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Variability of *Aspergillus flavus* Link Ex. Fries in groundnut

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Abstract
Groundnut (*Arachis hypogaea* L.) is an important food crop for vegetable oil production. Groundnut suffers from many diseases among them *Aspergillus flavus* has the potential to infect in the field, Preharvest, postharvest, storage and during transit. They produce a potent toxin and carcinogenic substance called aflatoxin which has a great impact on human health. An experiment was conducted on the cultural, morphological and aflatoxigenic variability of *A. flavus* isolates from the seeds of different forty samples of groundnut and were named AF-1 to AF-40. Maximum mean colony diameter was recorded in eighteen isolates, whereas least in AF-37 after seven days of incubation. The colour of the mycelium ranged from yellowish green to dark green. The central part of the mycelium ranged from light green to dark green. The reverse site colony colour were light yellow, yellow, creamish yellow, greenish yellow, yellowish white and white. No sclerotia production was observed in any isolates of *A. flavus* in culture. The length of the conidiophore of *A. flavus* ranged from 240.6 to 1214.7 µm. Similarly, the width also ranges from 9.5 to 15.1 µm. Conidial diameter ranged from 3.4 to 7.5 µm. Conidial wall, observed from finely rough to smooth wall which was an important character in identification. Conidial shape was observed from globose to subglobose. During the aflatoxigenic test, isolates AF-6 was found highly toxigenic in nature and showed a dark red colour development.

Keywords: Variability, *Aspergillus flavus*, groundnut, aflatoxigenicity

Introduction
Groundnut (*Arachis hypogaea* L.) is an annual legume and also known as peanut. It is the thirteenth most important food crop of the world and third most important oil seed crop used for vegetable oil production. Groundnut is cultivated in the tropical and subtropical regions of the world. India stands first in area with 3.9 million hectares, second in production (6.7 million ton) and productivity of 1422 kgha^{-1}. In Gujarat, groundnut is grown on about 20.72 lakh hectares with a production of 54.64 lakh ton and an average productivity of 2637 kgha^{-1} (Anon., 2021) [3]. Groundnut seeds (kernels) contain 35.8- 54.2 per cent oil (Jambunathan *et al.*, 1985) [9], 16.2-36.0 per cent protein (Dwivedi *et al.*, 1990) [7], and 10-20 per cent carbohydrate (Salunkhe *et al.*, 1992) [15]. Groundnut crop suffers from many diseases, among the soil borne diseases, afla rot caused by *Aspergillus flavus* is an important disease in groundnut growing areas of the world (Klich, 2007) [10]. *A. flavus* is most common in warm temperate zones and environment with low water level and higher temperature.

Material and Methods
Collection of groundnut samples from North Gujarat Agroclimatic Zone
Forty samples of groundnut seeds were collected from different locations of groundnut growing areas of North Gujarat Agroclimatic Zone and brought to the laboratory for examination and isolation.

Isolation and purification of *A. flavus*
Forty different isolates of *A. flavus* were isolated from seeds of different samples of groundnut and named AF-1 to AF-40. The blotter paper technique was used for the isolation. For isolation, blotter paper was cut according to the size of Petri plate used and sterilized separately in autoclave at 121 ^{0}C (1.1 kgcm^{-2}) temperature and 15 lbs/psi vapour pressure for 20 min., placed at the bottom of each Petri plate aseptically with moistened by sterile water. Seeds were treated with 0.1 per cent mercuric chloride ($HgCl_2$) for one minute followed by three subsequent washes with sterile distilled water. Ten seeds were placed at equidistance in each Petri plate. These Petri plates were incubated at 27±2 ^{0}C.

After seven days of incubation, examined it and the typical growth of *A. flavus* developed on seed was transfer aseptically to Potato Dextrose Agar (PDA) slants. The fungal culture was purified by single spore isolation. The pure culture was maintained on PDA medium by periodic sub-culturing and storing it under refrigeration at 5 ^{0}C for further study.

