

Occurrence of Mycotoxins and Toxigenic Fungi in Groundnut (*Arachis hypogaea* L.) Seeds in Andhra Pradesh, India

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Andhra Pradesh is one of the major groundnut growing states in India. A total of 182 groundnut samples collected at harvesting from farmers fields in five districts, namely; Anantapur, Chittoor, Cuddapah, Kurnool, and Mahaboobnagar, during 1999 and 2000 rainy seasons were evaluated for the presence of mycotoxins (both aflatoxins and zearalenone) and toxigenic fungi. In samples collected from each district, average seed infection by *Aspergillus flavus* and *Fusarium* spp. was 11.9-18.3% and 5.6-12.8% in 1999, and 9.5-14.1% and 9.4-11.9% in 2000, respectively. Among the samples collected, 20.3% and 16.5% were contaminated with aflatoxin in 1999 and 2000, respectively, and in 11.4% and 8.7% of the seed samples collected in two seasons, the aflatoxin content was >30 µg/kg. An alarming aflatoxin content of 851.9 µg/kg was found in samples collected from Anantapur district during the rainy season in 1999. Zearalenone was not detected in any of the samples collected in 1999, while 2 out of 103 samples collected in 2000 were contaminated with 35.1 and 129.4 µg/kg. Under *in vitro* cultural conditions, 35.8% of the 173 *A. flavus* isolates collected from the groundnut samples produced aflatoxins at concentrations of 94.3-1598.6 ng/ml and 3% of the 266 *Fusarium* spp. isolates produced 98.1-847.3 µg/g of zearalenone. The results emphasize the need for a more systematic and regular monitoring of pre-harvest aflatoxin contamination.

Keywords : aflatoxin, *Arachis hypogaea*, *Aspergillus flavus*, *Fusarium*, zearalenone.

Mycotoxins, the secondary metabolites of toxigenic fungi, commonly occur in various agricultural products including food and feed stuffs and are a potential threat to the health of humans and animals. Because of the associated health risks of mycotoxin consumption, their presence in many foodstuffs has drawn the attention of scientists and the

general public in several parts of the world.

Groundnut is an important legume crop in many tropical and subtropical areas of the world, and mycotoxin contamination causes economic losses in groundnut processing and export. Aflatoxins, a group of mycotoxins produced by *Aspergillus flavus* Link ex Fries, are the major mycotoxins that contaminate groundnuts and affect their quality. Aflatoxins as potent carcinogenic metabolites are the most toxic among several mycotoxins contaminating food and feed commodities (Campbell and Stoloff, 1974). Aflatoxins are also hepatotoxic and teratogenic. Aflatoxins present in groundnuts are relatively heat stable and are not eliminated completely by ordinary cooking procedures (Lee et al., 1969). *A. flavus* invades groundnut seeds both at pre-harvest growth stages and at post-harvest drying/curing and storage, producing aflatoxins B₁, B₂, G₁, and G₂ (Keenan and Savage, 1994).

Zearalenone or F-2 toxin [6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic acid lactone] is a non-steroidal, oestrogenic toxin produced by various *Fusarium* spp. including *F. graminearum*, *F. oxysporum*, *F. roseum*, *F. semitectum*, and *F. tricinctum* in various feed stuffs, mainly cereals (Kim et al., 1993; Tanaka et al., 1988). Zearalenone causes hyperestrogenism in farm animals and humans (Goodman et al., 1987). It also causes noticeable histopathological changes in liver, kidneys, lungs, heart, adrenal glands, spleen, and uterus of farm animals. *Fusarium* spp. are important among the common flora of groundnut seeds, and causes pod rots (Mehan et al., 1981). The presence of zearalenone in groundnuts has been reported in the past (Mehan et al., 1985).

Andhra Pradesh is one of the biggest groundnut growing states in India. The crop is grown on a large scale in the four districts of Rayalaseema region, namely; Anantapur, Chittoor, Cuddapah and Kurnool, and in Mahaboobnagar district of Telangana region. The total groundnut producing area in these five districts is approximately 1.75 million ha and the crop production is approximately 1.7 million tons (Anonymous, 1998). Apart from its use as food and oil,

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groundnut is also an important source of cash and cattle feed for farmers in these districts. The crop is grown and managed mostly by resource-poor farmers. These farmers are usually not aware of appropriate cultural management practices, and use local cultivars that are highly susceptible to be attacked by both foliar and soil-borne fungal pathogens. The average rainfall in these districts is 570–1090 mm per year, making these areas prone to drought almost every year, a condition favorable to aflatoxin infection. Limited monitoring and surveillance activities for mycotoxin contamination have been conducted in these districts.

