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Aflatoxin Contamination of Major Groundnut Growing Districts of Odisha and Morphological Diversity of *Aspergillus flavus* isolates

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ABSTRACT: The groundnut (*Arachis hypogaea* L.) is one of the most significant edible crops in India, containing 46% oil and serving as a source of nutrition for humans and animals. Aflatoxin contamination is a severe issue that has a significant effect on the global groundnut industry and poses hazards to public health. The present investigation was carried out to know the occurrence of aflatoxin contamination in groundnut pod samples in Odisha. Study the *Aspergillus flavus* cultural variability. The survey conducted during rabi season 2017-18 from 40 blocks from 10 major groundnut growing districts of Odisha, there was a significant difference with respect to kernel aflatoxin content. Block-wise, kernel aflatoxin contamination was highest (1296 µg kg^{-1}) in kernels collected from Boden (Naupada) (OGS-21), followed by OGS-1 sample from Balangir (1235 µg kg^{1}) and OGS-32 sample from Sambalpur (1142 µg kg^{-1}). A total of 15 *A. flavus* isolates have been isolated using the agar plate method from infected groundnut kernels. The *Aspergillus flavus* Isolates showed variation in their cultural and morphological characters. The colony colour *of A. flavus* isolates ranged from dark green to light green and the colony margins ranged from green to white and from smooth to irregular in appearance. Four isolates only produced sclerotia exhibiting different sclerotial patterns, including concentric, irregular, and peripheral. There was also variation in the number of sclerotia per plate and the size of the sclerotia.

Keywords: *Aspergillus flavus*, sclerotia, aflatoxin contamination.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is considered to be one of the most significant oilseed crops in the world. It is ranked as the thirteenth most important food crop and the fourth most important oilseed crop in the world. Odisha's production area is 1200 ha, with productivity and output rates of 2300 t/ha and 1920 kg/ha, respectively. It is mostly produced in the sandy soils of the Bolangir, Dhenkanal, Angul, Ganjam, Kalahandi, Nuapda, and Bargarh districts of Odisha (Directorate of Economics and Statistics, 2015). *Aspergillus* spp. are the most problematic in terms of pre- and post-harvest losses in the field. *Aspergillus niger* causes root rot when combined with other fungi to form root rot complex and in storage conditions *Aspergillus flavus* causes losses by producing aflatoxins that render kernels unsuitable for consumption by both humans and livestock. This will undoubtedly have an effect on agricultural productivity, particularly for staple commodities such as maize and legumes, as well as the interface between plants, insect pests, and fungal infections of staple foods. In many crop soils, toxigenic and nontoxigenic strains of *A. flavus* coexist in the groundnut crop ecosystem. Due to the frequent occurrences of false positives and false negatives, a polyphasic approach would be the optimal method for accurate detection Almoammar *et al.* (2013). However, because the maintenance of molecular and analytical laboratories is costly and requires a high level of expertise, cultural methods of aflatoxigenic strain detection are acquiring momentum in many regions of the globe. Immunochemical methods employing antigen-antibody reactions are among the most selective, sensitive, and rapid techniques for detecting Aflatoxins, while also being relatively inexpensive. Recently, they are increasingly employed for the effect of *A. flavus* on seed quality, i.e., aflatoxin levels in groundnut seed samples, was determined using an indirect competitive ELISA method (Enzyme-Linked Immuno Sorbent Assay).

MATERIALS AND METHODS

Groundnut extracts were diluted to 10% and then used to generate AFB1 standards with concentrations ranging

from 0.1 to 50 ng/ml. These extracts did not contain any aflatoxin. Groundnut kernels that were healthy and free of aflatoxin were crushed into a powder and then extracted in 100 ml of methanol that contained 0.5% potassium chloride. After filtering, the extract was then diluted to a 1:10 ratio in PBST–BSA. This was used as a diluent for aflatoxin standards.

