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Natural occurrence of aflatoxin, aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* in groundnut seeds across India

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A survey across different agro-climatic regions of India was done and 38 groundnut seed samples were collected from various sources. Upon analysis, all samples were found infected with *Aspergillus flavus* ranging from 2 to 50% incidence with aflatoxin content of 0.0 to 28 ppb. Greenhouse studies revealed no correlation between incidence of *A. flavus* and aflatoxin content on seedling emergence, root length, shoot length and dry weight. Seeds were predominantly contaminated with aflatoxin B_1 followed by aflatoxin B_2. Among the tested *A. flavus* isolates, 31 were found aflatoxigenic and seven were non-aflatoxigenic when analyzed through cultural, thin layer chromatography, competitive direct enzyme linked immunosorbent assay and multiplex polymerase chain reaction. Present study reveals the current scenario of aflatoxin contamination, and aflatoxigenic and non-aflatoxigenic fungal infection in groundnut seeds collected across India.

Key words: Polymerase chain reaction (PCR), *Aspergillus flavus*, aflatoxin, enzyme-linked immuno sorbent assay (ELISA), groundnut.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the most important food and oilseed crops cultivated and consumed in most parts of the World. It is widely accepted as an excellent source of nutrition to both human and animals due to its high protein content. Groundnut is grown on nearly 23.95 million ha worldwide with the total production of 36.45 million tons with an average yield of 1520 kg/ha in 2009 (FAOSTAT, 2011). Preharvest and postharvest infection by storage mold infection and subsequent mycotoxin production in groundnut are serious problems in the tropical hot and humid climate of World (Keenan and Savage, 1994; Kishore et al., 2002). Storage conditions, including temperature and humidity and seed moisture level play a major role in biodeterioration of seeds which include; seed rots, moulding of seeds, pre and post-emergence damping off, low seed viability and poor seedling growth (Ojimelukwe, 1999; Kumar et al., 2008). Storage moulds are ubiquitus in nature and being a saprophyte grows on a wide variety of substrates, including decaying plant and animal debris under field conditions. The mycotoxins produced by these moulds are toxigenic contaminants of food and feeds that are frequently responsible for health and economic concerns in many countries (Bhatnagar et al., 2003).

Aflatoxin is a group of mycotoxins produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Aflatoxin, especially AFB_1 is the most potent toxic metabolite, which shows hepatotoxic teratogenic and mutagenic properties, causing such diseases to mammals as toxic hepatitis, hemorrhage, edema, immunosuppression and

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hepatic carcinoma. It has been classified as Class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002). In plant system, aflatoxin affects amylase activity in germinating seeds causing inhibition of starch hydrolysis and consequent unavailability of sucrose to the embryonic axis during imbibition. The embryoes of aflatoxin contaminated seeds remain alive with fairly high dehydrogenase activity and are capable of growth in culture when supplemented with sucrose (Chatterjee, 1988). Aflatoxin mediated seed quality deterioration are reported frequently in crops like maize, soybean, red gram, green gram, black gram, lettuce and cotton (Crisan, 1973; el-Naghy et al., 1999; Ahammed et al., 2008; Janardhan et al., 2011). Various surveys conducted in different parts of India (Sharma et al., 1994; Bhat et al., 1996; Sinha et al., 1999) have revealed that groundnuts and their products are high-risk commodities for aflatoxin contamination; this contamination affects 1.8 million tons of groundnuts each year (ICRISAT, 2009).

One of the ecofriendly method for managing these aflatoxigenic fungi and aflatoxin contamination in food crops is by using non-aflatoxigenic isolates which competitively inhibit toxigenic fungal strains (Dorner et al., 1999; Dharmaputra et al., 2001). In recent years, non-aflatoxigenic strains of *Aspergillus* sp. are being commercially used (Dorner and Lamb, 2006). Hence, present study was undertaken to get preliminary information about, aflatoxin contamination and the occurrence of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* sp. in groundnut seed samples from different agro-climatic regions of India.

MATERIALS AND METHODS

Collection of seed sample

A survey was conducted during January to September, 2009 across groundnut growing regions of India and 38 seed samples were collected from National and State seed corporations, Agricultural Universities and Research Institutes irrespective of their storage conditions. A minimum of 2.5 kg seeds were collected in each sample and labeled. Seeds were surface sterilized with 0.4% sodium hypochlorite for 5 min followed by thorough rinsing in distilled water before being used in the experiments.