The present study was undertaken to obtain preliminary information on aflatoxin and zearalenone contamination, and the presence of toxigenic *A. flavus* and *Fusarium* spp. in groundnuts from the five districts.

Materials and Methods

Sample collection. Seventy-nine and 103 groundnut pod samples were collected at harvest from the farmers' fields in Anantapur, Chittoor, Cuddapah, Kurnool, and Mahaboobnagar districts of Andhra Pradesh in 1999 and 2000, respectively. In each field, the sampled plants were randomly selected from five to six locations and the collected pods were pooled. The samples collected during the two crop seasons were numbered from GN 1 to GN 182. Approximately 1 kg of pods were collected from each field, brought to the laboratory, and dried in paper bags. Pods were then hand shelled and divided into two sub-samples. Sub-samples selected to examine for seed infection were stored at 4°C and sub-samples to be analyzed for mycotoxin contamination were stored at -18°C.

Determination of seed infection. Seeds were surface sterilized in 1% sodium hypochlorite solution for 10 minutes and then rinsed thrice with sterile distilled water (SDW). The surface sterilized seeds were aseptically plated on 90 mm diameter petri plates containing modified Czapek Dox agar medium with Rose bengal (30 mg/L) and streptomycin sulphate (0.2 g/L). Ten seeds were plated on each petri plate and a total of 50 seeds per sample were tested for seed infection. The plates were incubated at $28 \pm 1^\circ\text{C}$ for 7 days and then observed under a stereomicroscope for seed colonization by *Aspergillus flavus* or *Fusarium* spp. Based on cultural and morphological characteristics, the number of seeds colonized by *A. flavus* or *Fusarium* spp. was recorded separately.

Isolation and identification of *Aspergillus flavus* and *Fusarium* spp. strains. From each contaminated sample, one randomly selected *A. flavus* strain and one or more *Fusarium* spp. was isolated. A total of 173 *A. flavus* and 266 *Fusarium* spp isolates were obtained and single spore isolates were maintained on potato dextrose agar (PDA) at 4°C. *Fusarium* spp. was identified according to the classification scheme of Nelson et al. (1983).

Aflatoxin extraction and analysis. One hundred g of groundnut seed, 500 ml of methanol-water (55:45 v/v), 200 ml of hexane, and 4 g of NaCl were added and blended in a waring blender. The

mixture was allowed to settle and 25 ml of the aqueous methanol phase was transferred to a separating funnel, and extracted with 25 ml of chloroform followed by extraction with 10 ml of chloroform (Anonymous, 1975). The pooled chloroform phase was subjected to evaporation using a rotary vacuum evaporator and the final residue was dissolved in 100 µl of chloroform and used for thin-layer chromatography (TLC) analysis. From each sample collected, two sub-samples were analyzed for aflatoxin contamination. In this study, TLC was used for analysis of mycotoxins as it is simple, rapid, and less expensive (Soares, 1992).

Ten µl of the extracted samples were spotted on glass plates coated with silica gel G. Standard aflatoxin compounds (Sigma Chemical Co., St. Louis, MO, USA) dissolved in chloroform (10 µg/ml) were spotted along with the test samples. The plates were developed with chloroform:acetone (90:10, v/v), dried and observed for fluorescence under ultra violet (UV) light at 365 nm. Aflatoxin contamination in the test samples was detected by comparing the R_f values with standard aflatoxin compounds. Presence of aflatoxins was further confirmed by development of yellow spots upon spraying the TLC plates with 30% H_2SO_4 .

