Original Paper Open Access

Qualitative, quantitative and molecular detection of aflatoxins from maize grains in north-west India

Harleen Kaur[1]*, Harpreet Singh[2], Mandeep Singh Hunjan[2], Gaganjyot Kaur[3]

[1]Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana-141 004, India
[2]Department of Plant Pathology, Punjab Agricultural University, Ludhiana-141 004, India
[3]Department of Entomology, Punjab Agricultural University, Ludhiana-141 004, India
*Corresponding author: E-mail: harleenkaur@pau.edu

Abstract

Thirty three isolates of *Aspergillus flavus* collected from different maize growing areas from Indian Punjab were screened for aflatoxin production using qualitative and quantitative methods. Qualitatively, bioassay on okra revealed 11 isolates to be positive for aflatoxin production with chlorosis index ranging from 0.14 to 0.33. The isolate Af 4 produced highest chlorosis index (1.33) thereby causing maximum reduction in germination of okra seedlings (20%). Concurrently, ammonia vapour test however, revealed 6 isolates positive for aflatoxin production, with one isolate Af 29 being highly toxigenic showing dark red colour development. In total, twelve isolates of *A. flavus* were observed to be positive using qualitative detection methods. These were further subjected to quantitative (HPLC) and molecular analysis. Out of these 12, eight isolates produced aflatoxin B_2 (AFB_2) in the range of 89 - 3269.2 µg kg^{-1}, with highest concentration of AFB_2 detected in Af 5 sample of Ludhiana district. These isolates were confirmed positive using aflatoxin gene specific primers OmtB, Omt1 and Nor1. Sequence analysis of the *Nor1* gene amplified from six isolates with different levels of toxin production showed high homology among themselves. The amino acid sequence analysis showed that protein sequences were highly conserved with 99% similarity among them and with other strains in database.

Keywords: aflatoxin, *Aspergillus flavus*, HPLC, isolates, maize, *Nor1* gene

Introduction

Maize (*Zea mays* L), an important cereal is used as source of food, feed ingredients, forage for livestock and for other industrial maize by-products. Maize grain is a good substrate for mould infection particularly in those areas where rainfall is above normal from silking to harvest. In Punjab, the north-western part of India, maize is traditionally grown as summer (rainy) season crop (Kaur et al, 2014). Hot and humid conditions prevailing during this season favour the ear rot development. A large number of fungi are associated with grain moldiness, but the most common are *Aspergillus flavus*, *A. parasiticus*, *Fusarium graminearum*, *F. verticillioides*, *Penicillium spp.*, and *Diplodia maydis* (Gonzaslez et al, 2003; Kpodo et al, 2000). These fungi may produce different types of mycotoxins if storage conditions are poorly managed, and subsequent consumption of mycotoxin laden grains may lead to severe health hazards.

Among these mycotoxigenic fungi, aflatoxin-producing fungi have the highest frequency of occurrence and aflatoxins have been known to be the most common mycotoxins produced by these fungi (Kumar et al, 2014). More than 20 different types of aflatoxins are identified, but the major and most toxic forms are B_1 (AFB_1), B_2 (AFB_2), G_1, and G_2 (Pittet, 1998). AFB_1 is the most potent mutagenic and carcinogenic metabolite known and ranked as class I human carcinogen (Ahsan et al, 2010). Maximum levels of total aflatoxins in maize have been set at 10 µg kg^{-1}, whereas maximum level of aflatoxin B_1 has been set at 5 µg kg^{-1} as given in Regulation (EC) No 1881/2006 amended by commission regulation (EU) No 165/2010 (CAST, 2003). When seasons were compared, the incidence of aflatoxins was found more in samples of the Kharif (rainy season) crop (47%) than samples of the Rabi crop (17%) (Sinha, 1990).

The diseases caused by aflatoxin consumption in man and animals are usually called aflatoxicosis. Aflatoxicosis cause acute liver damage, liver cirrhosis, induction of tumors, impaired central nervous system, skin disorder and hormonal defects (Peraica et al, 1999; Amaike and Keller, 2011). The first report of aflatoxin epidemic in India was from Banswara (Rajasthan) and Panchmahals (Gujarat) in 1975 among tribal group of Bhils who had consumed maize grains heavily contaminated with *A. flavus* (Reddy and Raghavender, 2007). Further, during 1986, an outbreak of aflatoxicosis due to AFB_1 occurred in an experimental pig farm in Meghalaya (Ghosh et al, 1988) and in ducklings in Tripura, India (Roy et al, 1989). Comprehensive surveys on aflatoxin contaminating maize in developing countries of Asia and Africa are rare, although the losses in these countries are considered to be more severe than in developed countries (Yu et al, 2005). The average economic loss due to mycotoxin contamination is estimated at approximately one billion dollars in the United States,

 RECEIVED 01/27/2017

(Amaike and Keller, 2011) and the management and monitoring costs for mycotoxins are estimated at $0.5 million to > $1.5 billion for aflatoxin in maize and peanuts, fumonisin in maize, and deoxynivalenol in wheat (CAST, 2003).