Screening of seed samples for seed-borne fungal incidence

Groundnut seeds were subjected to standard blotter method (SBM) (ISTA, 2003) to analyze seed-borne storage mold and field fungi. Fungi developed on each seed were examined under different magnifications of a stereomicroscope and identified based on the way they grow on seeds; “habit characters” (Singh et al., 1991; Mathur and Kongsdal, 2003). Suspected *Aspergillus* spp. were isolated, identified as *A. flavus* by its conidial characters under compound microscope and also by growing these isolates on *A. flavus* - *parasiticus* differentiating medium. All *A. flavus* isolates were pure cultured onto potato dextrose agar (PDA) slants and maintained at 4°C until further use.

Quantification of aflatoxin in groundnut seed samples

Aflatoxin from 100 g of seed samples was extracted following the procedure explained by Kumar et al. (2007). The extract was concentrated on a rotor vapor (Buchi, Germany) and used for TLC as explained below. The total AF content was estimated from seed samples by competitive indirect ELISA as explained by Reddy et al. (2009) with minor modifications. AFB_1-oxime and AFB_1-OVA conjugate were prepared following the standard procedures (Kolosova et al., 2006). To perform ciELISA, the wells of microtitre plates (Maxisorp F96, Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with 100 µL of AFB_1-OVA conjugate in carbonate buffer (1 mg/ml), at pH 9.6, and then washed thrice with PBS containing 0.05% Tween 20 (PBST). AFB_1 standards (Sigma) in 10% (v/v) methanol-PBS (50 µL), or different dilutions of samples (50 µL), were added to the wells. After 15 min incubation at 37°C, 100 µl of anti-AFB_1 antibody (Sigma) (1:10000) in PBS were added and incubated at 37°C for 45 min. The plates were washed with PBST 4 to 5 times. Subsequently, 100 µL of secondary antibody conjugated with horseradish peroxidase (HRP) (1:10000) (Bangalore Genei) in PBS was added and incubated at 37°C for 45 min. At the end of the incubation period, the plates were washed 4 to 5 times with PBST. 100 µl of substrate (TMB-H_2O_2) (Bangalore Genei) were added and incubated at room temperature for 10 to 15 min. The reaction was stopped by adding 100 µL of stop solution (2 M H_2SO_4) and the colour developed was read at 450 nm. Standard curves using absorbance (A) vs. logarithm of analyte concentration were plotted. Total aflatoxins in the samples were determined from the standard curve and expressed in ppb.

Quality parameter analysis of groundnut seeds

Seed samples which showed different degrees of *A. flavus* incidence and aflatoxin contamination were evaluated for the seed quality variables which included seedling emergence, root length, shoot length and dry weight (ISTA, 2003). Seedlings dry weight was determined by drying in hot air oven at 60°C for 2 days. The experiment was performed in triplicates and repeated twice.

Differentiation of toxigenic and nontoxigenic *Aspergillus flavus* isolates

Ultra violet (UV) and ammonia vapor tests were performed following the standard procedures of Hara et al. (1974) and Saito and Machida (1999), respectively. Thin layer chromatography differentiation of aflatoxigenic and non aflatoxigenic fungi was done by growing the *A. flavus* isolates on PDA and YESA medium for 7 days at 28 ± 2°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 50 µL chloroform and applied onto TLC plate as explained below (Criseo et al., 2001). 5 µL of aflatoxin extract along with aflatoxin standard (Sigma Aldrich, USA) were spotted on precoated TLC plates (20x20 Merck, Germany). Plates were developed in mobile phase chloroform:acetone (9:1). Plates were dried and observed under long wavelength UV (365 nm) light. The blue (AFB_1 and AFB_2) and green (AFG_1 and AFG_2) florescent spots on TLC plates were identified by comparing them with the standard aflatoxins (Sigma).

For Competitive indirect ELISA, *A. flavus* isolates were grown on PDB (50 ml) in 100 ml conical flasks for 7 days at 28 ± 2°C. At the end of incubation period, different dilutions of culture filtrate (1:1, 1:10 and 1:100) with methanol:water (7:3) were analyzed for the presence of total aflatoxin as explained earlier.

To perform multiplex polymerase chain reaction (PCR), DNA was isolated from fungal strains according to the method of Yelton et al. (1984). Multiplex PCR reaction was performed according to the method of Farber et al. (1997) with specific primers for aflatoxin biosynthetic genes. The sequences of primers used were: nor1, 5'-ACCGCTACGCCGGCACTCTCGGCAC-3', nor2, 5'- GTTGGCCGC-

CAGCTTCGA CACTCCG-3' enclosing a fragment of 400 bp from nucleotide 501-900 of the *A. parasiticus* nor-1 gene; ver1, 5'-GCCGCAGGCCGCGGAGAAAGTGGT-3', ver2, 5'-GGGGATATACTCCCGCGACACAGCC-3', enclosing a fragment of 537 bp from nucleotide 623-1160 of the *A. parasiticus* ver-1 gene; omt1, 5'-GTGGACGGACCTAGTCCGA CATCAC-3', omt2, 5'-GTCGGGCGCCACGCACTGGGTTGGGG-3', enclosing a fragment of 797 bp from nucleotide 301-1098 of the *A. parasiticus* omt-A gene; aflR1,5'-TATCTCCCCC CGGGCATCTCCCGG-3',aflR2, 5'-CCGTCAGACAGCCACTGGACAGCGG -3', enclosing a fragment of 1032 bp from nucleotide 450-1482 of the *A. parasiticus aflR* gene.