Identification of *A. flavus*

Identification of *A. flavus* was carried out by studying the cultural and morphological characters. The cultural characters were recorded right from initiation of the growth till the formation of conidia. The morphological characters of pathogen were carried out by observing slides stained with cotton blue under the compound microscope. Measurement of fungal hyphae and conidia as shape, length and breadth were recorded and necessary microphotograph was taken with the help of special attachment camera.

Ammonia vapour test for aflatoxigenicity

The colonies of each isolate were grown separately on PDA as a single colony in the centre of Petri plates. After seven days of incubation, the plates were inverted and 2 ml of concentrated ammonia solution (extra pure AR grade) were poured on the inside of the lid. Within few minutes, various degrees of colour development were observed and as a result the strains were categorized as highly, moderately, mildly and non-toxic (Saito and Machida, 1999 [14] and Kumar *et al.*, 2007 [12]).

Results and Discussion

Cultural characters: Macroscopic growth features *viz.*, mean colony diameter (mm) and cultural characteristics of *A. flavus* isolates on PDA medium in Petri plates were recorded (Table 1). Since *A. flavus* grows well on PDA medium, all the isolates produced colonies with a diameter ranging from 67.50 to 90.00 mm after seven days of incubation. Maximum mean colony diameter of 90.00 mm was recorded in AF-5, AF-6, AF-8, AF-11, AF-12, AF-13, AF-14, AF-15, AF-16, AF-18, AF-20, AF-21, AF-22, AF-27, AF-29, AF-31, AF-39 and AF-40. Least mean colony diameter of 67.50 mm was recorded in AF-32 followed by AF-37 which produced 75.00 mm colony diameter.

Table 1: Cultural characters of *A. flavus* on PDA medium

Sr. No.	Isolates	Mean colony diameter (mm)	Macroscopic growth features			
			Front site			Reverse site
			Colony colour	Colony centre colour	Growth pattern at margin	
1	AF-1	85.50	Light green	Light green	Irregular	Light yellow
2	AF-2	86.70	Dark green	Green	Irregular	Light yellow
3	AF-3	85.00	Light green	Light green	Irregular	White
4	AF-4	89.00	Light green	Light green	Regular	Light yellow
5	AF-5	90.00	Dark green	Dark green	Irregular	Creamish yellow
6	AF-6	90.00	Dark green	Dark green	Regular	Creamish yellow
7	AF-7	85.00	Dark green	Dark green	Irregular	Creamish yellow
8	AF-8	90.00	Light green	Dark green	Regular	White
9	AF-9	80.00	Dark green	Dark green	Irregular	Light yellow
10	AF-10	84.00	Dark green	Dark green	Irregular	Light yellow
11	AF-11	90.00	Dark green	Dark green	Irregular	Yellow
12	AF-12	90.00	Light green	Green	Regular	White
13	AF-13	90.00	Light green	Dark green	Irregular	Creamish yellowish white
14	AF-14	90.00	Dark green	Dark green	Irregular	Light yellow
15	AF-15	90.00	Dark green	Dark green	Regular	Greenish yellow
16	AF-16	90.00	Green	Green	Irregular	Light yellow
17	AF-17	88.00	Dark green	Dark green	Regular	Greenish yellow
18	AF-18	90.00	Dark green	Dark green	Irregular	Yellowish green
19	AF-19	85.50	Light green	Light green	Irregular	White
20	AF-20	90.00	Dark green	Dark green	Irregular	White
21	AF-21	90.00	Dark green	Dark green	Regular	Creamish yellowish white
22	AF-22	90.00	Dark green	Dark green	Irregular	Light yellow
23	AF-23	85.00	Dark green	Dark green	Irregular	Greenish yellow
24	AF-24	78.00	Light green	Light green	Irregular	White
25	AF-25	85.70	Light green	Green	Irregular	White
26	AF-26	83.30	Light green	Green	Irregular	Yellowish white
27	AF-27	90.00	Green	Dark green	Irregular	White
28	AF-28	88.00	Dark green	Green	Irregular	White
29	AF-29	90.00	Dark green	Green	Regular	White
30	AF-30	87.50	Light green	Dark green	Irregular	White
31	AF-31	90.00	Light green	Green	Regular	White
32	AF-32	67.50	Dark green	Dark green	Irregular	Yellow
33	AF-33	86.50	Dark green	Dark green	Irregular	Creamish white
34	AF-34	78.00	Dark green	Dark green	Irregular	Yellow
35	AF-35	80.00	Light green	Light green	Irregular	White
36	AF-36	85.00	Yellowish green	Light green	Irregular	Yellowish white
37	AF-37	75.00	Light green	Green	Irregular	White
38	AF-38	89.00	Dark green	Dark green	Irregular	Greenish yellow
39	AF-39	90.00	Yellowish green	Green	Regular	Yellow
40	AF-40	90.00	Yellowish green	Green	Irregular	Yellowish white

