academicJournals

Vol. 12(40), pp. 5839-5844, 2 October, 2013
DOI: 10.5897/AJB12.2366
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Occurrence of aflatoxin contamination in maize kernels and molecular characterization of the producing organism, *Aspergillus*

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Accepted 24 August, 2012

Aflatoxins are toxic metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 (AFB1) is a potent carcinogen, teratogen and mutagen. 660 pre- and post- harvest maize samples were collected from major maize growing areas in Tamil Nadu, India. Aflatoxin contamination was observed in 40.22% of the samples tested of which, 22.97% of pre-harvest and 53.93% post-harvest maize samples were found to be infected with AFB1 and 12.05% of the total samples exceeded WHO permissible limit of 20 µg/kg. AFB1 contamination ranged from 0 to 149.32 µg/kg. 28 *A. flavus* isolates were isolated and grouped into three sets based on aflatoxin producing potential viz., highly aflatoxin producing isolates, medium producing isolates and no aflatoxin producer or traces of aflatoxin producing isolates. The genetic coefficient matrix analysis using random amplified polymorphic DNA (RAPD) with ten random primers revealed minimum and maximum percent similarities among the tested *A. flavus* strains ranging from 35 to 89%. Cluster analysis separated the three sets of isolates into two groups (groups I and II) with each two subgroup confirming the genetic diversity among the *A. flavus* isolates from maize.

Key words: Maize, survey, *Aspergillus flavus*, aflatoxin, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Aflatoxins are a group of closely related heterocyclic compounds produced predominantly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin contaminates a vast array of food and agricultural commodities. *Aspergillus* species are capable of growing on a variety of substrates and under a variety of environmental conditions. Maize (*Zea mays* L.) is one of the most widely distributed food plants in the world and its infection by this fungus can result to mycotoxin contamination during the growing, harvesting, storage, transporting and processing stages (Bradburn et al., 1993). The economic consequences of mycotoxin contamination are profound, as the crops contaminated with high levels of mycotoxin are often destroyed. The affected crops are sometimes diverted to animal feed, resulting in reduced growth rates and illness of animal consuming contaminated feeds and result in meat and milk containing toxic contaminates. Globally, high levels of aflatoxin contamination of dietary staples have been reported (Bhat et al., 1997; Setamou et al., 1997; Kpodo et al., 1996; Wild and Turner, 2002). In India, Waliyar et al. (2003) reported that 43% of maize samples were contaminated with aflatoxin with the highest AFB1 level of 806 g/kg.

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Currently, there is a limited understanding of the details of the molecular variability among the isolates of *Aspergillus* spp. especially from maize. The use of non-toxigenic strains for the biological control of toxigenic ones has already been suggested by Dorner (2009). It is important to better understand the genetic diversity within this fungal group and the critical factors for retention or loss of characteristics such as toxigenic capacity and virulence to plants. Variability in aflatoxin production potential of *A. flavus* isolates have been reported (Karthikeyan et al., 2009). This is why the ability to distinguish between the various *Aspergillus* species may have diagnostic value. The analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive and reliable method for determining genetic relationships among pathogenic microorganisms (Zhang et al., 2004; Khoodoo and Jaufeerally-Fakim, 2004).

The objectives of the present study was to determine the levels of aflatoxin in maize grains collected from different maize growing areas of Tamil Nadu, India, and study the genetic variability among the isolates of *A. flavus* from the maize .

MATERIALS AND METHODS

Survey and collection of samples

A survey was conducted in different agro-ecological zones comprising of 16 districts viz., Coimbatore, Dindigul, Madurai, Salem, Theni, Trichy, Namakkal, Perambalur, Erode, Thirunelveli, Thiruvannamalai, Vellore, Villupuram, Virudunagar, Sivagangai and Thoothukudi districts of Tamil Nadu, India over three years (2009 to 2011) in order to understand the magnitude of aflatoxin contamination in maize. Pre- and post-harvest maize samples were collected from farmers' fields and stores.