Statistical analysis

All data from laboratory and greenhouse experiments were analyzed separately for each experiment and were subjected to arcsine transformation and analysis of variance (ANOVA) (SPSS, version 16). Significant effects of treatments were determined by the F values ($P \leq 0.05$). Treatment means were separated using Turkey's HSD test.

RESULTS

Seed infection by *Aspergillus flavus* and aflatoxin contamination

All the 38 seed samples collected were found infested with *A. flavus*. Other storage molds detected were *A. niger*, *A. tamari*, *A. terrues*, *A. fumigatus*, *Penicillium* spp., *Fusarium graminearum*, *F. solani* and *F. oxysporum* (Table 1). However, none of the samples had *A. parasiticus*. *Aspergillus flavus* incidence was highest in groundnut seed samples as compared to other storage fungi. Highest incidence of *A. flavus* (50%) was recorded in sample G37 collected from Gujarat, and the least (2%) was in samples GS17 (Karnataka), G62 and G71 (Rajasthan) (Table 1). A total of 38 *A. flavus* isolates were isolated from groundnut seed samples collected from various sources and they were pure cultured onto PDA slants. When aflatoxin levels in seed samples were quantified through ciELISA, sample G62 was free from any kind of aflatoxin contamination. Highest concentration of 28 ppb of aflatoxin was recorded in sample G57 (Karnataka) and least concentration of 0.03 ppb was in sample G16 (Rajasthan). AFB_1 was the predominant aflatoxin observed in most of the samples followed by AFB_2 and sample G65 showed presence of both AFB_1 and AFG_1 (Table 2).

Seed samples with varied levels of storage mold and field fungal incidence showed different symptoms and abnormal growth depending on the extent of infection which included, aflaroot symptoms, damping-off and seed /seedling rotting (Figure 1). Analysis of seed quality factors (% seedling emergence, root length, shoot length and dry weight) revealed that these parameters had no correlation with the percent incidence of *A. flavus* or aflatoxin concentration. This indicates that other storage molds and field fungi might be responsible for the seed quality variables (Tables 1 and 2).

Differentiation of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates

The results obtained from the cultural, TLC, ELISA and molecular methods are presented in Table 3. Among the 38 strains, seven strains (AFG15, AFG20, AFG26, AFG48, AFG64, AFG65 and AFG71) were non-aflatoxigenic. Ultra violet and ammonia vapour tests showed variable results with aflatoxigenic strains upon repeating the experiments. But with non-aflatoxigenic strains, the result was reproducible. TLC analysis showed a clear demarcation between aflatoxin producing and non producing strains of *A. flavus*. Among 29 aflatoxigenic isolates, AFB_1 was predominantly produced followed by AFB_2, and few strains produced only AFB_1 or AFB_2 (Table 3). But ciELISA results showed that, 31 isolates produced aflatoxin. Two *A. flavus* isolates (AFG19 and AFG39) were found positive for aflatoxin production but was reported as non aflatoxin producer on TLC analysis which is due to higher sensitivity of ciELISA (Table 3).

Results of multiplex PCR analysis is shown in Figure 2 and Table 3. Bands of the fragments *nor-1, ver-1, omt-A* and *aflR* genes can be visualized at 400, 537, 797 and 1032 bp, respectively (Figure 2). 32 strains showed quadruplex pattern indicating the presence of four genes of the aflatoxin biosynthesis pathway whereas isolates AFG20, AFG26, AFG48, AFG64, AFG65 and AFG71 showed the banding pattern in which one or two bands were missing. Whereas strain AFG15 which was negative for aflatoxin production as detected in other methods showed presence of all the four bands (Figure 2).

DISCUSSION

Most of the studies regarding the aflatoxin contamination in groundnut are confined to the grain samples which are used as food or feed. But limited information is available on the *A. flavus* incidence and subsequent aflatoxin contamination of groundnut seeds used for sowing across India. The main source of plant propagation in groundnut is through seeds and maintaining its health is important to improve quality and quantity of the crop and its product. In the present investigation, we observed varied levels of storage and field fungal incidence and also its adverse effect on seed quality variables (Figure 1, Table 1 and 2). *A. flavus* and *A. parasiticus* infection and aflatoxin production in groundnut was reported by several early workers across under Indian conditions. Groundnut samples collected from Andhra Pradesh, Gujarat and Tamil Nadu were contaminated to the extent of 20 to 40% with aflatoxin (Anonymous, 1967). Similarly, Kishore et al. (2002) evaluated a total of 182 groundnut samples collected at harvest from farmers fields in Andhra Pradesh and reported

Table 1. Screening of seed samples to analyze seed-borne incidence of *Aspergillus flavus*.