A. Indirect competitive ELISA method

There are 5 steps in indirect competitive ELISA test *viz*.,

(i) Coating. Loading ELISA (all 60 wells) with 150µl/well of AfB1-BSA conjugate. Incubate it overnight at 37°C for 1 hour. Wash the plate twice with PBS-T.

(ii) Blocking. Dilute healthy groundnut extract (HGN) with 0.2 per cent BSA (10 times). Simultaneously prepare pure toxin (AfB1) by diluting with the above prepared HGN extract. Add 100µl of diluted pure toxin to first two columns and first two rows. Then add 100µl of diluted HGN extract to remaining wells of first two rows. Mix the solution from 3rd well to 20th well (serial dilution) to reduce pure toxin concentration from 50 mg to 0.1 mg. Add 90µl of 0.2 per cent BSA to the remaining wells. Then add 10 µl of seed sample extract to wells from 21-60 (in replicates). Then add 50 µl of antiserum (prepared in step II) to all wells. Incubate at 37°C for 1 hour. Wash the plate thrice with PBS-T.

(iii) Conjugation. Load the plate with 150 µl goat antirabbit IgG (1:2000 dilution in 0.2% BSA) per well. Incubate at 37°C for 1 hour. Wash the plate thrice with PBS-T.

(iv) Substrate. Load 150 µl of substrate buffer per well (5 mg PNPP per 10 ml of 10 per cent diethanolamine) to all wells. Simultaneously add substrate alone (10% diethanolamine) to A1 well as blank. Incubate at room temperature under dark condition for 1 hour.

Measure absorbance at 405 nm in ELISA reader.

Plot the negative longer theme graph mean of A and B taking absorbance values on X – axis and toxin concentration (µg/ml) on Y-axis.

AfB1 in sample was calculated by using the formula.

AfB1 (µg/kg or ppb) = (A x D x E) / G

A: Value on Y-axis

D: Times dilution with buffer (0.2% BSA) (10)

E: Extraction solvent volume used (methanol) (100)

S: Sample weight (20 g)

B. Isolation, purification and maintenance of isolates of Aspergillus spp.

Pathogens associated with groundnuts were isolated from infected samples collected from various groundnut-growing states. Groundnut kernels were surface-sterilized with 1% sodium hypochlorite for one minute and subsequently rinsed twice with distilled water. Four surface-sterilized groundnut kernels were placed equidistantly on potato dextrose agar (PDA) medium in each Petri dish under strict aseptic conditions (Inside the laminar air flow). For four to five days, plates were incubated at 25±2 °C in a BOD incubator to obtain fungal growth. Different isolates of *Aspergillus flavus* were purified using the single spore isolation method. After four days of culture using an inoculation cycle, a single spore of culture was extracted and inoculated into another Petri dish. For each culture, the inoculation loop was completely **i**ncinerated to prevent cross-contamination. For further research, the purified cultures were sub cultured on PDA slant. A total of 15 *Aspergillus flavus* isolates were obtained.

C. Morphological characterization

(i) Morphological characteristics. Comparison of morphological characteristics of *Aspergillus flavus* was done by sub culturing the isolates of damaged groundnut samples on PDA medium in Petri plates. Actively growing 5mm mycelial disc obtained from 4 days old culture of *Aspergillus flavus*. were used to inoculate Petri plates contained PDA medium. Each isolate inoculated separately and incubated at 25±2 °C. Each isolate was replicated thrice. The observations on colour and pigmentation of colony, size of the conidia and conidiophores were recorded up to seven days at regular intervals of 24 hours

(ii) Measurement of conidia, conidiophore and sclerotiasize. The size of conidia and conidiophore were measured by growing each isolate on Petri dish containing PDA medium and kept in incubator at 25±2 °C for 7 days. Slide was prepared in sterile water after 7 days of incubation and observed under different magnification under compound microscope. Image analysis software was used to measure size of conidia and conidiophores of each isolate separately. The size of conidia and conidiophores were measured at 400X and 40X and mean value was recorded