Different methods including qualitative (Abbas et al, 2004), quantitative (Nilufer and Boyacioglu, 2002) and molecular (Scherm et al, 2005) have been regularly used for the detection of aflatoxin contamination in grain samples. Since maize is the third most important cereal crop of India, there is a serious concern about the quality and hence the safe consumption of maize grains. The present studies were conducted to investigate the presence and magnitude of aflatoxins in maize produced in north-western India using both qualitative and quantitative methods.

Materials and Methods

Fungal isolates, their isolation and maintenance

Thirty three isolates of *A. flavus* used in this study were obtained from infected ear rot samples collected from five different maize growing districts of Punjab *viz*. Ludhiana, Jalandhar, Hoshiarpur, Gurdaspur and Kapurthala during Kharif 2013 and spring 2014 (Supplementary Figure 1). These isolates belonging to two different agro-ecological zones *viz*. sub-mountaneous undulating plain zone and central plain zone of Punjab have been given in Supplementary Table 1. All the isolates were identified, purified and maintained on PDA slants below 4°C for further screening of aflatoxin production.

Qualitative Methods

Bioassay on okra

The bioassay on okra was carried out according to the method described by Kang (1970). The assay was based on chlorophyll inhibition in cotyledonary leaves of okra seedlings. Individual isolates were grown on potato dextrose broth for 10 days at 25 ± 1°C. The culture was syringe-filtrated to eliminate the fungal hyphae and spores. Seeds of okra variety Punjab-8 were surface sterilized with 2% sodium hypochlorite for 2 min and rinsed thoroughly in sterile water. The seeds were further soaked in culture filtrate of the test isolates for atleast 12 h before sowing, air dried and sown in sterilized soil in 10" diameter earthen pots. Okra seeds soaked in sterilized PDB medium served as control. Five seeds were grown in each pot and all the treatments were replicated thrice. Observations on chlorosis of leaves were recorded after 7 days of sowing. Chlorosis index of the seedlings was determined following 0-3 scale as given by Singh (1985).

Ammonia vapour test

The ammonia vapour test was carried out according to the method described by Saito and Machida (1999). Different isolates of *Aspergillus spp*. were grown individually in 90 mm Petri dishes at 25 ± 10°C for seven days. After incubation, the Petri dishes were inverted and 2 ml of concentrated ammonium hydroxide solution was poured onto the inside of the lid. Varying degree of color changes in the mycelium and medium after exposure to ammonia vapour was observed on the underside of the Petri dish and categorized as highly, moderately and mildly toxigenic on the basis of intensity.

Quantitative methods

The healthy maize grains of cv. PMH-1 were artificially inoculated with the isolates of *A. flavus* which were found positive with qualitative detection methods and aflatoxins from these grain samples were quantified through HPLC. Analysis of these samples was carried out in Pesticide Residue Lab, Department of Entomology, Punjab Agricultural University Ludhiana, Punjab.

Preparation of standards

The analytical standards of AFB_1 and AFB_2 were obtained from HiMedia™ laboratories (Mumbai, India). The standard solutions of AFB_1 and AFB_2 were prepared by dissolving the solid commercial toxin in HPLC grade acetonitrile to a concentration of 1 mg ml^{-1}. The standards required for constructing a calibration curve (0.1, 0.2, 0.3, 0.4, 0.5 µg ml^{-1}) were prepared from stock solution by serial dilutions with acetonitrile. Standard solutions were stored at -20ºC and warmed to room temperature before use.

Apparatus

The high performance liquid chromatograph apparatus (Model DGU-2045, Shimadzu Corporation, Japan) equipped with reverse phase (RP) C18 column, photo diode array (PDA) detector and dual pump was used. The analytical column was a Luna 5 µm C18 column (250 × 4.6 mm size, 5.20±0.30 µm particle size, 2.20 ± 0.30 (90% / 10%) particle distribution, 95 ± 15 Å pore diameter, 430 ± 40 m^2 g^{-1} surface area, < 55.0 ppm metal content, 19.00 ± 0.70% total carbon and 3.25 ± 0.50 µmoles m^{-2} surface coverage) obtained from Spincotech Pvt Ltd, Chennai, India. The mobile phase consisted of mixture of acetonitrile:water (60:40, v/v) at the flow rate of 0.30 ml min^{-1}. The pressure of pump was set at 1200 - 1300 psi and wavelength of detector used was 254 nm. The aflatoxins were quantified by using the formula given below:

$$Residue\ level\left(mg\ kg^{-1}\right) = \frac{ng\ of\ the\ standard\ injected}{peak\ height\ /\ area\ of\ the\ standard} x \frac{Peak\ height\ /\ area\ of\ the\ sample}{\mu l\ of\ sample\ injected} x \frac{Final\ volume\ of\ sample\ (ml)}{Weight\ of\ the\ sample\ (g)}$$

Aflatoxin extraction from artificially inoculated maize grains

The analysis method used for aflatoxins in maize grain samples was made following the procedure of Fu et al (2008). The analytic method was assessed for recovery before the sample analysis. Samples of healthy maize grains were fortified with AFB_1 and

AFB_2 at level of 30 µg kg^{-1}, 20 µg kg^{-1}, and 10 µg kg^{-1}, respectively.