Detection of AFB1 by indirect competitive ELISA

Samples (2 g) were powdered in a coffee grinder and then mixed with 10 ml of solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl. The mixture was incubated on a rotary shaker for 30 min at 250 rpm at room temperature (28±2°C). The extract was filtered through Whatman No. 41 filter paper and the filtrate was used for determination of AFB1 content by indirect competitive ELISA following the method of Reddy et al. (2001). Briefly, the wells of microtiter plates were coated with 150 µl per well of AFB-BSA at a concentration of 100 µg/ml in carbonate coating buffer. The plates were washed with phosphate buffered saline (PBS) containing Tween-20 (PBST) and treated with PBST-BSA. 100 µl of sample extract or AFB1 standard was mixed with 50 µl of AFB-BSA antiserum (1:6 000) in 0.2% PBST-BSA and added into the wells. This step was followed by the addition of alkaline phosphatase labelled goat antirabbit IgG conjugate diluted to 1:4 000 in PBST-BSA. *P*-nitrophenyl phosphate prepared in 10% diethanolamine was used as substrate. The plates were incubated at room temperature and then read in an ELISA reader at 405 nm. The concentration of AFB in the samples was calculated based on the absorbance of the AFB1 standard.

Isolation of pathogen

Aspergillus sp. were isolated from the infected maize samples collected from farmers fields and stored using the potato dextrose agar (PDA) medium under laboratory conditions. The *Aspergillus* cultures were identified as species using a taxonomic key and species descriptions (McClenny, 2005).

Aflatoxin (AFB1) productivity by *A. flavus* isolates

All the isolates were screened *in vitro* for their aflatoxin production potential in potato dextrose broth. The cultures were grown in 250 ml conical flasks containing 100 ml of potato dextrose broth at room temperature (28 ± 2°C) for 10 days. At the end of incubation period, culture filtrate was collected by filtering through two layers of muslin cloth. The culture filtrate was extracted with chloroform (1:1 v/v). The chloroform fraction was concentrated to 0.1 ml *in vacuo*. Further, quantification of aflatoxin contamination was carried out using ELISA method.

Molecular characterization

DNA isolation

Extraction of DNA from the aflatoxin producing *A. flavus* isolates was carried out using CTAB method (Henrion et al., 1992) with slight modification. To extract the DNA, the mycelial mat was ground to a fine powder using a pestle and mortar by the addition of liquid nitrogen. The resulting powder (200 to 300 mg) was mixed with 500 µl extraction buffer and incubated at 65°C for 30 to 60 min in water bath. Equal volume of phenol-chloroform-isoamyl alcohol was added and mixed well by inversion and centrifugation at 13000 rpm for 5 to 10 min and the supernatant was transferred into a new tube. The solution was then mixed with equal volume of isopropanol, followed by gentle mixing. The mixture was incubated for 1 h at -70 or -20°C for 2 h. DNA was then pelleted by centrifugation at 13000 rpm for 10 min, washed with 70% ethanol, pelleted again and dried at room temperature. Finally, the DNA pellet was rehydrated in 30 to 50 µl of 10 mM TE buffer and stored at -20°C until use. RAPD work was performed with Opron Primers viz., OPR2 (5'CACAGCTGCC3'), OPR8 (5'CCCGTTGCCT3'), OPR13 (5'GGACGACAAG3'), OPP12 (5'AAGGGCGAGT3'), OPP17 (5'TGACCCGCCT3'), OPP19 (5'GGGAAGGACA3'), OPC 02 (5'GTGAGGCGTC3'), OPC 08 (5'TGGACCGGTG3'), OPC 11 (5'AAAGCTGCGG3'), OPC 15 (5'GACGGATCAG3') and the resulted gels were scored via computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a genotype, it was designated as '1' and if absent, it was designated as '0'.

The RAPD data were analysed using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) computer package18. The data were used to generate Jaccard's similarity coefficients for RAPD bands. 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). The amplifications were carried out twice to check for reproducibility.

RESULTS AND DISCUSSION

Survey and collection of *Aspergillus* isolates

The results shown in Tables 1 and 2 indicate that aflatoxin contamination in maize samples was observed in 40.22% of the samples tested. AFB1 was detected in 22.97% of pre-harvest and 53.93% post-harvest maize

Table 1. Level of aflatoxin contamination (µg/kg) in maize samples in different districts of Tamil Nadu.