State	Sample Code	Incidence percentage (%) of seed-borne fungi									
		Af	***An***	***Ata***	***Ate***	***Afu***	***Afcol***	***Pen***	***Fg***	***Fso***	***Fo***
Karnataka	G1	8	30	-	-	-	-	17	-	1	-
Karnataka	G8	17	22	1	-	-	-	24	2	-	-
Karnataka	G11	23	9	-	-	-	-	5	7	1	2
Karnataka	G12	18	27	-	-	-	-	-	-	-	-
Karnataka	G13	14	26	-	1	1	-	23	-	-	-
Karnataka	G14	20	7	-	-	-	-	22	11	-	7
Tamil Nadu	G15	24	6	-	-	-	8	-	-	-	-
Karnataka	G16	19	22	-	-	-	-	12	-	-	-
Tamil Nadu	G18	31	16	1	-	-	7	7	4	-	-
Tamil Nadu	G19	07	3	-	1	1	6	14	-	1	1
Tamil Nadu	G20	20	-	-	-	-	12	-	-	-	-
Tamil Nadu	G24	40	10	1	-	-	-	10	1	-	-
Tamil Nadu	G25	3	25	-	-	-	-	12	-	-	-
Karnataka	G26	22	37	-	1	2	1	17	-	1	1
Karnataka	G27	38	16	-	2	1	1	19	2	1	1
Gujarat	G28	30	15	-	-	-	5	21	-	-	-
Andhra Pradesh	G29	18	20	-	-	-	-	20	-	-	-
West Bengal	G30	47	32	1	-	-	1	-	-	-	-
Rajasthan	G33	16	5	-	-	-	-	10	-	-	-
Gujarat	G35	20	36	-	-	-	6	36	-	-	-
Gujarat	G37	50	29	-	-	-	2	19	-	-	-
Andhra Pradesh	G39	36	3	1	1	-	2	3	-	3	-
Karnataka	G48	25	2	2	-	-	8	3	-	-	-
Karnataka	G49	12	22	-	-	-	-	24	-	-	-
Karnataka	G50	9	4	-	-	-	4	8	8	-	-
Rajasthan	G52	20	-	-	-	4	-	76	-	-	-
Rajasthan	G54	25	10	-	-	2	-	1	-	-	-
Karnataka	G57	19	7	-	-	-	2	10	-	-	-
Rajasthan	G62	2	-	-	-	-	-	1	-	-	-
Rajasthan	G64	14	6	-	-	-	-	2	-	-	-
Rajasthan	G65	16	4	-	-	-	-	6	-	-	-
Rajasthan	G66	6	8	-	-	2	4	4	-	-	-
Rajasthan	G69	12	4	-	-	-	-	2	-	-	-
Rajasthan	G71	2	8	-	-	-	-	8	-	-	-
Karnataka	GS15	12	22	-	-	-	-	4	-	-	-

Table 1. Contd.

State	Sample Code	Incidence % of seed-borne fungi									
		Af	*An*	*Ata*	*Ate*	*Afu*	*Afcol*	*Pen*	*Fg*	*Fso*	*Fo*
Karnataka	GS17	2	2	-	-	-	-	-	-	-	-
Karnataka	GS31	24	30	-	-	-	6	2	-	-	-
Karnataka	GS33	4	8	-	-	-	-	34	-	-	-

Af, *Aspergillus flavus*; An, *Aspergillus niger*; Ata, *Aspergillus tamarii*, Ate, *Aspergillus terreus*, Afu, *Aspergillus fumigatus*; Afcol, *Aspergillus flavus columnaris*; Pen, *Penicillium* species; Fg, *Fusarium graminearum*; Fso, *Fusarium solani*; Fo, *Fusarium oxysporum*.

Table 2. Aflatoxin content in groundnut seed samples and their seed quality variables.