RESULT AND DISCUSSION

A. Survey and assessment of Aflatoxin contamination in groundnut samples

The survey was conducted during the rabi of 2018 to collect the groundnut samples from various farmers' fields from different districts of Odisha, Total of 40 samples were collected from all the places. Among 40 samples 15 isolates of *Aspergillus flavus* and 3 isolates of *Aspergillus niger* isolated by the agar plate method. 15 isolates were subjected to cultural and microscopic examinations in order to confirm species identification. All of the isolates were grown in Petri plates on the PDA media to observe colony colour and pigmentation. Isolates were identified morphologically with the help of Laboratory Guide to Common *Aspergillus* speci because they were found to be non-toxigenic, they were not continued on with the experiment. It was determined that all 15 isolates were *Aspergillus*, and while there was minimal variance in colony colour among them, there was some diversity in colour such as yellow green, and dark green with or without white perimeter. During rabi season survey conducted from 40 blocks from 10 major groundnut growing districts of Odisha, there was a significant difference with respect to kernel aflatoxin content (P 0.0035). Block-wise, kernal aflatoxin contamination was highest (1296 µg kg^{-1}) in kernels collected from Boden (Naupada) (OGS-21), followed by OGS-1 sample from Balangir (1235 µg kg^{-1}) and OGS-32 sample from Sambalpur (1142 µg

kg^{-1}) (Table 1). Overall, the aflatoxin levels in surveyed farmers field from Odisha ranging from 0 µg kg^{-1} to 1296 µg kg kg^{-1}. The pod samples from Maneswar, Jajpur, Delong were recorded low aflatoxin levels (0 µg kg^{-1}) followed by Pipilli 1.27µg kg^{-1}. The findings are supported by findings of Coulibaly *et al.* (2008); Hong *et al.* (2010); Chena *et al.* (2013); Sudini *et al.* (2015).

Table 1: Extent of aflatoxin contamination by *Aspergillus flavus* from important groundnut growing districts of Odisha.

District	Block	Sample	Total No. of Pods collected	Number of damaged pods	Number of Healthy pods	Pod Damage %	Isolation result*	Aflatoxin (µg/kg)
Balangir	Balangiri	OGS1	539	158	381	29.31	+	1235
	Belpara	OGS2	378	78	300	20.63	+	451
	Saintatal	OGS3	157	21	136	13.38	+	24
	Patnagarh	OGS4	283	17	266	6.01	-	1.34
Nabrongpur	Nabrongpur	OGS5	279	23	256	8.24	+	228
	Raighar	OGS6	289	17	272	5.88	-	2.01
	Umarkote	OGS7	317	21	296	6.62	+	66
	Nandahandi	OGS8	329	13	316	3.95	-	19
Bhadarak	Chandabali	OGS9	367	39	328	10.63	+	547
	Tihidi	OGS10	264	36	228	13.64	+	173
	Basudebpur	OGS11	489	16	473	3.27	-	1.56
	Bhadrak	OGS12	632	19	613	3.01	-	1.32
Jajpur	Jajpur	OGS13	329	16	313	4.86	-	0
	Bari	OGS14	625	179	446	28.64	+	716
	Rasulpur	OGS15	357	8	349	2.24	-	2.1
	Badachana	OGS16	264	9	255	3.41	-	1.3
Ganjam	Chhatarpur	OGS17	284	47	237	16.55	-	4.06
	Ganjam	OGS18	472	38	434	8.05	-	4.19
	Rengelunda	OGS19	289	31	258	10.73	+	4.28
	Khalikote	OGS20	356	13	343	3.65	-	4.1
Naupada	Boden	OGS21	578	181	397	31.31	+	1296
	Khariar	OGS22	289	48	241	16.61	-	353
	Sinapadi	OGS23	279	15	264	5.38	+	737
	Naupada	OGS24	189	9	180	4.76	-	27
Malakangir	Khairapur	OGS25	237	7	230	2.95	-	1.37
	Korkunda	OGS26	284	12	272	4.23	-	10
	Mathili	OGS27	118	6	112	5.08	-	11
	Kalimela	OGS28	183	50	133	27.32	+	1067
Sambalpur	Jojumurua	OGS29	183	7	176	3.83	-	21
	Rairakho	OGS30	289	48	241	16.61	+	298
	Maneswar	OGS31	293	4	289	1.37	-	0
	Sambalpur	OGS32	409	89	320	21.76	+	1142
Puri	Nimapara	OGS33	247	11	236	4.45	-	1.38
	Gop	OGS34	293	18	275	6.14	+	3.36
	Pipili	OGS35	258	13	245	5.04	-	1.27
	Delonga	OGS36	268	26	242	9.70	-	0.00
Mayurbhanj	Katipada	OGS37	269	16	253	5.95	-	12
	Udala	OGS38	283	14	269	4.95	-	1.39
	Khunta	OGS39	236	13	223	5.51	-	1.56
	Murunda	OGS40	416	28	388	6.73	-	1.67