S/N	District	Pre-harvest		Post-harvest	
		Total sample	Aflatoxin infected	Total sample	Aflatoxin infected
1	Coimbatore	35	10	40	24
2	Dindigul	27	9	35	18
3	Madurai	25	7	37	26
4	Erode	18	4	26	12
5	Salem	20	6	28	16
6	Namakkal	19	7	24	17
7	Karur	22	6	24	13
8	Virudhunagar	29	7	19	11
9	Sivagangai	18	2	27	14
10	Thirunelveli	25	2	25	10
11	Thiruvannamalai	22	2	23	12
12	Vellore	12	2	23	10
13	Villupuram	11	1	25	9
	Total	283	65	356	192

Table 2. Aflatoxin B1 content in maize samples assessed by indirect competitive ELISA.

Sample	Number of sample analyzed	Number of samples with aflatoxin B1 (µg/kg)		
		0	1-20	>20
Pre-harvest maize kernels	283	218	43	22
Post-harvest maize kernels	356	164	137	55
Sub total	639	382	180	77

samples and 12.05% of the total samples exceeded 20 µg/kg. In total, AFB1 was detected in 257 out of 639 samples with amounts ranging from 0.4 to 149.32 µg/kg. It was evident from this study that post-harvest stage is the favorable stage for infection by *Aspergillus* spp. and aflatoxin production.

The occurrence of high levels of AFB1 in food and feed stuffs has been reported by several workers (Reddy et al., 1984; Singh et al., 1984; Balasubramanian, 1985; Selvasubramanian et al., 1987; Dhavan and Chaudary, 1995; Dutta and Das, 2001). Dutta and Das (2001) reported that AFB1 content in feed samples collected from different parts of Northern India were very high with an average of 0.412 to 0.514 ppm. It is well known that growth of *Aspergillus* spp. and subsequent production of aflatoxins in maize is dependent on a number of factors such as temperature, humidity, insect injury, handling during harvest and storage (Hell et al., 2003). A significant positive correlation between moisture content of maize seeds and *A. flavus* population and aflatoxin production has been reported (Oyebanji and Efiuvwevwere, 1999). Karthikeyan et al. (2009) reported wide variability in aflatoxin production potential of different isolates of *A. flavus* from maize. Vijayasamundeeswari et al. (2009) reported that AFB1 was detected in 61.3% of the maize kernel samples and the levels of AFB1 in 26% of the pre- and post-harvest maize kernels exceeded 20 µg/kg. Hence, the variations in aflatoxin content among different samples may have been as a result of unsatisfactory storage conditions, collection of samples from different regions, high moisture content, stages of samples, seasons of collection and the occurrence of aflatoxigenic fungi.

Aflatoxin (AFB1) productivity by *A. flavus* isolates

All *Aspergillus* fungi isolated from infected maize seed were found to be *A. flavus*. When they were tested for their ability to produce aflatoxin, all the isolates varied in their level of toxin production. The amount of AFB1 produced by the toxigenic isolates of *A. flavus* ranged from 0 to 58.53 ng/ml. Among the various isolates of *A. flavus*, the isolate AFM2 produced the highest amount of (58.53 ng/ml) AFB1 (Table 3).

Based on these results, the isolates were grouped into three categories viz., no aflatoxin producer or traces of aflatoxin producer (0 to trace ng/ml), medium aflatoxin producer (1 to 10 ng/ml) and high aflatoxin producer (>10 ng/ml).

Table 3. Aflatoxin (AFB1) productivity by *A. flavus* isolates.