Molecular methods

DNA extraction

Twelve isolates (Af 1, Af 2, Af 4, Af 5, Af 9, Af 11, Af 14, Af 26, Af 29, Af 30, Af 31, and Af 33) found positive in either or both qualitative and quantitative analyses were selected for molecular studies. Genomic DNA from these twelve aflatoxin positive isolates along with five aflatoxin negative isolates (Af 20, Af 21, Af 22, Af 23, and Af 28) was isolated using Easy DNA kit (Invitrogen Inc) as per manufacturer's protocol. Quantity and quality of DNA was checked on TECAN 200 nanoquant plate reader. The DNA of all the samples was diluted to 25 ng $µl^{-1}$ and stored at -20°C for further use.

PCR amplification

Three primer sets specific for aflatoxin biosynthesis viz. Nor1, OmtB and Omt1 (Scherm et al, 2005) were used for confirmation of toxigenic *A. flavus* isolates. The housekeeping gene tub1 (β-tubulin) based primer Tub1 was also used. The list of primers along with their sequence is depicted in Supplementary Table 2. *In vitro* amplification using polymerase chain reaction (PCR) was performed in a 96 well PCR plate (Thermo Fischer Inc) in Eppendorf Master Cycler ProS. PCR amplification was carried out in a final reaction volume of 30 µl, containing 2.0 mM $MgCl_2$, 2 units Taq DNA polymerase, 0.2 mM dNTPs, 1x PCR buffer, 1.5 µl of each primer and 50 ng of genomic DNA. PCR amplification cycles were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s, extension at 72°C for 90 s and final extension at 72°C for 7 minutes. The amplified products were electrophoresed in 1% agarose gel in 0.5 X TAE buffer, stained with ethidium bromide at a concentration of 5.0 µl 100 ml^{-1} of buffer. The gels were visualized under UV light and photographed using SYNGENE gel documentation system with GeneSnap software programme.

Sequencing and data analysis

Based on the differential toxigenic reaction of Aspergillus isolates in different methods, six isolates (Af 9, Af 30, Af 5, Af 29, Af 28, and Af 20) were selected for sequencing. The typical 990 bp band representing *aflD* gene was eluted from the agarose gel using Wizard® SV gel and PCR clean-up system (Promega Inc). Double pass sequencing was outsourced from Avantor Performance Material India Ltd. After trimming the primer sequences, the contigs were generated using the sequence analysis software BioEdit sequence alignment editor 2.0. The final sequences thus obtained were compared with similar sequences available in GenBank using BLASTn. The translated nucleotides were also compared with protein database available in GeneBank using BLASTp. The sequence analysis was conducted using MEGA version 4 and phylogenetic trees were prepared by the neighbour-joining method. Bootstrap values were calculated from 1,000 replications of the bootstrap procedure using programs within MEGA4 package which refers to tests of the reliability of an inferred tree.

Results

Confirmation of* A. flavus *isolates

Primer combinationTub1, based on housekeeping gene β-tubulin was used for the confirmation of *A. flavus* isolates. All the isolates yielded an amplicon of 1,498 bp (Figure 1) with Tub1 primer irrespective of their toxicity confirming their identity to be *A. flavus*. Toxigenic isolates were further identified using qualitative, quantitative and molecular detection methods.

Qualitative methods

Bioassay on okra

Out of 33 isolates, 11 isolates of *A. flavus* were observed to be toxigenic based on bioassay on okra (Table 1). Two isolates *viz*. Af 4 and Af 11 obtained from the Ludhiana district central were highly toxigenic having mean chlorosis index of more than 1.0. The isolate Af 4 was the most potent of all producing the highest chlorosis index of 1.33 and caused maximum reduction in germination percentage of okra seeds (up to 80%). Three isolates (Af 5, Af 14, and Af 30) were moderately toxigenic with chlorosis index ranging from 0.6-1.0 and the rest of the six isolates (Af 1, Af 2, Af 9, Af 26, Af 29, and Af 33) were mildly toxigenic having 0.1-0.5 chlorosis index. The remaining isolates having chlorosis index of <0.1 were designated as non-toxigenic.