*(+/- indicates isolated and not isolated respectively)

Table 2: Categized different blocks based on risk factor.

Categorization	Blocks
Very low risk (0-10 ppb)	Patnagarh, Raighar, Bhadrak, Basudebpur, Jajpur, Rasulpur, Badachana, Chhatarpur, Ganjam, Rengelunda, Khalikote, Khairapur, Korkunda, Maneswar, Nimapara, Gop, Pipili, Delonga Udala, Khunta, Murunda
Low risk (11-30 ppb)	Nandahandi, Saintatal, Naupada, Mathili, Jojumurua, Katipada
Moderate risk (30-100 ppb)	Umarkote
High risk (>100 ppb)	Balangiri, Belpara, Saintatal, Nabrongpur, Chandabali, Kalimela, Khariar, Rairakho, Sambalpur

B. Categorized blocks based on risk factor

In state of Odisha, based on aflatoxin contamination by *A. flavus*, the blocks wereBalangiri,Belpara, Saintatal, Nabrongpur, Chandabali, Kalimela, Khariar, Rairakho, Sambalpur categorized as high-risk zone. The blocks were Patnagarh, Raighar, Bhadrak, Basudebpur, Jajpur, Rasulpur, Badachana, Chhatarpur, Ganjam, Rengelunda, Khalikote, Khairapur, Korkunda, Maneswar, Nimapara, Gop, Pipili, Delonga Udala, Khunta, Murunda categorized as Very low risk (0-10 ppb) zone (Table 2).

Table 3: Designation of isolates obtained from different samples.

Sr. No.	Sample	Isolate designation	Block	District
1.	OGS1	oaf1	Balangiri	Balangir
2.	OGS2	oaf2	Belpara	Balangir
3.	OGS3	oaf3	Saintatal	Balangir
4.	OGS5	oaf4	Nabrongpur	Nabrongpur
5.	OGS7	oaf5	Umarkote	Nabrongpur
6.	OGS8	oaf6	Chandabali	Bhadarak
7.	OGS9	oaf7	Tihidi	Bhadarak
8.	OGS13	oaf8	Jajpur	Jajpur
9.	OGS14	oaf9	Bari	Jajpur
10.	OGS18	oaf10	Rengelunda	Ganjam
11.	OGS19	oaf11	Boden	Naupada
12.	OGS20	oaf12	Sinapadi	Naupada
13.	OGS34	oaf13	Kalimela	Malakangir
14.	OGS36	oaf14	Rairakho	Sambalpur
15.	OGS40	oaf15	Sambalpur	Sambalpur