A. flavus isolate	Locality	Aflatoxin B1 (ng/ml)
AFM1	Coimbatore	0
AFM2	Coimbatore	58.53
AFM3	Coimbatore	9.02
AFM4	Erode	0
AFM5	Erode	0
AFM6	Erode	5.5
AFM7	Salem	79.5
AFM8	Salem	0
AFM9	Salem	08.48
AFM10	Dindigul	3.65
AFM11	Dindigul	68.48
AFM12	Dindigul	5.45
AFM13	Madurai	0
AFM14	Madurai	7.23
AFM15	Madurai	7.90
AFM16	Namakkal	9.58
AFM17	Namakkal	77.95
AFM18	Namakkal	0
AFM19	Karur	9.95
AFM20	Karur	0
AFM21	Karur	0
AFM22	Virudhunagar	52.15
AFM23	Sivagangai	0
AFM24	Thirunelveli	44.75
AFM25	Thiruvannamalai	53.46
AFM27	Vellore	59.84
AFM28	Villupuram	0

Molecular characterization

Molecular characterization was performed on AFM2, AFM7, AFM11, AFM17 (high aflatoxin producing isolates), AFM3, AFM6, AFM10, AFG12 (medium aflatoxin producing isolates) and AFM1, AFM4, AFM5, AFG8 (no aflatoxin or traces of aflatoxin producing isolates). A total of 71 bands were amplified with the 10 primers and 12 isolate. The pairwise Jaccard's coefficients for the genetic similarities among the 12 isolates are presented in Table 4. The cluster analysis of the distribution of 71 RAPD bands is shown as a dendrogram (Figure 1). All 12 accessions were distinguished from each other. The distribution of the bands isolates of three different aflatoxin producing groups merged together.

Other workers (Bayman and Cotty, 1993; Croft and Varga, 1994; Jovita and Bainbridge, 1996; Tran-Dinh et al., 1999; Lourenco et al., 2007) have reported no correlation between DNA band profiles and toxin production using RAPD. Egel et al. (1994) grouped strains with similar toxigenic capacities, in a more subtle differentiation than the simple classification of toxin producers and non producers. Other researchers working with different nycotoxin producing fungi have reported that no correlation could be observed between clustering of the isolates of these fungi based on RAPD and their mycotoxin-producing abilities or aggressiveness (Toth et al., 2004). The total sizes of the restriction fragments in each enzyme digest in the present study exceeded the apparent size of the ITS-PCR product. This difference in size of the fragments could be attributed to the presence of multiple forms of the rDNA-ITS gene cluster in single isolates. This phenomenon is common in fungi and has been reported in *Sclerotium rolfsii* (Harlton et al., 1995), *Ascochyta* sp. (Fatehi and Bridge, 1998) and *Phytophthora* sp. (Appiah et al., 2004).

Aflatoxin affects the quality of the commodities and there by hitting the export trade in the international market. The present study reveals that the level of AFB1 contamination is more than 26% of the pre- and post-harvest maize kernel samples and exceeded the permissible level of 20 µg/kg, the tolerance level fixed by the World Health Organization (WHO, 1991) and US National Grain and Feed Association (US NGFA, 2009). The presence of aflatoxin in maize kernels and feeds presents a risk for human and animal health. Therefore, proper

Table 4. Similarity matrix for Jaccard's coefficients for 12 *A. flavus* isolates.

	AFM1	AFM 4	AFM5	AFM8	AFM3	AFM6	AFM10	AFM12	AFM 2	AFM7	AFM11	AFM17
AFM 1	1.00											
AFM 4	0.83	1.00										
AFM 5	0.68	0.76	1.00									
AFM 8	0.62	0.70	0.67	1.00								
AFM 3	0.57	0.70	0.61	0.72	1.00							
AFM 6	0.59	0.67	0.55	0.57	0.72	1.00						
AFM 10	0.48	0.60	0.52	0.48	0.53	0.56	1.00					
AFM 12	0.35	0.50	0.39	0.46	0.51	0.42	0.62	1.00				
AFM 2	0.38	0.50	0.46	0.52	0.50	0.41	0.54	0.73	1.00			
AFM 7	0.47	0.61	0.48	0.51	0.49	0.41	0.58	0.63	0.58	1.00		
AFM 11	0.58	0.68	0.57	0.47	0.47	0.58	0.62	0.54	0.47	0.71	1.00	
AFM 17	0.52	0.62	0.49	0.43	0.43	0.52	0.62	0.55	0.48	0.65	0.89	1.00

