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Morphological Credentials of Afla-Toxigenic and Non-Toxigenic *Aspergillus* Using Polyphasic Taxonomy

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A B S T R A C T

The study was performed for development of macro and micro morphological characters of *Aspergillus* isolated from groundnut seeds, groundnut cake and soil using cultural and microscopoic methods. Fungus colonies was inoculated in different media like Potato Dextrose Agar (PDA) and Yeast Extract Sucrose (YES) Agar and compared with standard cultures. The *Aspergillus* isolates grown on both medium were identified using macroscopic characteristics such as colony color, colony reverse color, colony edge, mycelia color, conidation, shape of conidia, conidial wall, mycelium growth, mycelium growth rate and microscopic characteristics including vesicle diameter, conidiophore width, length of conidia head and ascospore diameter. According to colony colour, pigmentation and length of conidial head, the 21 isolates were identified as *Aspergillus* species *viz., A. flavus* (06) (200-800µm conidial head length), *A. tamarii* (05) (250-700 µm), *A. flavus var. columnaris* (05) (500-1200 µm), *A. sojae* (02) (300-900 µm), *A. parasiticus* (01) (350-900 µm), *A. niveus* (01) (250-350 µm) and *A. fumigatus* (01) (900-950 µm). This is the first report on identification and characterization of *Aspergillus* strains based on length of conidial head on PDA medium. Fungal toxicity was anticipated based on aflatoxin detection using biochemical test. Out of 21, 08 isolates were found to be afla-toxigenic based on biochemical test.

Introduction

The groundnut, or peanut (*Arachis hypogaea*), is a species in the legume or "bean" family (Fabaceae). Groundnut is grown in nearly 100 countries. China leads in production of groundnut, having a share of about 41.5% of overall world production, followed by India (18.2%) and the United States of America (6.8%). In Europe, the leading producer is Greece, at roughly 2000 tons per year. India is one of the major exporting countries of groundnuts after china (Anonymous, 2013).

Groundnut contaminated with the mold *Aspergillus flavus* which produces a carcinogenic substance called aflatoxin. *Aspergillus* is a large genus composed of more than 180 accepted anamorphic species (Pitt *et al.*, 2000), with teleomorphs described in nine different genera (Pitt *et al.*, 2000). The genus is subdivided in 7 subgenera, which in turn are further divided into sections (Klich, 2007).

As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia.

Micromorphological characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores (Klich, 2002). Furthermore, all these morphological features have to be determined under standardized laboratory conditions (Okuda *et al*., 2000), in order to obtain an accurate identification. In this contest several *Aspergillus* taxonomic keys and guides are available (Klich, 2002).

Aspergillus Subgenus *Circumdati* Section *Flavi*, also refered to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided in two groups of species.

One includes the aflatoxigenic species *A. flavus*, *A. parasiticus* and *A. nomius*, which cause serious problems worldwide in agricultural commodities, and the other includes the nonaflatoxigenic species *A. oryzae*, *A. sojae* and *A. tamarii*, traditionally used for production of fermented foods in Asia (Kumeda and Asao, 2001).

The objectives of the study were to investigate and characterize the diversity and distribution of aflatoxigenic and non aflatoxigenic *Aspergillus* fungi associated with groundnut seeds, groundnut cake and soil.

Materials and Methods

Collection of groundnut seeds, cakes and soil samples

Groundnut seeds were collected from farmers fields of Saurastra region, Gujarat and groundnut cake obtained from oil industries for isolation of aflatoxin and non aflatoxin producing *Aspergillus.* Soil samples were collected from healthy and infected groundnut fields of Junagadh Agricultural University, Junagadh.

Isolation of *Aspergillus* from ground nut seeds and cake

For isolation of *Aspergillus*, groundnut seeds and cake were surface sterilizing with 0.5 % sodium hypochloride solution. After surface sterilization samples were cut into two halves with sterile scrapple and inoculated onto specific medium (K_2HPo_4 0.5 g L^{-1}, $MgSo_4$ 0.5 g L^{-1},Peptone 0.5 g L^{-1}, Yeast 0.5 g L^{-1},Sucrose 20 g L^{-1},Agar 17 g L^{-1}, after autoclaving add 25 mg L^{-1} antibiotic (Streptomycin sulfate) and Rose Bengal dye (Manjusha and Anita, 2013), while groundnut cake were inoculated as such on same media so as to grow *Aspergillus* only. The yellow/green colour grown colonies from plates were inoculated onto centre of fresh PDA medium to obtain pure culture of fungus.

Isolation of fungi from soil sample

One gram of soil was added into the tube containing 9mL of sterile distilled water to obtain 1/10 dilution (stock solution) and a series of 1/00, 1/1000, 1/10,000, and 1/100,000 dilutions was prepared by adding 1mL of solution to 9 ml of sterile distilled water respectively (Waksman and Fred, 1922). The one ml of suspension from each dilution was transferred onto specific

medium. The yellow/green colour grown colonies from plates were inoculated onto centre of fresh PDA medium to obtain pure culture of fungus (Johnston and Booth, 1983). Plates were incubated at room temperature for 7 days at 28^0C. All the pure cultures were derived by subsequent transformation of pure mycelia on PDA media. Pure cultures were maintained fresh and viable by periodic transfer on PDA medium (Schindler *et al*., 1967).