Sample code	Seed quality variable				Aflatoxin	
	Mean root length (cm)	Mean shoot length (cm)	Emergence (%)	Dry wt/Seedling (g)	TLC	ELISA (ppb)
G1	10.36 ± 0.057^{g}	10.36 ± 0.034^{lm}	95 ± 1.154^{a}	0.24 ± 0.011^{fghi}	-	0.19 ± 0.005^{j}
G8	12.4 ± 0.115^{c}	12.4 ± 0.173^{de}	80 ± 1.154^{efgh}	0.21 ± 0.017^{hijk}	B1, B2	12.0 ± 1.732^{c}
G11	12.44 ± 0.023^{c}	12.38 ± 0.011^{de}	90 ± 0.5773^{ab}	0.23 ± 0.011^{ghij}	B1, B2	8.5 ± 0.115^{d}
G12	11.77 ± 0.011^{d}	11.22 ± 0.011^{hi}	90 ± 1.154^{ab}	0.27 ± 0.017^{defg}	B1, B2	4.0 ± 0.115^{hi}
G13	13.8 ± 0.115^{a}	12.2 ± 0.057^{e}	85 ± 1.732^{bcd}	0.24 ± 0.017^{efghi}	B1, B2	7.2 ± 0.057^{e}
G14	12.88 ± 0.017^{b}	11.5 ± 0.173^{g}	85 ± 1.452^{bcd}	0.28 ± 0.011^{cdef}	B1, B2	16.0 ± 0.115^{b}
G15	6.29 ± 0.023^{r}	4.02 ± 0.011^{s}	82 ± 1.732^{defg}	0.19 ± 0.005^{ijk}	B1	0.26 ± 0.017^{j}
G16	9.95 ± 0.023^{hi}	10.8 ± 0.173^{jk}	54 ± 2.027^{pq}	0.21 ± 0.005^{hijk}	B1	0.03 ± 0.005^{j}
G18	7.9 ± 0.057^{o}	3.24 ± 0.023^{t}	50 ± 1.732^{q}	0.17 ± 0.011^{k}	B1, B2	11.0 ± 0.173^{c}
G19	7.5 ± 0.115^{p}	$9.7\pm0.173m^{n}$	70 ± 0.577^{lmn}	0.18 ± 0.011^{jk}	-	0.16 ± 0.005^{j}
G20	9.23 ± 0.017^{jk}	11.05 ± 0.028^{ij}	56 ± 1.154^{p}	0.27 ± 0.020^{defg}	B1, B2	3.0 ± 0.115^{i}
G24	11.45 ± 0.028^{e}	10.23 ± 0.017^{m}	67 ± 1.1547^{no}	0.30 ± 0.011^{bcd}	B1, B2	5.0 ± 0.057^{gh}
G25	10.65 ± 0.017^{f}	10.15 ± 0.011^{m}	80 ± 1.154^{efgh}	0.28 ± 0.005^{cdef}	-	0.22 ± 0.011^{j}
G26	8.26 ± 0.034^{n}	9.34 ± 0.023^{o}	83 ± 1.732^{cdef}	0.26 ± 0.005^{defg}	B1, B2	12.0 ± 0.173^{c}
G27	5.93 ± 0.017^{s}	7.33 ± 0.011^{r}	87 ± 0.577^{bcde}	0.24 ± 0.011^{efghi}	-	0.15 ± 0.011^{j}
G28	6.67 ± 0.057^{q}	8.89 ± 0.057^{p}	73 ± 1.732^{jklm}	0.26 ± 0.005^{defg}	B1, B2	6.0 ± 0.230^{efg}
G29	8.07 ± 0.017^{no}	11.57 ± 0.017^{g}	77 ± 1.154^{ghij}	0.27 ± 0.011^{defg}	B1, B2	3.0 ± 0.115^{i}
G30	7.89 ± 0.051^{o}	10.97 ± 0.057^{ij}	82 ± 1.732^{defg}	0.30 ± 0.011^{bcd}	-	0.13 ± 0.017^{j}
G33	8.8 ± 0.115^{m}	9.65 ± 0.028^{mn}	81 ± 0.577^{efgh}	0.29 ± 0.026^{bcde}	-	0.18 ± 0.011^{j}
G35	10.10 ± 0.057^{h}	11.39 ± 0.023^{gh}	79 ± 0.577^{efgh}	0.32 ± 0.011^{abc}	B1	5.0 ± 0.115^{gh}
G37	9.78 ± 0.046^{i}	12.78 ± 0.046^{c}	68 ± 1.154^{mn}	0.28 ± 0.011^{cdef}	B1, B2	15 ± 0.173^{b}
G39	11.58 ± 0.023^{de}	12.64 ± 0.023^{cd}	88 ± 1.732^{bcd}	0.30 ± 0.011^{bcd}	B1	0.30 ± 0.017^{j}

Table 2. Contd.