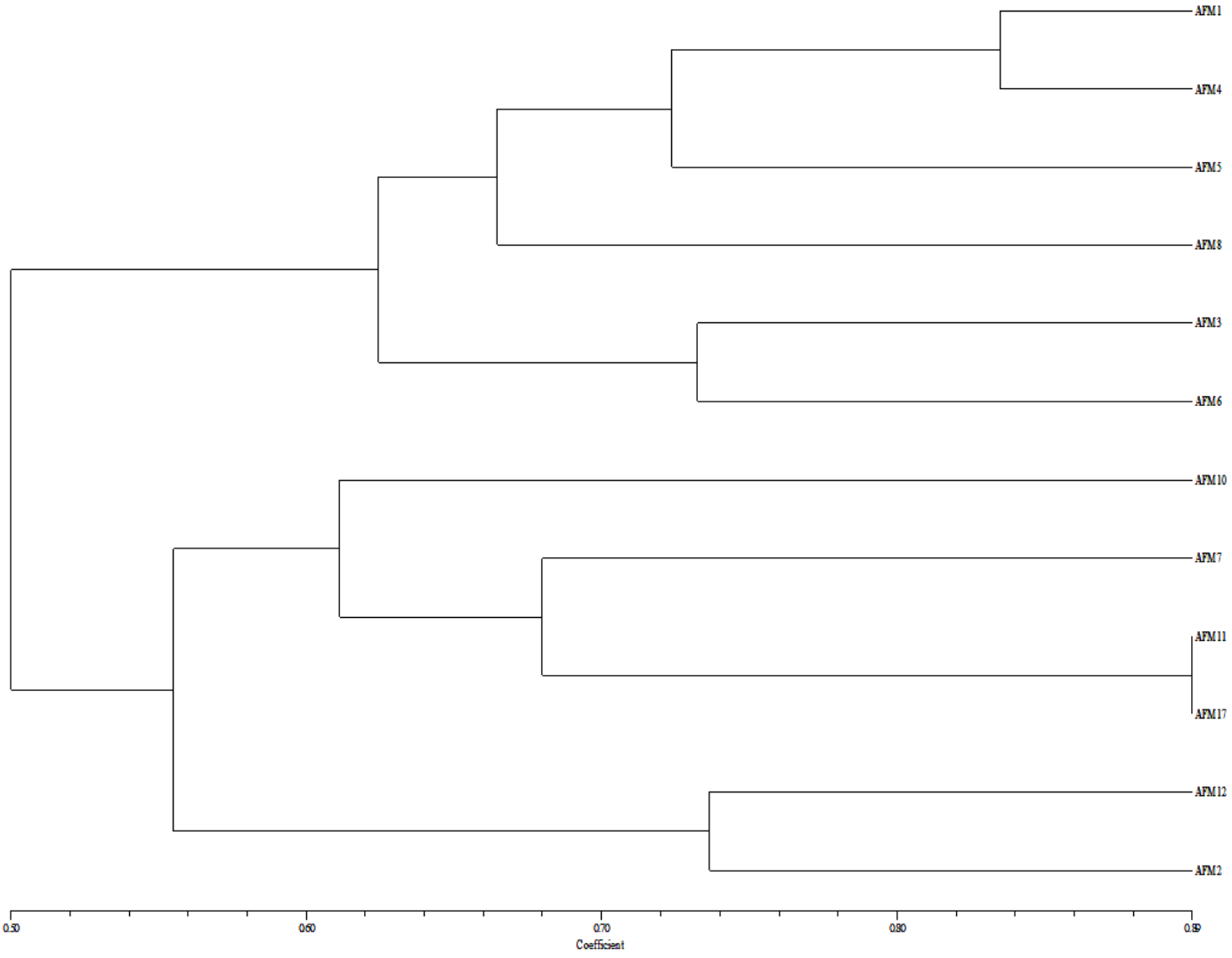

Figure 1. Dendrogram of *A. flavus* isolates based on 71 PCR bands amplified by 10 RAPD primers. The bar on the bottom represents similarity index based on Jaccard's coefficients.

post-harvest handling of maize and proper storage of feeds can greatly help in reducing infection by *Aspergillus* spp. and subsequent contamination with aflatoxins and the variability among *A. flavus* isolates in aflatoxin production potential, and genetic makeup may present a problem for the control of *A. flavus* infection.

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