Ammonia vapour test

Based on ammonia vapour test, only 5 isolates viz. Af 2, Af 4, Af 5, Af 9 and Af 31 were found moderately toxigenic with development of light red color, whereas one isolate Af 29 was found highly toxigenic with dark red color development (Table 1; Supplementary Figure 2). Remaining 33 isolates were shown non-toxigenic in this method with no color change. All moderately toxigenic isolates were from Ludhiana, while one highly toxigenic isolate (Af 29) was from Kapurthala district.

Data of qualitative methods revealed that frequency of toxigenic isolates of *A. flavus* were more in Ludhiana and Kapurthala districts of Punjab. Okra

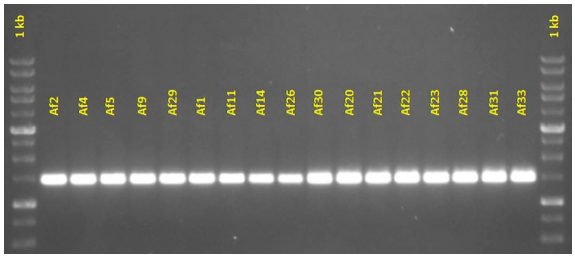

Figure 1 - A characteristic band of ~1,500 bp long amplified in all the isolates, using primers Tub1, confirmed them to be *Aspergillus flavus*. Lane 1 and Lane 19 contains 1kb DNA ladder.

Table 1 - Qualitative and quantitative analysis of aflatoxins from maize grains.

Isolate/s	Qualitative method				Quantitative method	
	Bioassay on okra		Ammonia vapour test		AFB_1 ($\mu g\ kg^{-1}$)	AFB_2 ($\mu g\ kg^{-1}$)
	AF production	*Mean chlorosis index	AF production	Color		
Af 1	+	0.17	-	-	BDL**	BDL
Af 2	+	0.14	+	Light red	BDL	BDL
Af 4	+	1.33	+	Light red	BDL	BDL
Af 5	+	0.71	+	Light red	BDL	3,269.2
Af 9	+	0.22	+	Light red	BDL	BDL
Af11	+	1.29	-	-	BDL	189
Af 14	+	0.63	-	-	BDL	124
Af 26	+	0.22	-	-	BDL	2,180
Af 29	+	0.50	+	Dark red	BDL	188
Af 30	+	0.63	-	-	BDL	BDL
Af 31	-	-	+	Light red	BDL	89
Af 33	+	0.17	-	-	BDL	1,60

*mean of three replications, **BDL (Below detection limit) = 10.0 $\mu g\ kg^{-1}$

test revealed that maximum isolates producing aflatoxins were from Ludhiana (54.5%) district followed by Kapurthala (75%) district. However ammonia vapour test showed aflatoxin producing isolates were from Kapurthala and Ludhiana districts having frequency of 50% and 36.6%, respectively.

Quantitative method

Detection of aflatoxins from artificially contaminated maize grains

The twelve isolates found positive by either or both the qualitative methods were further analyzed by HPLC for quantification of aflatoxins produced by them on artificially contaminated maize grains. Results obtained in the maize grain samples were expressed as such and not corrected for recovery as the analytical method gave consistently good recoveries at each level of fortification with AFB_1 and AFB_2 both (Supplementary Table 3).

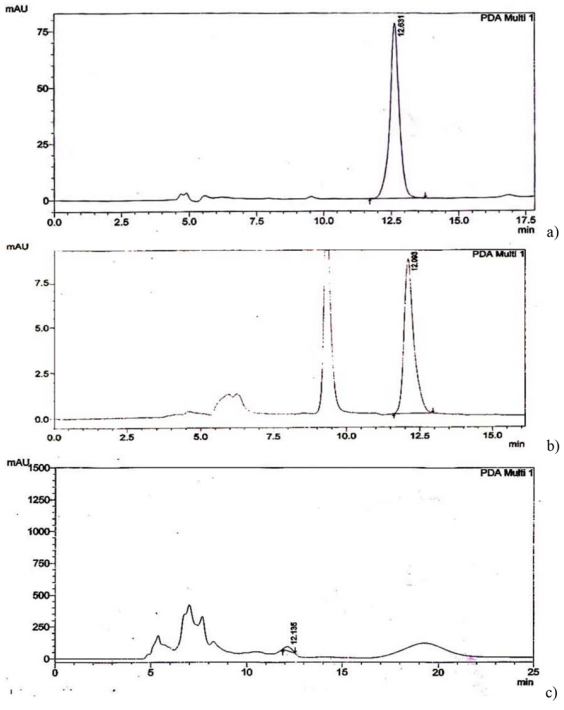

Figure 2 - HPLC chromatogram of **a**) standard AFB_1 ; **b**) standard AFB_2; **c**) sample producing maximum amount of aflatoxin (Isolate Af 5).