G48	7.98±0.017^{o}	9.21±0.005^{o}	84±1.154^{bcde}	0.26±0.005d^{efg}	B1	0.55±0.028^{j}
G49	8.92±0.01^{1m}	10.67±0.040^{k}	62±0.577^{o}	0.27±0.017d^{efg}	B2	0.70±0.115^{j}
G50	9.76±0.034^{i}	11.07±0.040^{ij}	76±0.577^{hijk}	0.31±0.005^{bcd}	B1	0.16±0.008^{j}
G52	6.66±0.023^{q}	8.43±0.017^{q}	71±1.154^{klmn}	0.22±0.005^{hijk}	B1	0.16±0.011^{j}
G54	7.56±0.034^{p}	8.88±0.023^{p}	78±0.577^{fghi}	0.23±0.017^{ghij}	B1	0.20±0.011^{j}
G57	8.91±0.017^{m}	9.43±0.017^{no}	83±0.577^{cdef}	0.25±0.014^{efgh}	B1, B2	28.0±0.288^{a}
G62	11.43±0.017^{e}	13.83±0.017^{b}	87±0.577^{bcd}	0.33±0.011^{ab}	B1	0^{j}
G64	9.35±0.028^{j}	10.57±0.017^{kl}	80±1.154^{efgh}	0.28±0.017^{cdef}	B1	5.75±0.028^{fg}
G65	8.87±0.017^{m}	9.69±0.023^{mn}	76±1.154^{hijk}	0.24±0.005^{fghi}	B_1, G_1	6.00±0.090^{efg}
G66	10.39±0.051^{g}	11.52±0.017^{g}	87±1.154^{bcd}	0.30±0.017^{bcd}	-	0.17±0.005^{j}
G69	12.93±0.017^{b}	14.29±0.023^{a}	90±1.154^{ab}	0.35±0.011^{a}	B1, B2	9.00±0.173^{d}
G71	9.18±0.011^{jkl}	10.59±0.005^{kl}	83±1.732^{cdef}	0.32±0.005^{abc}	B1, B2	6.50±0.115^{ef}
GS15	8.28±0.011^{n}	9.76±0.005^{m}	77±0.577^{ghij}	0.26±0.023^{defg}	-	0.16±0.011^{j}
GS17	7.86±0.012^{o}	9.16±0.023^{op}	75±1.154^{ijkl}	0.25±0.005^{efgh}	B1, B2	0.30±0.005^{j}
GS31	9.08±0.017^{klm}	11.36±0.034^{gh}	85±1.732^{bcde}	0.28±0.017^{cdef}	B1, B2	5.00±0.115^{gh}
GS33	10.47±0.017^{fg}	11.91±0.005^{f}	89±1.154^{bc}	0.30±0.011^{bcd}	-	0.24±0.023^{j}

TLC, Thin Layer Chromatography, ELISA, Enzyme Linked Immuno Sorbent Assay. Values are the mean with in the column sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$.

Table 3. Detection of aflatoxin producing ability of *Aspergillus flavus* isolates.

Isolate code	Conventional Methods		TLC	ELISA	Quadruplex PCR				Aflatoxin production
	UV Test	AV Test			*aflR*	*omt-A*	*ver-1*	*nor-1*	
AFG1	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG8	+	+	B_1	+	+	+	+	+	Positive
AFG11	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG12	+	V	B_1, B_2	+	+	+	+	+	Positive
AFG13	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG14	+	+	B_2	+	+	+	+	+	Positive
AFG15	-	-	-	-	+	+	+	+	Negative
AFG16	+	+	B_1	+	+	+	+	+	Positive
AFG18	+	+	B_1	+	+	+	+	+	Positive
AFG19	+	+	-	+	+	+	+	+	Positive
AFG20	-	-	-	-	-	+	+	+	Negative
AFG24	V	V	B_1, B_2	+	+	+	+	+	Positive

Table 3. Contd.

Isolate code	Conventional Methods		TLC	ELISA	Quadruplex PCR				Aflatoxin production
	UV Test	AV Test			*aflR*	*omt-A*	*ver-1*	*nor-1*	
AFG25	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG26	-	-	-	-	-	+	+	+	Negative
AFG27	+	+	B_1	+	+	+	+	+	Positive
AFG28	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG29	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG30	+	+	B_1	+	+	+	+	+	Positive
AFG33	+	+	B_1	+	+	+	+	+	Positive
AFG35	+	V	B_1, B_2	+	+	+	+	+	Positive
AFG37	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG39	+	+	-	+	+	+	+	+	Positive
AFG48	-	-	-	-	-	+	+	+	Negative
AFG49	+	+	B_1	+	+	+	+	+	Positive
AFG50	+	+	B_1	+	+	+	+	+	Positive
AFG52	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG54	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG57	V	V	B_1, B_2	+	+	+	+	+	Positive
AFG62	+	V	B_1, B_2	+	+	+	+	+	Positive
AFG64	-	-	-	-	-	+	-	+	Negative
AFG65	-	-	-	-	-	+	+	-	Negative
AFG66	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG69	+	+	B_1, B_2	+	+	+	+	+	Positive
AFG71	-	-	-	-	-	+	+	+	Negative
AFGS15	+	+	B_1, B_2	+	+	+	+	+	Positive
AFGS17	+	+	-	+	+	+	+	+	Positive
AFGS31	+	+	B_1, B_2	+	+	+	+	+	Positive
AFGS33	+	+	B_1	+	+	+	+	+	Positive