C. Qualitative and quantitative characters of different isolates of Aspergillus spp.

(i) Qualitative characters. The data in the Table 4 indicates the colony colour and pigmentation produced by them. All the isolates were of light green in colour except the isolates oaf 1, oaf 3, oaf 8 and oaf 11 which are dark green. All the isolates colony margin green in colour except isolate oaf 14 which is white. Reverse pigmentation of 15 isolates varies lightly with light creamish to creamish colour. green with white periphery. The pigmentation of all the isolates varied between creamish yellow to creamish in colour. The isolates oaf 1, oaf 3, oaf 11 and oaf 12 have creamish yellow and rest of the isolates shown creamish colour pigmentation. The texture of sporulation varies from smooth to coarse. oaf 1, oaf 2, oaf 3, oaf 4, oaf 6, oaf 7, oaf 8, oaf 10, oaf 11, oaf 12, oaf 13, oaf 15 Smooth sporulation while isolates oaf 5, oaf 9 and oaf 14 coarse sporulation (Table 4). The current results are consistent with those of Guruprasad *et al.* (2014).

Table 4: Cultural (Qualitative) characters of *Aspergillus flavus* isolates.

Isolate	Colony colour	Colony reverse Pigmentation	Colour of the margin	Texture Sporulation
OAF1	Dark Green	Creamish	Green	Smooth
OAF2	Light Green	Creamish	Green	Smooth
OAF3	Dark Green	Creamish	Green	Smooth
OAF4	Light Green	Creamish	Green	Smooth
OAF5	Light Green	Creamish yellow	Green	Coarse
OAF6	Light Green	Creamish	Green	Smooth
OAF7	Light Green	Creamish	Green	Smooth
OAF8	Dark Green	Creamish	Green	Smooth
OAF9	Light Green	Creamish yellow	Green	Coarse
OAF10	Light Green	Creamish yellow	Green	Smooth
OAF11	Dark Green	Creamish	Green	Smooth
OAF12	Light Green	Creamish	Green	Smooth
OAF13	Light Green	Creamish	Green	Smooth
OAF14	Light Green	Creamish	White	Coarse
OAF15	Light Green	Creamish yellow	Green	Smooth

D. Quantitative characters

(i) Size of conidia and conidiophores. All 15 isolates were grown on PDA media for seven days and measurements were obtained using micrometry at 400X and 40X magnification to determine the size of conidia and conidiophores. Conidia were numerous in all isolates, and they were single celled, spherical in form, and transparent. The vesicle carries a chain of conidia on biseriate sterigmata, and the conidiophores were long with variable lengths. Conidia diameter of *A. flavus* varied from 19.75 µm to 31.95µm, according to the data provided in Table 4. The isolate oaf 3 had the largest conidial diameter of 31.95µm, followed by the isolates oaf 13 and oaf 12, which had 30.85µm and 30.65µm conidial diameters, respectively. The smallest conidial diameter of 19.75µm was observed in the isolate oaf 12 and it was followed by the isolates oaf 7 and oaf 4 having the conidial diameter of 20.00 µm and 21.55 µm

respectively. Along with the width of the conidiophore, the length of the conidiophore differed considerably across the isolates. All of the isolates' conidiophore lengths varied from 462.1µm to 1278.0µm. The isolate oaf 5 had the longest conidiophore length of 1278.0 µm, followed by oaf 15 and oaf13, which had conidiophore lengths of 1079.25 µm and 925.6µm, respectively. Isolate oaf8 has the shortest conidial length of 462.1µm, followed by isolates oaf 6 and oaf14, which have the conidiophore lengths of 497.8µm and 519.7µm, respectively. Similar to conidiophore length, conidiophore width varied from 15.95 µm to 25.7µm, with the isolate oaf 8 having the widest conidiophore width of 25.7 µm followed by oaf 12 and oaf 14 with conidiophore widths of 24.85 µm and 24.6 µm, respectively), and the isolates oaf 1 and oaf 7 with lowest conidiophore widths of 14.7 µm and 15.65 µm respectively (Table 5).