Out of twelve maize samples artificially contaminated with different isolates of *A. flavus*, AFB_2 could be detected in 58 per cent (Table 1) of the samples. Chromatographs obtained after injection of standard solution of AFB_1 and AFB_2 and sample producing highest amount of AFB_2 are given in Figure 2. The levels for AFB_2 ranged from 89 to 3,269.2 µg kg^{-1}. The highest concentration of AFB_2 was detected in the isolate Af 5 from Ludhiana district (Af 5) having AFB_2 level of 3,269.2 µg kg^{-1}, followed by Af 26 that produced 2,180 µg kg^{-1} AFB_2. AFB_2 was not detected in five samples *viz*. Af 1, Af 2, Af 4, Af 9, and Af 30 by HPLC. However, these samples were found positive by either of the other two qualitative methods of detection. AFB_1 was not detected in any of the tested samples by HPLC (Table 1).

Molecular methods

Confirmation of toxigenic isolates of A. flavus

The confirmation of toxigenic isolates of *A. flavus* was done with aflatoxin gene specific primers Nor 1, Omt B, and Omt 1 based on the genes responsible for aflatoxin biosynthesis. The amplification pattern of these genes from toxigenic and non-toxigenic isolates has been presented in Table 2. All the three primers *viz*. Nor 1, Omt B, and Omt 1 based on aflatoxin biosynthesis genes *aflD*, *aflO*, and *aflP* respectively, were amplified in all the twelve toxigenic isolates (Af 1, Af 2, Af 4, Af 5, Af 9, Af 11, Af 14, Af 26, Af 29, Af 30, Af 31, and Af 33). Primer OmtB (Figure 3a) and Omt1 (Figure 3b) gave amplicons of 1,333 bp and 1,490 bp respectively. The amplification of *aflD* gene with Nor1 primer yielded amplicon of 990 bp in all the toxigenic isolates and in two non-toxigenic isolates (Af 28 and Af 20, Figure 3c).

However, the pattern of amplification of these genes in non-toxigenic isolates (Af 20, Af 21, Af 22, Af 23, and Af 28) of *A. flavus* varied. It was observed that in non-toxigenic isolate, Af 20, all the genes were

Table 2 - Amplification of different aflatoxin genes involved in aflatoxin biosynthesis pathway.

Isolate	Gene locus amplified by PCR		
	aflO	aflP	aflD
Af 1	+	+	+
Af 2	+	+	+
Af 4	+	+	+
Af 5	+	+	+
Af 9	+	+	+
Af 11	+	+	+
Af 14	+	+	+
Af 20	+	-	+
Af 21	+	-	-
Af 22	-	-	-
Af 23	-	-	-
Af 26	+	+	+
Af 28	+	+	+
Af 29	+	+	+
Af 30	+	+	+
Af 31	+	+	+
Af 33	+	+	+
Af 26	+	-	-

«+» denotes amplification of desired product length, «-» denotes no amplification

amplified. However in Af 21 and Af 28, either one or two genes out of three were amplified, respectively. In Af 22 and Af 23, none of the three genes *viz*. *aflO*, *aflP,* and *aflD* were amplified (Table 2, Figure 3).

Sequencing data

Sequencing was done from the amplified *aflD* gene (using Nor1 primer) from six isolates (Af 9, Af 30, Af 5, Af 29, Af 20, and Af 28). These six isolates were selected on the basis of their toxigenic reaction identified by different detection methods. Out of these six, Af 9 and Af 30 were found positive by qualitative method, Af 5 and Af 29 were positive by both qualitative and quantitative methods and Af 28 and Af 20 were characterized non-toxigenic by both qualitative and quantitative methods.

The sequenced data was matched with database at GenBank, where it was observed that isolates Af 5 and Af 29, that produced higher amount of AFB2 were grouped together (Figure 4). One isolate, Af 9 was grouped with *A. oryzae* strain RIB40 (with 99% homology and 99% query cover) and it did not produce any AFB_2. Other two isolates, Af 20 and Af28, found non-toxigenic, also stayed in one group. The nucleotides of six isolates were further translated and individual ORFs were further searched against database using BLASTp. Approximately 225 amino acid long polypeptide (aflatoxin biosynthesis ketoreductase) was detected from all the six isolates of *A. flavus*. The protein sequences were found to be highly conserved with almost 100% similarity (Figure 5). The protein sequences thus retrieved also matched at >99% similarity with the similar proteins from *A. flavus* in the database. The phylogenetic tree generated from the protein blast sequence data placed all the 6 isolates in one group along with *A. flavus* strains.

Figure 3 - PCR amplification products of *A. flavus* isolates obtained by housekeeping gene primer **a**) OmtB, *b*) Omt1, and **c**) Nor1. Lane 1 and Lane 19 contain 1kb DNA ladder.

Discussion

In the present study, we used different methods (qualitative, quantitative and molecular techniques) to determine the toxigenicity of isolates of *A. flavus* from maize samples collected from fields and markets of major maize growing regions of North India. The majority of the isolates (63.6%) of *A. flavus* from different maize growing areas of Punjab were found non-toxigenic based on the qualitative methods. Among qualitative methods, bioassay on okra revealed 11 isolates positive for aflatoxin production and ammonia vapour test showed only 6 isolates positive for aflatoxin production. Both the methods share positive results in 5 isolates.

