearly 80% of farmers were unaware. This wide gap needs to be filled by conducting awareness campaigns among producers and stakeholders. The study showed a varied distribution across the zones in India of fungi that are responsible for aflatoxin contamination. It concludes that occurrence of fungi/moulds is governed by prevailing environmental factors and post-harvest operations like harvesting of cobs, drying, sorting, processing and

storage where farmers can play a major role. Also, genetic variability among the isolates of *A. flavus* can be used for designing primers for its early detection. The identified atoxigenic isolates (AF-9, AF-36, and AF-39) can act as a potential tool for the biocontrol of aflatoxin contamination of maize in India which has to be further validated.

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