The isolates also varied widely in their cultural characteristics (Table 2). The colour of the mycelium ranged from yellowish green to dark green.

Table 2: Categorization of *A. flavus* based on colony colour

Sr. No.	Colony colour	No. of isolates	Per cent isolates	Name of isolates
1	Yellowish green	03	07.5	AF-36, AF-39 and AF-40
2	Light green	14	35.0	AF-1, AF-3, AF-4, AF-8, AF-12, AF-13, AF-19, AF-24, AF-25, AF-26, AF-30, AF-31, AF-35 and AF-37
3	Green	02	05.0	AF-16 and AF-27
4	Dark green	21	52.5	AF-2, AF-5, AF-6, AF-7, AF-9, AF-10, AF-11, AF-14, AF-15, AF-17, AF-18, AF-20, AF-21, AF-22, AF-23, AF-28, AF-29, AF-32, AF-33, AF-34 and AF-38

Three isolates *viz.*, AF-36, AF-39 and AF-40 produced yellowish green mycelium; fourteen isolates *viz.*, AF-1, AF-3, AF-4, AF-8, AF-12, AF-13, AF-19, AF-24, AF-25, AF-26, AF-30, AF-31, AF-35 and AF-37 produced light green mycelium; two isolates AF-16 and AF-27 produced green mycelium, whereas remaining twenty-one isolates produced dark green colour mycelium. Frequency distribution of the *A. flavus* isolates on the basis of colony colour showed that 7.5 per cent belonged to yellowish green, 35.0 per cent belong to light green, 5.0 per cent belong to green and the rest of the isolates belonged to the dark green colour (52.5%).

The central part of the mycelium colour ranged from light green to dark green (Table 3).

Table 3: Categorization of *A. flavus* based on colony centre colour

Sr. No.	Colony colour	No. of isolates	Per cent isolates	Name of isolates
1	Light green	07	17.5	AF-1, AF-3, AF-4, AF-19, AF-24, AF-35 and AF-36
2	Green	11	27.5	AF-2, AF-12, AF-16, AF-25, AF-26, AF-28, AF-29, AF-31, AF-37, AF-39 and AF-40
3	Dark green	22	55.0	AF-5, AF-6, AF-7, AF-8, AF-9, AF-10, AF-11, AF-13, AF-14, AF-15, AF-17, AF-18, AF-20, AF-21, AF-22, AF-23, AF-27, AF-30, AF-32, AF-33, AF-34 and AF-38

Seven isolates *viz.*, AF-1, AF-3, AF-4, AF-19, AF-24, AF-35 and AF-36 produced light green mycelium; eleven isolates *viz.*, AF-2, AF-12, AF-16, AF-25, AF-26, AF-28, AF-29, AF-31, AF-37, AF-39 and AF-40 produced green mycelium, whereas remaining twenty-two isolates produced dark green colour mycelium. Frequency distribution of the *A. flavus* isolates on the basis of colony centre colour showed that 17.5 per cent belonged to light green, 27.5 per cent belong to green and the rest of the isolates belonged to the dark green colour (55.0%).