For quantitative determination of aflatoxins, a separate set of TLC plates spotted with the test samples was developed using the method previously described. Silica gel of each spot was scrapped by observing under UV light and collected individually in clean dry centrifuge tubes. Aflatoxins were extracted into methanol by adding 5 ml of cold methanol to each tube, and these tubes were then centrifuged at 5000 rpm for 3 minutes. The optical density of the methanolic solution was measured at 360 nm for estimation of aflatoxins B_1 and G_1 and at 362 nm for estimation of aflatoxins B_2 and G_2 . Aflatoxin content in each sample was calculated by using the formula: aflatoxin concentration = $(D \times M \times 10^6) / (E \times L \times 1000)$ mg/ml, where D is the optical density; M is the molecular weight of aflatoxin; E is the molar extinction coefficient; and L is the path length of the cubet (Nabney and Nesbitt, 1965).

Toxigenicity of *A. flavus* strains. Spores from 8-day-old cultures of each *A. flavus* isolate grown on PDA were washed into SDW containing 0.01% Tween 80. The spore concentration was adjusted to 10^5 spores/ml using a haemocytometer, and 500 µl of this spore suspension was added to 50 ml of semi-synthetic liquid medium in 250 ml Erlenmeyer flasks. One liter of the semi-synthetic liquid medium contained the following: sucrose, 200 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KNO_3 , 3.0 g; and yeast extract, 7.0 g (Diener and Davis, 1966). The flasks were incubated at $28 \pm 1^\circ\text{C}$ on a mechanical shaker at 120 rpm for 20 days. The cultures were then filtered through Whatman's filter paper No. 1 and the culture filtrates were collected. Three flasks were maintained for each *A. flavus* isolate and the experiment was repeated once. To 40 ml of culture filtrate, 20 ml of chloroform was added and shaken for 5 minutes in a separating funnel. The chloroform layer was collected and washed with 10 ml of hexane. Finally, the chloroform was evaporated to dryness on a rotary vacuum evaporator and the residue was dissolved in 100 µl of chloroform and used for analysis of aflatoxins by TLC.

Analysis of zearalenone contamination. Two replicates of seed samples 100 g each were finely ground in a waring blender for 5

minutes and moistened with 30 ml of water. The moistened sample was extracted thrice with 250 ml ethyl acetate for 30 minutes with occasional shaking. The ethyl acetate extract was filtered, evaporated, and dissolved in 25 ml of chloroform (Mirocha et al., 1974). The samples were defatted by partitioning between acetonitrile and petroleum ether. Finally, the chloroform was evaporated to near dryness, then the residue was dissolved in 100 µl acetone and used for TLC analysis.

Ten µl of each sample was spotted on glass plates coated with activated silica gel G and the plates were developed with chloroform: ethanol (97:3). The R_f values of separated spots in each test sample were compared with that of standard zearalenone compound (Sigma Chemical Co., St. Louis, MO, USA) dissolved in acetone (1 mg/ml) and the plates were observed for fluorescence under UV light. Zearalenone fluoresces a blue-green color at 365 nm and greenish at 254 nm. Zearalenone spots in sample extracts were further confirmed by the development of an intense blue color after spraying with a mixture of 1% $K_3Fe(CN)_6$ and 2% $FeCl_3$ (1:1, v/v) solutions followed by 2 N HCl (Mirocha et al., 1974).

For quantification of zearalenone, the fluorescent zone from the plates developed with chloroform:ethanol (97:3, v/v) was removed, extracted with acetone, and evaporated to dryness. The residue was dissolved in 2.5 ml of ethanol (95%) and the spectrum was recorded between 220 and 330 nm. Zearalenone exhibits characteristic peaks at 236, 274 and 314 nm. The amount of zearalenone in each sample was determined from a standard curve prepared by plotting the absorbance of different concentrations of zearalenone at 274 nm.

Toxicogenicity of *Fusarium* spp. isolates. One ml conidial suspension (10^5 conidia/ml) of each isolate of *Fusarium* spp. obtained by washing the cultures grown on PDA for 8 days at 25°C with SDW containing 0.01% Tween 80 was inoculated into 30 g of autoclaved polished rice (with 45% moisture content before autoclaving) in 250 ml Erlenmeyer flasks (Mehan et al., 1985). The flasks were incubated at 25°C for 15 days, and then at

15°C for 30 days, with gentle shaking daily for the first 7 days. After incubation, the moldy rice in the flasks was dried at 40°C, powdered in a waring blender, and used for detection and quantification of zearalenone as described above. Three replicates were maintained for each isolate and the experiment was repeated once.