V, Variable; UV, Ultra Violet test; AV, Ammonia Vapor test; TLC ,Thin Layer Chromatography; ELISA, Enzyme Linked Immuno Sorbent Assay; PCR, Polymerase Chain Reaction; AF, *Aspergillus flavus*.

that 20.3 and 16.5% of seed samples were contaminated with aflatoxin in 1999 and 2000, respectively. A highest content of 851.9 µg/kg of aflatoxin was recorded in sample collected from Anantapur district.

Similarly, we observed a varied degree of *A. flavus* infection and aflatoxin contamination in groundnut seeds collected from all over India under varied climatic conditions. From our results, seeds from Gujarat (G37) and Tamil Nadu (G24) showed highest *A. flavus* incidence but highest aflatoxin contamination of 28 ppb, was recorded in seeds collected from Karnataka (G57). However, we could not observe any correlation between *A. flavus* infection and aflatoxin production among

Figure 1. Groundnut with varied degree of seed-borne fungal infection showing various seedlings abnormalities. **a, b and c.** Cotyledons of emerging seedlings colonized by *A. niger* and *A. flavus*. **d – g.** Abnormal root formation, h. seed and seedling rot.

different seed samples collected. This is because the standard blotter method (SBM) which we followed did not give information about the intensity of *A. flavus* infection in seeds.

According to Crisan (1973), aflatoxin does not affect seed germination but it is inhibitory to hypocotyl elongation in lettuce and *Lepidium* sp. Some researchers reported that aflatoxin affects certain plants by inhibiting seed germination (Schoental and White, 1965), elongation of the hypocotyls or roots of developing seedlings or both (Reiss, 1971), by interference with chlorophyll synthesis (Schoental and White, 1965; Slowatizky et al., 1969) and by inhibiting the DNA-dependent RNA synthesis (Tripathi and Misra, 1981). Recently, Janardhan et al. (2011) observed reduced seed quality parameters in bean, red gram, green gram and black gram treated with culture filtrate of aflatoxigenic *A. flavus*. As we analyzed naturally infected samples, we did not observe any relation between *A. flavus* infection, aflatoxin contamination and seed quality variables in groundnut varieties. This is probably due to these seeds also got infected with some other storage and field fungi which affected seed quality variables (Table 2).

In nature, all *A. flavus* and *A. parasiticus* isolates are not aflatoxin producers. Because of mutations in the genes involved in the biosynthesis pathway of aflatoxin, several strains exist as non-aflatoxigenic (Criseo et al., 2001). Several methods are being used to differentiate between toxigenic and non toxigenic fungi which include cultural, analytical, immunological and molecular methods (Hara et al., 1974; Lemke et al., 1989). Competitive indirect ELISA distinguishes aflatoxigenic and non-aflatoxigenic strains precisely as it is known for its specificity and sensitivity. This method can detect aflatoxin as low as 0·2 to 2 ng/0·5 ml sample (Chu and Ueno, 1977), and in dot-ELISA, the sensitivity was improved from 500 pg to 1 pg by including an additional step of pre-incubation (Shashidhar and Rao, 1988). Hence in the present study, strains AFG19 and AFG39 were grouped under non-