The reverse site colony colour were light yellow (AF-1, AF-2, AF-4, AF-9, AF-10, AF-14, AF-16 and AF-22), yellow (AF-11, AF-32, AF-34 and AF-39), creamy yellow (AF-5, AF-6 and AF-7), creamish yellow white (AF-13 and AF-21), creamy white (AF-33), yellowish green (AF-18), greenish yellow (AF-15, AF-17, AF-23 and AF-38), yellowish white (AF-26, AF-36 and AF-40) and white (AF-3, AF-8, AF-12, AF-19, AF-20, AF-24, AF-25, AF-27, AF-28, AF-29, AF-30, AF-31, AF-35 and AF-37) (Table 4).

Table 4: Categorization of *A. flavus* based on reverse site colony colour

Sr. No.	Reverse site colony colour	No. of isolates	Per cent isolates	Name of isolates
1	Light yellow	08	20.0	AF-1, AF-2, AF-4, AF-9, AF-10, AF-14, AF-16 and AF-22
2	Yellow	04	10.0	AF-11, AF-32, AF-34 and AF-39
3	Creamy yellow	03	07.5	AF-5, AF-6 and AF-7
4	Creamish yellow white	02	05.0	AF-13 and AF-21
5	Creamy white	01	02.5	AF-33
6	Yellowish green	01	02.5	AF-18
7	Greenish yellow	04	10.0	AF-15, AF-17, AF-23 and AF-38
8	Yellowish white	03	07.5	AF-26, AF-36 and AF-40
9	White	14	35.0	AF-3, AF-8, AF-12, AF-19, AF-20, AF-24, AF-25, AF-27, AF-28, AF-29, AF-30, AF-31, AF-35 and AF-37

Frequency distribution of the *A. flavus* isolates on the basis of reverse site colony colour showed that 20.0 per cent belonged to light yellow, 10.0 per cent belong to yellow, 7.5 per cent belonged to creamish yellow, 5.0 per cent belonged to creamish yellow white, 2.5 per cent belonged to creamy white, 2.5 per cent belonged to yellowish green, 10.0 per cent belonged to greenish yellow, 7.5 per cent belonged to yellowish white and the rest of the isolates belonged to the white colour (35.0%).

No sclerotia production was observed in any isolates of *A. flavus* in culture.

Morphological character

Morphological characters of forty isolates of *A. flavus* were studied and the data presented in the Table 5 indicated that conidiophores were long with varying lengths and the vesicle bears a chain of conidia on Biseriate Sterigmata. The size of the conidiophore length also varied greatly among the isolates along with the width of the conidiophore. The length of the conidiophore of all the isolates ranged from 240.6 µm to 1214.7 µm. The isolate AF-13 having the highest conidiophore length of 1214.7 µm followed by AF-36 and AF-19 having the conidiophore length of 948.4 µm and 808.3

µm, respectively. The lowest conidial length (240.6 µm) observed in AF-15 followed by AF-33 (275.1 µm) and AF-38 (402.8 µm). Similar to conidiophore length, the width of the conidiophore also varied with the range of 9.5 µm to 15.1 µm, the isolates AF-7 having the highest conidiophore width of 15.1 µm followed by isolate AF-16 having the conidiophore width of 14.8 µm. The smallest conidiophore width 9.5 µm were observed in the isolate AF-5 followed by isolate AF-23 (9.7 µm).

Conidia diameter of *A. flavus* ranged from 3.4 µm to 7.5 µm, the highest conidial diameter of 7.5 µm were observed in the isolate AF-16 and AF-32 followed by AF-8 and AF-5 having 7.0 µm and 6.6 µm, respectively. The smallest conidial diameter of 3.4 µm was observed in AF-30 and it was followed by AF-28 and AF-29 having 3.6 µm and 3.7 µm, respectively.