Results

Seed infection by *Aspergillus flavus* and *Fusarium* spp.

In individual samples evaluated, seed infection by *A. flavus* and *Fusarium* spp. ranged from 0-38% and 0-24%, respectively. The average seed infection by *A. flavus* was 14.8% and 12.4%, and by *Fusarium* spp., 9.1% and 10.3%, in samples collected during 1999 and 2000 rainy seasons, respectively. The mean seed infection by *A. flavus* and *Fusarium* spp. in samples collected from individual districts during the two crop seasons was 9.5-18.3% and 5.6-12.8%, respectively (Table 1).

Mycotoxin contamination. Aflatoxins were detected in samples collected from all five districts during the two crop seasons. Among the samples collected during 1999 and 2000 rainy seasons, 20.3% and 16.5% were contaminated with aflatoxins, and 11.4% and 8.7% contain aflatoxins at concentrations of over 30 µg/kg (Table 1). The total aflatoxin content in contaminated samples ranged from 4.2-851.9 µg/kg with different levels of aflatoxins B_1 , B_2 , G_1 , and G_2 . However aflatoxin B_1 was detected in all the contaminated samples (Table 2). Zearalenone was not detected in any of the samples collected in 1999. Two samples each collected from Cuddapah and Mahaboobnagar districts in 2000 contain zearalenone at concentrations of 35.1 and 129.4 µg/kg (Tables 1 and 3).

Table 1. Seed infection by *Aspergillus flavus* and *Fusarium* spp., and occurrence of pre-harvest mycotoxin contamination in groundnuts collected from five major groundnut growing districts (Anantapur, Chittoor, Cuddapah, Kurnool, and Mahaboobnagar) of Andhra Pradesh, India, during 1999 and 2000 rainy seasons

Year and season	District	Number of seed samples analyzed (Isolate no.)	Mean percent of seeds contaminated with		Number of samples contaminated with			
			<i>A. flavus</i>	<i>Fusarium</i> spp.	aflatoxins (µg/kg)			zearalenone (µg/kg)
					<30	30-100	>100	
1999 rainy season	Anantapur	22 (GN 1-GN 22)	18.3	12.8				
	Chittoor	16 (GN 23-GN 38)	16.7	10.8	1	2	1	0
	Cuddapah	12 (GN 39-GN 50)	14.1	8.5	2	0	0	0
	Kurnool	14 (GN 51-GN 64)	12.8	7.8	2	0	1	0
	Mahaboobnagar	15 (GN 65-GN 79)	11.9	5.6	1	0	1	0
2000 rainy season	Anantapur	41 (GN 80-GN 120)	14.1	9.4	3	1	2	0
	Chittoor	21 (GN 121-GN 141)	13.8	11.9	2	1	1	0
	Cuddapah	15 (GN 142-GN 156)	9.5	10.3	1	0	1	1
	Kurnool	12 (GN 157-GN 168)	11.4	9.4	1	1	0	0
	Mahaboobnagar	14 (GN 169-GN 182)	13.1	10.3	1	0	2	1

Table 2. Levels of pre-harvest aflatoxin contamination in groundnuts collected from five major groundnut growing districts (Anantapur, Chittoor, Cuddapha, Kurnool, and Mahaboobnagar) of Andhra Pradesh, India, during 1999 and 2000 rainy seasons

Samples contaminated with aflatoxins	Range of aflatoxin contamination ($\mu\text{g/kg}$)	No. of samples containing aflatoxin				Range of aflatoxin contamination ($\mu\text{g/kg}$) ^a			
		B ₁	B ₂	G ₁	G ₂	B ₁	B ₂	G ₁	G ₂
GN 5, GN 27, GN 39, GN 46, GN 53, GN 64, GN 72, GN 81, GN 86, GN 88, GN 128, GN 135, GN 155, GN 159 and GN 179	<30	15	1	0	0	4.2-27.9	0-4.8	0	0
GN 12, GN 15, GN 27, GN 28, GN 118, GN 130 and GN 163	30-100	7	3	2	1	28.6-84.3	0-19.3	0-24.6	0-4.9
GN 9, GN 10, GN 35, GN 62, GN 75, GN 92, GN 108, GN 122, GN 147, GN 172 and GN 174	>100	11	7	5	2	110.6-608.2	0-159.8	0-259.8	0-42.3

^aThe values presented are the means of two replicates. Thin layer chromatography was used to determine the aflatoxin contamination.