These tests did not allow true characterization of aflatoxin-producing and non-producing *A. flavus* and may generate false positives or false negatives in the tested isolates. However, this approach still represents a simple test for the initial screening of isolates (Singh, 1985; Kang, 1970). Saito and Machida (1999) reported that the ammonium hydroxide vapour test gave 11% false positive and 6% false negative results for aflatoxigenicity. Kumar et al (2007) observed 92% efficacy for the ammonium vapour test thereby generating 8% false negatives. The recent development of alternative substrates may improve the reliability of this method. Abbas et al (2004) reported that on using a yellow pigmentation combined with the ammonium hydroxide vapour test reduced false negatives to 7%.

Among quantitative methods, HPLC is the most popular method for the analysis of mycotoxins in foods and feeds. During the last fifteen years, there

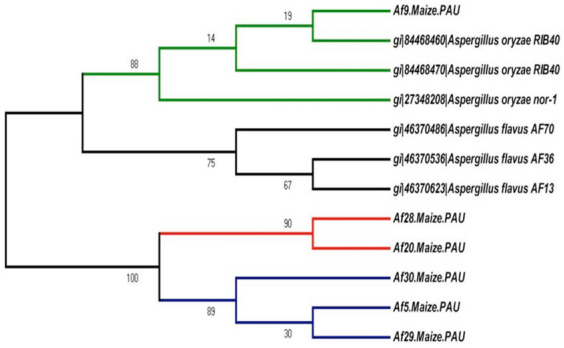

Figure 4 - Phylogenetic tree generated from the sequence data.

are several reports of successful application of HPLC techniques for the analysis of mycotoxins in grains (Li et al, 2001; Younis and Malik, 2003; Chan et al, 2004; Gobel and Lusky, 2004; Ahsan et al, 2010; Garrido et al, 2012; Ramesh et al, 2013). In this study, at least 7 isolates of *A. flavus* could produce aflatoxin levels that were above the safe limits prescribed as per Indian and USFDA standards, and thus, these grains were unsafe for consumption. Our results are in consistent with those of Ramesh et al (2013) and Ahsan et al (2010) who have also reported samples of maize contaminated with aflatoxins above the safe limits. Currently, nucleic acid based methods have replaced time consuming microbiological analysis by amplification of specific genomic markers.

During the last decade, PCR-based detection systems have been developed and routinely used for the detection of major species and groups of aflatoxigenic fungi (Rodriguez et al, 2012). The generally accepted pathway for aflatoxin biosynthesis involves at least 25 genes and most of the corresponding genes have been isolated and characterized (Yu et al, 2004; Hicks et al, 2002; Payne and Brown, 1998; Woloshuk and Prieto, 1998; Townsend, 1997). The *aflO* gene regulates the conversion of demethylsterigmatocystin into sterigmatocystin and *aflP* gene converts sterigmatocystin into O-methylsterigmatocystin (Scherm et al, 2005). These two steps lay at the end of the biosynthetic pathway of aflatoxin. The current work was more focused on *aflD* gene because product of this gene converts norsolorinic acid into averantin representing one of the first steps in biosynthetic pathway of aflatoxins (Hicks et al, 2002). In this study, both toxigenic and non-toxigenic isolates were screened for the presence of three genes (*aflO*, *aflP*, and *aflD*) of the aflatoxin cluster.

The aflatoxin producing isolates exhibited the presence of all the three genes, whereas the non-aflatoxigenic isolates lacked one or more genes. Chang et al (2005) characterized deletions of a part or the entire gene cluster in non-aflatoxigenic isolates of *A. flavus* supporting the hypothesis that the loss of aflatoxin-producing ability could be associated with deletions or mutations in the related genes. Gallo et al (2012) also correlated lack of amplification of aflatoxin biosynthesis genes in non-toxigenic isolates to non-aflatoxigenicity. The results obtained in our study confirmed that lack of amplification of aflatoxin