Conidial wall, observed from finely rough to smooth wall which was an important character in *A. flavus* identification. Twenty-two isolates *viz.*, AF-1, AF-4, AF-8, AF-9, AF-10, AF-12, AF-17, AF-21, AF-23, AF-24, AF-25, AF-27, AF-28, AF-30, AF-31, AF-32, AF-34, AF-35, AF-36, AF-37, AF-39 and AF-40 produced finely rough and eighteen isolates *viz.*, AF-2, AF-3, AF-5, AF-6, AF-7, AF-11, AF-13, AF-14, AF-15, AF-16, AF-18, AF-19, AF-20, AF-22, AF-26, AF-29, AF-33 and AF-38 produced smooth conidia wall. Frequency distribution of the *A. flavus* isolates on the basis of conidia wall showed that 55.0 per cent belong to finely rough and 45.0 per cent belong to smooth conidia wall (Table 6).

Conidia shape was observed from globose to subglobose. Twenty-three isolates *viz.*, AF-1, AF-2, AF-3, AF-5, AF-6, AF-7, AF-8, AF-13, AF-15, AF-16, AF-17, AF-21, AF-25, AF-26, AF-27, AF-28, AF-29, AF-30, AF-33, AF-34, AF-35, AF-36 and AF-38 produced globose and seventeen isolates *viz.*, AF-4, AF-9, AF-10, AF-11, AF-12, AF-14, AF-18, AF-19, AF-20, AF-22, AF-23, AF-24, AF-31, AF-31, AF-37, AF-39 and AF-40 produced subglobose shape conidia. Frequency distribution of the *A. flavus* isolates on the basis of conidia shape showed that 57.5 per cent belong to globose and 42.5 per cent belong to subglobose conidia shape (Table 6).

Similar results were obtained with the study conducted by different workers. According to Raper and Fennel (1965) [13] there was a variation in the conidiophore length and conidial size. Govrama and Bullerman (1995) [8] reported that the colour of colony is initially yellow and turns in to yellow green and old colony appears dark green colour. Klich *et al.*, (2002) [11] reported that conidia are smooth to finely roughened, globose to subglobose, 3.6 µm in diameter conidiophore are coarsely roughened, 800 µm long × 15.20 µm wide. Dube (2005) [6] reported that the colour of conidia blue, green, black or yellow gives the colony colour and is a useful tool for identification of species.

Table 5: Morphological characters of different isolates of *A. flavus* on PDA medium