Table 3. Levels of pre-harvest zearalenone contamination in groundnuts collected from five major groundnut growing districts (Anantapur, Chittoor, Cuddapha, Kurnool, and Mahaboobnagar) of Andhra Pradesh, India, during 1999 and 2000 rainy seasons

Groundnut sample	Zearalenone contamination ($\mu\text{g/kg}$) ^a
GN 148	35.1 \pm 5.8
GN 176	129.4 \pm 14.3

^aThe values presented are the means of two replicates. Thin layer chromatography was used to determine the zearalenone content in groundnut seeds. Zearalenone was not detected in any of the samples collected during 1999 rainy season.

Toxigenic potential of *A. flavus* and *Fusarium* spp. isolates. Of the 173 *A. flavus* isolates tested for *in vitro* toxigenicity, 111 (64.2%) were observed to be non-toxicogenic and 62 isolates (35.8%) produced aflatoxins (Table 4). Of the 62 toxigenic isolates, all produced aflatoxin B₁, 39 isolates (62.9%) produced aflatoxin B₂, 27 isolates (43.5%) produced aflatoxin G₁, and 19 strains (30.6%) produced aflatoxin G₂. The toxigenic potential of these

strains ranged from 94.3-1598.6 ng/ml, and the mean aflatoxin production is 1029.3 ng/ml (Table 4). Of the 266 *Fusarium* spp. isolated from the groundnut seeds, only 8 strains (3%) produced zearalenone *in vitro* and were identified as *F. oxysporum* and *F. moniliforme*. The production of zearalenone by these strains was in the range of 98.1-847.3 $\mu\text{g/g}$ (Table 5).

Discussion

In the present investigation, 20.3% and 16.5% samples collected during 1999 and 2000 rainy seasons were contaminated with aflatoxins. Pre-harvest aflatoxin contamination of groundnuts was reported to occur commonly in several parts of the world (Joffe, 1970). Little information is available on the aflatoxin contamination of groundnuts grown in Andhra Pradesh. In a previous study, it was observed that 20-40% of the groundnut samples collected from Andhra Pradesh, Gujarat, and Tamilnadu states of India were aflatoxin contaminated (Anonymous, 1967).

Table 4. *In vitro* toxigenicity of *Aspergillus flavus* isolates grown on semi-synthetic liquid medium at 120 rpm for 20 days at 28 \pm 1°C

Number of isolates tested	Number of isolates producing					Total aflatoxins
	No aflatoxin	Aflatoxin B ₁	Aflatoxin B ₂	Aflatoxin G ₁	Aflatoxin G ₂	
173	111	62	39	27	19	
Production range (ng/ml) ^a		83.9-823.3	7.8-168.3	15.8-792.3	5.6-121.9	94.3-1598.6
Average production (ng/ml)		628.2 \pm 328.9	89.3 \pm 63.9	429.1 \pm 329.8	52.9 \pm 50.8	1029.3 \pm 789.2

^aThe values presented are the means of six replicates in two separate sets of experiments.

Table 5. *In vitro* toxigenicity of *Fusarium* spp. isolated from groundnut seed and grown on polished rice

No. of isolates tested	No. of isolates producing zearalenone	Identification	Production range ($\mu\text{g/g}$) ^a	Average production ($\mu\text{g/g}$) ^a
266	3	<i>F. moniliforme</i>	128.4-565.3	342.8 \pm 158.7
	5	<i>F. oxysporum</i>	98.1-847.3	593.1 \pm 228.5

^aThe values presented are the means of six replicates in two separate sets of experiments.

Bhat et al. (1996) reported that among the samples collected from 11 states of India, 44.9% and 37.4% samples contain aflatoxins of over 5 µg/kg and 15 µg/kg, respectively, and among the samples collected from Andhra Pradesh, 15.2% were contaminated with aflatoxins. Results of this study support these earlier findings.