(ii) Sclerotial production. *A. flavus* isolates grown on PDA medium showed variation in sclerotia production. It was observed that 4 isolates produced sclerotia and remaining 11 isolates did not produce sclerotia). Variation in sclerotial pattern was observed among the isolates of *A. flavus*. The sclerotial pattern was classified as concentric, irregular to peripheral based on arrangement of sclerotia on Petri dish. Concentric type of sclerotial pattern was observed in *Aspergillus flavus* oaf 1 isolate viz., oaf 3 and oaf 9 showed peripheral pattern of sclerotia formation (n=2) whereas irregular type of sclerotia pattern was observed in isolate oaf 11. The number of sclerotia produced by *A. flavus* isolates varied from 38 to 198 with isolate oaf 11 produced highest number of sclerotia (198) and oaf 3 isolate produced lowest sclerotia number (38). The sclerotia were brown in colour. The *A. flavus* isolates showed variation in size of sclerotia with a range of 198.60 µm to 403.7 µm (Table 6). Based on sclerotial size, *A. flavus* isolates classified as L strain (> 400 µm) and S strain (< 400 µm). In the present study, the isolates *viz*., oaf 3, oaf 9, oaf 11 produced small sized sclerotia (< than 400 µm) whereas large sized sclerotia was observed only in oaf 1(403.7 µm) (Table 6). These results are similar to the findings of several scientists Singh *et al*. (2015); Gurupraad *et al*. (2014); Jamali *et al*. (2012).

Table 5: Quantitative characteristics of different isolates of *Aspergillus flavus* on potato dextrose agar medium.

Isolates	Conidia (µm)		Conidiopbore Size(µm)			
			**Length		*Width	
	Range	Mean	Range	Mean	Range	Mean
OAF1	25.6-33.6	29.6	835.7-978.9	907.3	12.6-16.8	14.7
OAF2	26.3-31.7	29	867.5-938.9	903.2	21.7-25.9	23.8
OAF3	28.4-35.5	31.95	521.8-658.9	590.35	18.9-24.8	21.85
OAF4	18.4-24.7	21.55	489.5-601.6	545.55	21.3-28.9	25.1
OAF5	25.6-31.3	28.45	1267-1289	1278	11.8-21.9	16.85
OAF6	25.7-28.4	27.05	482-513.6	497.8	13.7-23.9	18.8
OAF7	17.6-22.4	20	614-723.9	668.95	11.6-19.7	15.65
OAF8	24.7-31.4	28.05	418.6-505.6	462.1	22.6-28.8	25.7
OAF9	22.4-31.5	26.95	1132.8-608.9	870.85	15.6-19.87	17.735
OAF10	19.4-25.9	22.65	538.8-616.9	577.85	18.6-26.2	22.4
OAF11	17.8-21.7	19.75	525.8-632.1	578.95	14.8-21.8	18.3
OAF12	26.5-34.5	30.65	483.9-539.6	511.75	22.8-26.9	24.85
OAF13	27.8-33.9	30.85	867.3-983.9	925.6	13-18.9	15.95
OAF14	24.6-36.3	30.45	942.9-96.5	519.7	22.3-26.9	24.6
OAF15	17.4-26.7	22.05	1023.6-1134.9	1079.25	11.8-19.8	15.8

Table 6: Sclerotia characters of *Aspergilus flavus* isolates.

Isolate	Number	Pattern	Size (µm)	Colour
oaf1	89	Concentric	403.7	Brown
oaf3	38	Peripheral	328.7	Brown
oaf9	42	Peripheral	268.6	Brown
oaf11	198	Irregular	198.6	Brown

CONCLUSIONS

A high prevalence of kernel aflatoxin contamination was found in groundnut farmer seed samples. In state of Odisha, based on aflatoxin contamination by *A. flavus*, the blocks were Balangiri, Belpara, Saintatal, Nabrongpur, Chandabali, Kalimela, Khariar, Rairakho, Sambalpur categorized as high-risk zone (>100µg kg^{-1}). *A. flavus* isolates showing diversity in morphological characteristics.

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Conflict of Interest. None.

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