Sr. No.	Isolates	Conidiophore size (µm)				Conidia diameter (µm)		Conidia wall	Conidia shape
		Length		Width					
		Range	Mean	Range	Mean	Range	Mean		
1	AF-1	568.5-642.6	605.6	9.9-10.4	10.2	3.5-4.5	4.0	Finely rough	Globose
2	AF-2	586.7-676.8	631.8	10.3-12.2	11.3	3.8-4.5	4.2	Smooth	Globose
3	AF-3	734.8-807.9	771.3	9.5-10.2	9.9	4.1-5.3	4.7	Smooth	Globose
4	AF-4	615.8-735.5	675.7	12.4-15.0	13.7	5.7-5.8	5.8	Finely rough	Subglobose
5	AF-5	624.7-663.4	644.1	9.0-10.0	9.5	6.0-7.2	6.6	Smooth	Globose
6	AF-6	479.7-482.3	481.0	12.9-14.9	13.9	3.7-4.2	4.0	Smooth	Globose
7	AF-7	601.5-581.7	591.6	14.7-15.4	15.1	4.9-6.0	5.5	Smooth	Globose
8	AF-8	577.3-592.8	585.1	11.7-14.0	12.8	6.3-7.7	7.0	Finely rough	Globose
9	AF-9	658.5-704.8	681.7	10.3-12.5	11.4	5.5-6.2	5.9	Finely rough	Subglobose
10	AF-10	437.3-519.5	478.4	10.6-14.7	12.6	5.9-7.0	6.5	Finely rough	Subglobose
11	AF-11	368.4-462.7	415.6	9.9-11.9	10.9	3.4-4.4	3.9	Smooth	Subglobose
12	AF-12	600.0-700.0	650.0	9.5-10.7	10.1	3.9-4.6	4.3	Finely rough	Subglobose
13	AF-13	1210.7-1218.6	1214.7	9.9-12.5	11.2	5.6-6.0	5.8	Smooth	Globose
14	AF-14	688.7-773.1	730.9	13.8-12.5	13.1	4.8-5.5	5.2	Smooth	Subglobose
15	AF-15	228.7-252.5	240.6	11.0-9.4	10.2	3.7-5.0	4.4	Smooth	Globose
16	AF-16	627.8-726.8	677.3	14.0-15.7	14.8	7.0-8.0	7.5	Smooth	Globose
17	AF-17	784.4-765.5	775.0	12.5-10.5	11.5	3.7-4.2	4.0	Finely rough	Globose
18	AF-18	600.0-730.0	665.0	10.5-12.4	11.4	3.0-4.5	3.8	Smooth	Subglobose
19	AF-19	750.0-866.6	808.3	12.0-15.0	13.5	3.5-4.0	3.8	Smooth	Subglobose
20	AF-20	610.0-700.0	655.0	8.9-10.8	9.8	5.0-6.0	5.5	Smooth	Subglobose
21	AF-21	580.0-670.0	575.0	12.4-14.7	13.5	3.8-4.9	4.4	Finely rough	Globose
22	AF-22	780.8-800.0	790.4	11.0-12.9	11.9	5.0-6.5	5.8	Smooth	Subglobose
23	AF-23	600.0-710.0	655.0	9.2-10.2	9.7	5.0-6.3	5.7	Finely rough	Subglobose
24	AF-24	654.0-754.0	704.0	11.2-10.7	10.9	5.0-6.0	5.5	Finely rough	Subglobose
25	AF-25	600.0-750.0	675.0	9.8-10.5	10.2	5.0-8.0	6.5	Finely rough	Globose
26	AF-26	630.3-754.5	692.4	12.3-13.2	12.8	5.0-8.0	6.5	Smooth	Globose
27	AF-27	500.0-650.0	575.0	12.0-14.0	13.0	4.0-5.0	4.5	Finely rough	Globose
28	AF-28	400.0-500.0	450.0	11.6-14.0	12.8	3.3-3.9	3.6	Finely rough	Globose
29	AF-29	455.3-543.3	499.3	9.7-12.9	11.3	3.5-3.9	3.7	Smooth	Globose
30	AF-30	610.8-703.9	657.4	12.3-14.3	13.3	3.2-3.5	3.4	Finely rough	Globose
31	AF-31	423.9-543.9	483.9	11.5-14.5	13.0	5.5-7.2	6.3	Finely rough	Subglobose
32	AF-32	746.9-821.0	784.0	11.8-13.4	12.6	7.0-8.0	7.5	Finely rough	Subglobose
33	AF-33	229.9-250.2	275.1	13.-15.0	14.0	4.8-5.9	5.3	Smooth	Globose
34	AF-34	543.5-632.7	588.1	10.4-13.3	11.8	5.3-6.6	6.0	Finely rough	Globose