Aflatoxin contamination was more frequent in samples collected in 1999 than those in 2000. This could be due to the severe drought condition due to lack of rainfall from mid crop season until harvesting in 1999. The invasion of groundnut kernels by *A. flavus* and subsequent aflatoxin production was largely dependent on the soil temperature and moisture levels 30–40 days before harvesting (Hill et al., 1983). There was a positive correlation between the end-season drought and invasion of groundnut seeds by *A. flavus* and subsequent aflatoxin contamination (Diener et al., 1987). Apart from the end-season drought, some other factors like soil type, groundnut cultivar, seed size, period of sampling, etc. also influence the levels of aflatoxin contamination. In general, 11.4% and 8.7% of seed samples collected during 1999 and 2000 rainy seasons contained aflatoxins in excess of the tolerance level of 30 µg/kg set by the Government of India. These results emphasize the need to educate farmers to adopt crop management practices that can minimize aflatoxin contaminations. Though aflatoxins B₂, G₁, and G₂ were not present in all the contaminated samples, there is a possibility that these aflatoxins might be produced in post-harvest stages.

Further, it was observed that only 2 of the 182 samples were contaminated with zearalenone. This is the first report on the occurrence of zearalenone in groundnuts from these districts. The presence of zearalenone contamination in less than 2% of samples indicates that this toxin is of minor importance in groundnut in Andhra Pradesh. In a previous study, 4 of the 204 groundnut samples collected from farm fields of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh were reported to contain zearalenone in the range of 0.72–1.84 mg/kg (Mehan et al., 1985). This present and earlier studies confirm that zearalenone is currently not a potential toxin in groundnut.

Of all the *A. flavus* isolates collected and tested, 35.8% were toxigenic *in vitro*. Earlier, it was reported that 72 (30%) out of 240 *A. flavus* isolates from Indian groundnut samples were toxigenic (Subrahmanyam and Rao, 1974). Joffe (1969) observed that >90% of the *A. flavus* isolates evaluated were toxigenic. In the present study, each isolate was collected from an individual sample, and 62 (34.1%) samples were found to be contaminated with toxigenic *A. flavus* strains. However, aflatoxins were detected only in 33 (18.1%) samples. In a similar study, Sanders et al. (1981) observed the presence of high *A. flavus* populations without

the production of aflatoxins. These results imply that invasion, growth, and toxigenicity of *A. flavus* are separate processes or at least regulated in different ways, and that toxigenicity requires a narrower range of temperature and moisture condition for its growth. The toxigenic *A. flavus* strains present in certain samples may produce aflatoxins during post-harvest stages, depending on the conditions of drying and storage. Improper drying of groundnuts allowing them to retain high moisture content and poor storage conditions favors post-harvest aflatoxin contamination.

In this study, toxigenic *A. flavus* isolates differed in the quantity of aflatoxins produced and also in their potential to produce different types of aflatoxins. It was generally observed in several studies that *A. flavus* isolates differ in their potential to produce various aflatoxins, and that production also depends on the available substrate and environmental conditions. Of 1626 *A. flavus* isolates previously tested for *in vitro* toxigenicity, 1.7% produced only aflatoxin B₁, 95% produced both aflatoxins B₁ and B₂, and 8.4% produced aflatoxins B₁, B₂, G₁, and G₂ (Joffe, 1970).

Of the 266 isolates of *Fusarium* spp. tested *in vitro* for production of zearalenone, only 8 (3%) isolates, identified as *F. oxysporum* and *F. moniliforme*, produced zearalenone. These two species of *Fusarium* isolated from groundnut seeds were earlier reported to produce zearalenone (Mehan et al., 1985). The non-production of zearalenone by most of the isolates of *Fusarium* spp. is consistent with earlier reports. Of the 67 isolates of *Fusarium* spp. isolated from groundnut seed, only one isolate of *F. oxysporum* produced zearalenone (Mehan et al., 1985).

The preliminary information obtained in this study indicates that there is a need for a more systematic and regular evaluation of the occurrence of both aflatoxins and toxigenic *A. flavus* strains in groundnuts grown in the five major groundnut-growing districts. The information obtained in this study can be used to identify appropriate strategies for management of pre-harvest aflatoxin contamination. It was generally observed that most of the groundnut farmers in these districts are not aware of aflatoxin contamination and its associated health risks, and that they need to be educated on adopting integrated crop management practices to minimize aflatoxin contamination.

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