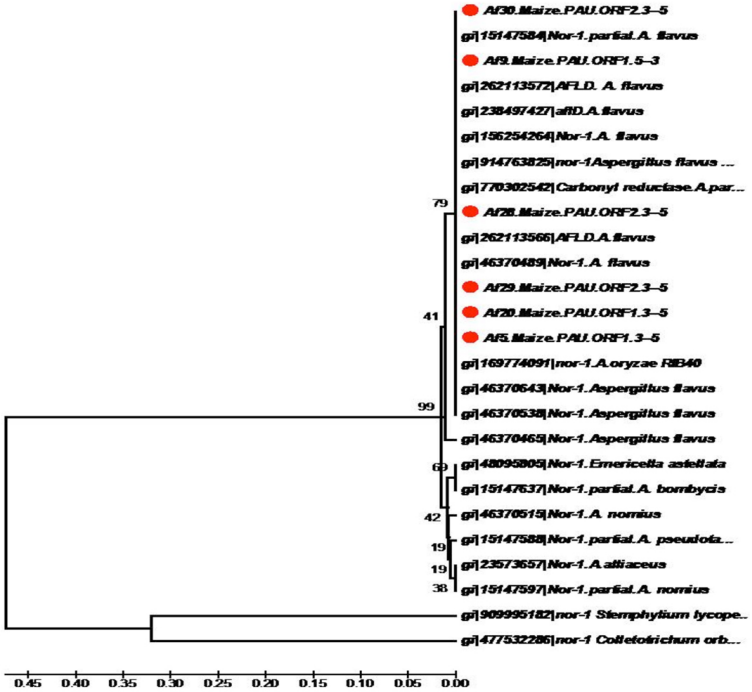

Figure 5 - Phylogenetic tree generated from the protein blast sequence data of *aflD* gene of 6 isolates of *A. flavus*. The tree was bootstrapped 1,000 times and the values are given at nodes.

biosynthetic genes is directly correlated with non-aflatoxigenicity in at least 60% of the *A. flavus* isolated from maize.

Similar reports were published by Criseo et al (2008) where they observed variable DNA banding patterns with one or more genes (*aflR*, *aflD*, *aflM*, *aflP*) missing specific to non-aflatoxigenic strains of *A. flavus*. In the present study, sequencing of product of aflD gene showed that non-toxigenic isolates (Af 28 and Af 20) were grouped with the toxigenic isolates. It is likely that one or more of the other genes involved in aflatoxin biosynthesis are lacking or carry some deletions in these isolates and thus, did not produce aflatoxins.

Furthermore, based on sequencing data of aflD gene, one toxigenic isolate found positive with qualitative method (Af 9) was grouped with *A. oryzae* RIB40 strain that did not produce aflatoxins. Tominaga et al (2006) analyzed the sequence of aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB40 and compared with *A. flavus* and *A. parasiticus*. They found that nor-1 gene was similar with *A. flavus*, but deletion and mutations in several other genes (*aflR*, *aflT*, *norA*, and *verA*) was there. Therefore, on the basis of *nor-1* or *aflD* gene, one isolate of *A. flavus* was grouped with *A. oryzae* RIB40 strain, which could be differentiated by sequencing and analysis of other genes involved in aflatoxin biosynthesis pathway.

This study demonstrated that inexpensive methods for detecting aflatoxin in cultures may be suitable when resources are limited. They would be ideal for ecological and genetic studies where a large population of Aspergillus isolates is characterized for aflatoxin production. Though percentage of false positives in qualitative methods may be higher but still could be used as preliminary indicators. In summary, all above-mentioned methods have their advantages and disadvantages, and their selection depends on objectives of the study, sample properties, environmental conditions and their cost. To ensure food safety, future projects should aim at multi-mycotoxin analyses, economizing quick and reliable detection techniques and portability for on-site aflatoxin testing from maize samples.

References

Abbas HK, Shier WT, Horn BW, Weaver MA, 2004. Cultural methods for aflatoxin detection. J Toxicol 23: 295-315

Ahsan S, Bhatti IA, Asif MR, Bhatti HN, Sheikh MA, 2010. Occurrence of aflatoxins in maize grains from central areas of Punjab, Pakistan. Int J Agric Biol 12: 571-575

Amaike S, Keller NP, 2011. *Aspergillus flavus*. Annu Rev Phytopathol 49:107-133

CAST, 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems. Task Force Report No. 139. Council for Agricultural Science and Technology, Ames, IA

Chang P, Horn BW, Dorner JW, 2005. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic Aspergillus flavus isolates. Fungal Genet Biol 42: 914-923

Chan D, MacDonald SJ, Boughtflower V, Brereton P, 2004. Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column cleanup and liquid chromatography-fluorescence detection. J Chromatogr A 1059:13-16

Criseo G, Racco C, Romeo O, 2008. High genetic variability in non-aflatoxigenic *A. flavus* strains by using Quadruplex PCR-based assay. Int J Food Microbiol 125: 341-343

Fu Z, Huang X, Min S, 2008. Rapid determination of aflatoxins in corn and peanuts. J Chromatogr A 1209: 271-274

Gallo A, Stea G, Battilani P, Logrieco AF, Perrone G, 2012. Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. Phytopathol Mediterr 51: 198-206

Garrido CE, Pezzani CH, Pacin A, 2012. Mycotoxins occurrence in Argentina's maize (*Zea mays* L.), from 1999 to 2010. Food Control 25: 660-665

Gonzaslez HHL, Resnik SL, Pacin AM, 2003. Mycoflora of freshly harvested flint corn from Northwestern Provinces in Argentina. Mycopathologia 155: 207–211