35	AF-35	699.6-774.7	737.2	13.5-14.0	13.7	3.9-4.4	4.2	Finely rough	Globose
36	AF-36	843.9-1052.8	948.4	9.7-13.0	11.4	5.8-6.0	5.9	Finely rough	Globose
37	AF-37	532.8-645.3	589.1	13.3-12.3	12.8	3.7-4.2	4.0	Finely rough	Subglobose
38	AF-38	354.8-450.8	402.8	10.4-13.6	12.0	4.0-5.0	4.5	Smooth	Globose
39	AF-39	585.2-643.5	614.4	12.2-14.0	13.1	4.8-5.0	4.9	Finely rough	Subglobose
40	AF-40	565.5-655.9	610.7	13.4-14.3	13.9	3.7-4.2	4.0	Finely rough	Subglobose

Table 6: Morphological characterization of different isolates of *A. flavus* grown on PDA

Sr. No.	Characters		No. of isolates	Per cent isolates	Name of isolates
1	Conidia wall				
	a.	Finely rough	22	55.0	AF-1, AF-4, AF-8, AF-9, AF-10, AF-12, AF-17, AF-21, AF-23, AF-24, AF-25, AF-27, AF-28, AF-30, AF-31, AF-32, AF-34, AF-35, AF-36, AF-37, AF-39 and AF-40
	b.	Smooth	18	45.0	AF-2, AF-3, AF-5, AF-6, AF-7, AF-11, AF-13, AF-14, AF-15, AF-16, AF-18, AF-19, AF-20, AF-22, AF-26, AF-29, AF-33 and AF-38
2	Conidia shape				
	a.	Globose	23	57.5	AF-1, AF-2, AF-3, AF-5, AF-6, AF-7, AF-8, AF-13, AF-15, AF-16, AF-17, AF-21, AF-25, AF-26, AF-27, AF-28, AF-29, AF-30, AF-33, AF-34, AF-35, AF-36 and AF-38
	b.	Subglobose	17	42.5	AF-4, AF-9, AF-10, AF-11, AF-12, AF-14, AF-18, AF-19, AF-20, AF-22, AF-23, AF-24, AF-31, AF-31, AF-37, AF-39 and AF-40

Bora (2008) [5] found that highest mycelial growth was observed on PDA. Bandh *et al.* (2012) [4] reported that conidium colour of the fungal colonies was green, brown, yellow, yellow-orange and reverse colour was white to cream, the colony diameter ranged between 19.00 to 50.00 mm. The conidia length, width, stipe length and stipe width varied between 2.5 to 3.5 µm, 2.2 to 3 µm, 28 to 350 µm and 2 to 3.5 µm, respectively and shape was globose, ellipsoid, subglobose and pyriform with ornamentation of conidia and stipe as smooth, coarsely roughened and finely roughened.

Ammonia vapour test for aflatoxigenicity

The colonies of forty isolates were grown on PDA medium as a single colony in the centre of Petri plates after seven days of incubation, ammonium vapour test was carried out described earlier.

The data in Table 7 indicated that, among forty isolates of *A. flavus*, five isolates *viz.*, AF-6, AF-5, AF-12, AF-10 and AF-20 in descending order were found highly toxigenic in nature and showed dark red colour development, while six isolates (AF-4, AF-13, AF-16, AF-21, AF-32 and AF-39) were found moderately toxigenic to moderate red colour and seven isolates *viz.*, AF-1, AF-2, AF-7, AF-14, AF-18, AF-34 and AF-36 exhibited light red colour which were mildly toxigenic, whereas remaining twenty-two isolates AF-3, AF-8, AF-9, AF-11, AF-15, AF-17, AF-19, AF-22, AF-23, AF-24, AF-25, AF-26, AF-27, AF-28, AF-29, AF-30, AF-31, AF-33, AF-35, AF-37, AF-38 and AF-40 were found to be non-toxigenic. Frequency distribution showed that 12.5 per cent belonged to high-toxic, 15.0 per cent moderately-toxic, 17.5 per cent mildly-toxic and the rest of the isolates were non-toxic (55.0%) (Table 8).

After treatment with glacial acetic acid, the colour of cultures turned back to normal, as before the ammonia vapour treatments.

Saito and Machida (1999) [14] reported that the colony reverse of aflatoxin producing strains of *A. flavus* turned pink when their cultures were exposed to ammonia vapor.