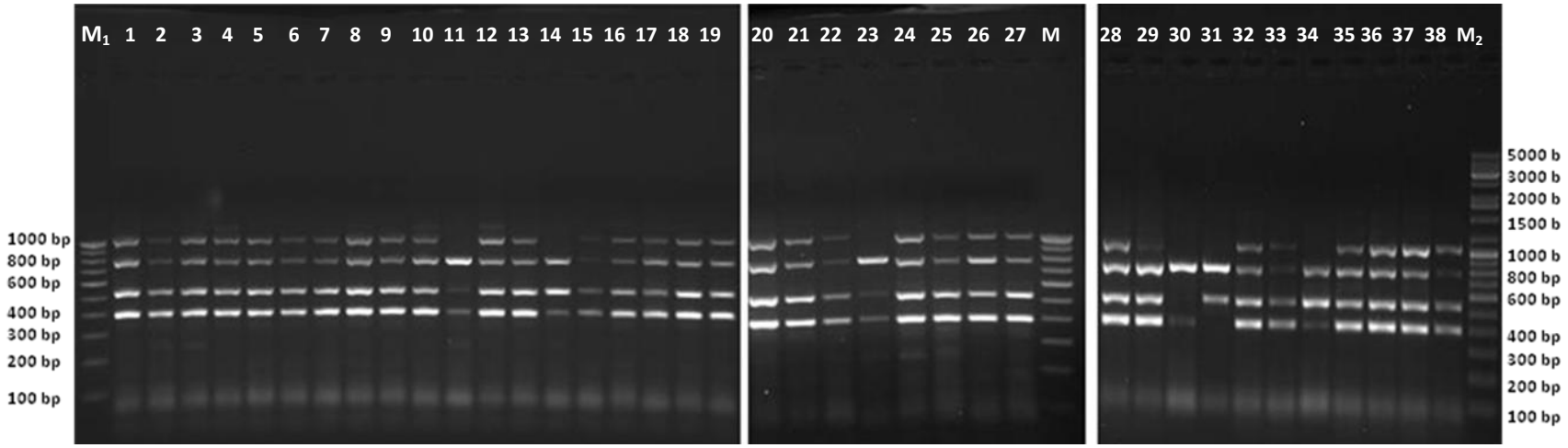

Figure 2. Agarose gel electrophoresis of Quadruplex PCR products. Lanes M_1: Molecular Marker (100 bp DNA Ladder) 1, G30/AF; 2, GS31/AF; 3, G1/AF; 4, G37/AF; 5 G19/AF; 6, G33/AF; 7, G29/AF; 8, G14/AF; 9 ,G25/AF; 10, G24/AF; 11, G27/AF; 12, G39/AF; 13, G16/AF; 14, G50/AF; 15, G54/AF; 16, G28/AF; 17, G48/AF; 18 ,G52/AF; 19, G49/AF; 20, G12/AF; 21, G35/AF; 22, G15/AF; 23, G13/AF; 24, G62/AF; 25, G8/AF; 26 ,G11/AF; 27, GS15/AF; M: molecular size markers (Genei 100 bp ladder); 28, GS17/AF; 29, G18/AF; 30, G57/AF; 31, G20/AF; 32, G65/AF; 33, G66/AF; 34, G64/AF; 35, G71/AF; 36, G69/AF; 37, GS33/AF; 38, G26/AF; M_2: Molecular Marker (Genei; medium range DNA ruler).

aflatoxigenic in TLC analysis which were found toxigenic when analyzed through ELISA. Multiplex PCR has proved to be very precise and a rapid bimolecular technique for detecting aflatoxigenic strain of *A. flavus* and *A. parastiticus.* But it does not always discriminate between aflatoxigenic and non aflatoxigenic strains. Criseo et al. (2008) observed some non-aflatoxigenic strains having complete pattern with four bands; could not distinguish these strains from the aflatoxigenic strain in which a quadruplex pattern is always present. With strain AFG15, we observed similar results, where all four bands were PCR amplified with specific primers but the strain was found negative for aflatoxin production when analyzed by cultural, TLC and ciELISA. Hence, to confirm the non toxigenicity of *Aspergillus* isolates, it is necessary to ascertain it by multiple methods rather than single method.

Preharvest aflatoxin contamination was reduced to between 80 to 95% in corn when non-aflatoxigenic strains were used to competitively excluse aflatoxigenic strains under field conditions. Further, the potential of these biocontrol non-aflatoxigenic strains were proved under storage conditions (Brown et al., 1991). The biocontrol strain K49 and AF36 were successfully used to reduce aflatoxin contamination of corn and cotton in field conditions (Abbas et al., 2006; Cotty, 1994). It was reported that, single nucleotide polymorphism (SNP) in polyketide synthase gene of aflatoxin biosynthesis gene cluster is responsible for non-aflatoxigenicity of strain AF36 and K49 (Ehrlich and Cotty, 2004; Chang et al., 2012). Afla-guard® a commercial product for the biocontrol of aflatoxin and aflatoxigenic fungus contains active ingredient *A. flavus* NRRL21882, which is a non-aflatoxigenic strain. Studies regarding the screening and characterization of potential atoxigenic strains and its further utilization in managing aflatoxin in ecomomically important crops under field and storage conditions are neglected in India.

From the above conducted survey and laboratory experiments, it was revealed that, seeds used for cultivation of groundnut in India is severely affected with aflatoxin and toxigenic fungi which play a major role in decreased quality and quantitiy of seed and grain production and adversely affect the human and animal health. In order to solve this problem, non-aflatoxigenic strains isolated in the present study will be further characterized,

studied under laboratory, greenhouse and field conditions for their biocontrol potential. Effective non-aflatoxigenci strains will be formulated and commercialized for wide applications.

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