Ghosh SS, Chattopadhyay SK, Borkataki P, Bujarbaruah KM, Saxena SC, 1988. An outbreak of aflatoxicosis in an organized pig farm in Meghalaya. Indian J Hill Farm 1: 45-48

Gobel R, Lusky K, 2004. Simultaneous determination of aflatoxins, ochratoxin A, and zearalenone in grains by new immunoaffinity column/liquid chromatography. J AOAC Int 87: 411–416

Hicks JK, Shimizu K, Keller NP, 2002. Genetics and biosynthesis of aflatoxins and sterigmatocystin, pp. 55– 69. In: The Mycota XI - Agricultural Applications. Kempken F ed. Springer-Verlag, Berlin Heidelberg

Kang MS, 1970. Pathogenesis in Groundnut (*Arachis hypogeal* L.) by *Aspergillus flavus* Link Ex Fries. Ph.D. dissertation. Punjab Agricultural University, Ludhiana, India

Kaur H, Mohan C, Vikal Y, Singh M, 2014. Pathogenic and molecular characterization of *Fusarium moniliforme* Sheld, the incitant of Fusarium maize stalk rot in the Punjab State of India. Maydica 59: 290-297

Kpodo K, Thrane U, Hald B, 2000. Fusaria and fumonisins in maize from Ghana and their co-occurrence with aflatoxins. Int J Food Microbiol 61: 147–157

Kumar S, Shekhar M, Khan AA, Sharma P, 2007. A rapid technique for detection of toxigenic and non-toxigenic strains of *Aspergillus flavus* from

maize grains. Indian Phytopathol 60: 31-34
Kumar MR, Sudhakar P, Santhoshi MVM, Krishna TG, Reddy KR, 2014. Cultural, morphological and pathological variability among isolates of *Aspergillus flavus* in maize collected from different parts of Andhra Pradesh. J Plant Pest Sci 1: 9-16
Li FQ, Yoshizawa T, Kawamura O, Luo XY, Li YW, 2001. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. J Agric Food Chem 49: 4122–4126
Nilufer D, Boyacioglu D, 2002. Comparative study of three different methods for the determination of aflatoxins in tahini. J Agric Food Chem 50: 3375-3379
Payne GA, Brown MP, 1998. Genetics and physiology of aflatoxin biosynthesis. Ann Rev Phytopath 36: 329-362
Peraica M, Radic B, Lucic A, Palovic M, 1999. Toxic effects of mycotoxins in humans. Bull World Health Organ 77: 754-766
Pittet A, 1998. Natural occurrence of mycotoxins in food and feeds an updated review. Rev Med Vet 6: 479-492
Ramesh J, Sarathchandra G, Surehkumar V, 2013. Survey of market samples of food grains and grain flour for aflatoxin B1 contamination. Int J Curr Microbiol App Sci 2: 184-188
Reddy BN, Raghavender CR, 2007. Outbreaks of aflatoxicoses in India. Afr J Food Agric Nutr Dev 7; 1-15
Rodriguez A, Rodriguez M, Luque MI, Martín A, Cordoba JJ, 2012. Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. Food Microbiol 31: 89-99
Roy AKB, Ghosh SS, Nanda SK, Mukit A, Choudhury GB, Deb PN, 1989. Aflatoxicosis in ducklings in North-Eastern Hill Region (Tripura). Indian Vet Med J 13: 210–212
Saito M, Machidan S, 1999. A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *Aspergillus parasiticus* by ammonia vapour. Mycoscience 40: 205-208
Scherm B, Palomba M, Serra D, Marcello A, Migheli Q, 2005. Detection of transcripts of the aflatoxin genes AflD, AflO, and AflP by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. Int J Food Microbiol 98: 201-210.
Singh D, 1985. Incidence and control of toxigenic strains of *Aspergillus flavus* Link ex Fr. on wheat grains stored under different storage conditions. M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India
Sinha KK, 1990. Incidence of mycotoxins in maize grains in Bihar State, India. Food Addit Contam 7: 55–61
Tominaga M, Lee Y, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, Gotoh K, Akita O, 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. Appl Environ Microbiol 72: 484-490
Townsend CA, 1997. Progress towards a biosynthetic rationale of the aflatoxin pathway. Pure Appl Chem 58: 227-238
Woloshuk CP, Prieto R, 1998. Genetic organization and function of aflatoxin B1 biosynthetic genes. FEMS Microbiology Letters 160: 169–176
Younis YMH, Malik KM, 2003. TLC and HPLC assays of aflatoxin contamination in Sudanese peanut and peanut products. Kuwait J Sci Eng 30: 79-94
Yu J, Cleveland TE, Tierman WC, Bennett JW, 2005. *Aspergillus flavus* genomics: gateway to human and animal health, food safety; and crop resistance to diseases. Rev Lberoam Micol 22: 192-202
Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW, 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol 70: 1253–1262