The yellow pigment and ammonium hydroxide vapor tests are based on the production of yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway. These compounds act as pH indicator dyes, which are more visible when they have turned red at alkaline pH (Abbas *et al.*, 2004a) [1]. Also, colour change after ammonium hydroxide vapor exposure was observed in *Aspergillus* isolates (Abbas *et al.*, 2004b) [2]. Kumar *et al.*, (2007) [12] reported that colour response to ammonia vapour was in agreement with the thin layer chromatography test.

Table 7: Determination of toxicity level of different isolates of *A. flavus* through ammonia vapour test

Isolates	Colour in media	Colour with ammonia	Toxicity level
AF-1	Light yellow	Light red	Mildly-toxic
AF-2	Light yellow	Light red	Mildly-toxic
AF-3	White	White	Non-toxic
AF-4	Light yellow	Moderate pink	Moderately-toxic
AF-5	Creamish yellow	Dark red	Highly-toxic
AF-6	Creamish yellow	Dark red	Highly-toxic
AF-7	Creamish yellow	Light red	Mildly-toxic
AF-8	White	White	Non-toxic
AF-9	Light yellow	Light yellow	Non-toxic
AF-10	Light yellow	Dark red	Highly-toxic
AF-11	Yellow	Yellow	Non-toxic
AF-12	White	Dark red	Highly-toxic
AF-13	Creamish yellowish white	Moderate pink	Moderately-toxic
AF-14	Light yellow	Light red	Mildly-toxic
AF-15	Greenish yellow	Greenish yellow	Non-toxic
AF-16	Light yellow	Moderate pink	Moderately-toxic
AF-17	Greenish yellow	Greenish yellow	Non-toxic

AF-18	Yellowish green	Light red	Mildly-toxic
AF-19	White	White	Non-toxic
AF-20	White	Dark red	Highly-toxic
AF-21	Creamish yellowish white	Moderate pink	Moderately-toxic
AF-22	Light yellow	Light yellow	Non-toxic
AF-23	Greenish yellow	Greenish yellow	Non-toxic
AF-24	White	White	Non-toxic
AF-25	White	White	Non-toxic
AF-26	Yellowish white	Yellowish white	Non-toxic
AF-27	White	White	Non-toxic
AF-28	White	White	Non-toxic
AF-29	White	White	Non-toxic
AF-30	White	White	Non-toxic
AF-31	White	White	Non-toxic
AF-32	Yellow	Moderate pink	Moderately-toxic
AF-33	Creamish white	Creamish white	Non-toxic
AF-34	Yellow	Light red	Mildly-toxic
AF-35	White	White	Non-toxic
AF-36	Yellowish white	Light red	Mildly-toxic
AF-37	White	White	Non-toxic
AF-38	Greenish yellow	Greenish yellow	Non-toxic
AF-39	Yellow	Moderate pink	Moderately-toxic
AF-40	Yellowish white	Yellowish white	Non-toxic

Table 8: Categorization of *A. flavus* isolates on the basis of their toxicity using ammonia vapour test

Toxicity level	Color development after exposure	No. of isolates	Per cent isolates	Isolates
High-toxic	Dark red	05	12.5	AF-5, AF-6, AF-10, AF-12 and AF-20
Moderately-toxic	Moderately red	06	15.0	AF-4, AF-13, AF-16, AF-21, AF-32 and AF-39
Mildly-toxic	Light red	07	17.5	AF-1, AF-2, AF-7, AF-14, AF-18, AF-34 and AF-36
Non-toxic	No colour	22	55.0	AF-3, AF-8, AF-9, AF-11, AF-15, AF-17, AF-19, AF-22, AF-23, AF-24, AF-25, AF-26, AF-27, AF-28, AF-29, AF-30, AF-31, AF-33, AF-35, AF-37, AF-38 and AF-40

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