Morphological characterization of *Aspergillus* species

Aspergillus species were characterized according the genus *Aspergilli* (Raper and Fennell, 1965; Diba *et al*., 2007) and classification system (Gams *et al*., 1985). Aspergillus species were cultured on two differential media i.e. Potato Dextrose Agar (PDA) (Salleh and Sulaiman, 1984) and Yeast Extract Agar (YEA), suitable for both production of aflatoxin (Davis, *et al.*, 1966). After seven days of incubation, plates (in triplicates) were observed for macroscopic characteristics such as colony diameter, colony colour, colony reverse colour colony edge, mycelial colour, conidiation, shape of conidia, conidiophore branching, Growth rate and conidial wall.

The microscopic characteristics including vesicle diameter, conidiophore width, conidia head and ascospore diameter. For microscopic characteristics slides were stained with cotton blue and mounted in lectophenol. Photographs were taken under Zeiss Axiocam Imager, model Z 2 microscope. A morphological examination of species was first made with naked eye and at low magnification power of microscope after that detailed examination were done according to Raper and Fennell (1965) and Gams *et al*. (1985). All the morphological observations were recorded in 5 replications and standard deviation calculated.

Screening of aflatoxigenic and non aflatoxigenic *Aspergillus* based on biochemical test

Biochemical test using ammonium hydroxide was done to identify toxic strain among 21 isolates. In this method, the reverse side of colonies of toxin producing strains on potato dextrose agar (PDA) medium turns from yellow to pink immediately after exposure to ammonium hydroxide vapor (Saito and Machida, 1999).

Results and Discussion

A total of 21 isolates of microscopic fungi consisting of *Aspergillus* species were obtained from the ground nut seeds, cake and soil and colony morphology were recorded (Table 1). Based on microscopic characters, the isolates were identified as *Aspergillus* species, *viz. A. flavus (06), A. tamarii (05), A. flavus var. columnaris (05), A. sojae (02), A. parasiticus (01), A.niveus (01), and A. fumigatus (01)* (Fig. 8). The identified isolates were found similar in characters like smooth in colony edge, white mycelia colour, round conidia shape and smooth conidial wall on both PDA and YES media.

Macro and micro-morphological descriptors of *Aspergillus*

A. flavus

Colonies on PDA showed slow growth rate and attend 75 mm in diameter after seven days at 28^0C. Colony color on PDA showed variation in different strains, yellow to green, or dark green, reverse white or yellow (Fig. 1A-B). Colonies on YES showed fast growth rate and attend 90 mm in diameter after seven days at 28^0C. Colony color on YES yellowish green, reverse whitish orange or orange (Fig. 1 E-F) (Table 1).

The *Aspergillus* isolates were subjected to observations under Zeiss Axiocam Imager, model Z 2 microscope. Conidial heads on PDA media were found to be 250-700 µm in diameter and Conidiophore 3.50-11.50 µm in width. Vesicle upon maturity were globose with 30-65 µm in diameter. Ascospores were ranged from 2.0 -8.0 µm in diameter with smooth cell wall (Fig. 1 C-D). Conidial heads on YES media were found to be 165-900 µm in diameter and Conidiophore 5.00-16.00 µm in width. Vesicle upon maturity were globose with diameter 23-80 µm in diameter. Ascospore were ranged from diameter 3.60-10.30µm in diameter with smooth cell wall (Fig. 1 G-H) (Table 2).

A. tamari

A. tamari colonies on PDA showed medium growth rate and attend 65 mm in diameter after seven days at 28^0C. Colony color on PDA was visualized as green or brown with mycelium white to dull white in colour. Reverse side of colonies were found white or cream in colour. (Fig. 2 A-B). *A. tamari* colonies on YES showed medium growth rate and attend 80 to 90 mm in diameter after seven days at 28^0C. Colony color on YES was visualized as greenish yellow or white or brown with reverse side orange or yellow or cream (Fig. 2 E-F) (Table 1).

Microscopy of *Aspergillus* isolates was performed under Zeiss Axiocam Imager, model Z 2 which measured Conidial heads on PDA media from 600-920µm in diameter and Conidiophore ranged from 5.50-12.50 µm in width. Vesicle appeared to be subglobose to globose with 37.00-68.00 µm in diameter. Ascospore resembled spherical, smooth and measured 4.00-8.10 µm in diameter (Fig. 2 C-D). Microscopy of *Aspergillus* isolates was performed under Zeiss Axiocam Imager, model Z 2 which measured Conidial heads on YES media from 240-2380µm in diameter and Conidiophore ranged from 6.00-15.50µm in width. Vesicle size was measured 31.00-80.00µm in diameter. Ascospore resembled spherical, smooth and measured 4.00-9.20µm in diameter (Fig. 2 G-H) (Table 2).

A. flavus var. columnaris

Colonies on PDA showed fast growth rate and attend 85 mm in diameter after seven days at 28^0C. Colony color on PDA showed variation in different strains, green or brown, reverse white or cream (Fig. 3A-B). Colonies on YES showed fast growth rate and attend 90 mm in diameter after seven days at 28^0C. Colony color on YES yellowish creamiest brown or yellowish green, reverse whitish orange or yellow (Fig. 3 E-F) (Table 1).

A. flavus var. columnaris isolates were subjected to observations under Zeiss Axiocam Imager, model Z 2 microscope. Conidial heads on PDA media measures 600-920 µm in diameter where as Conidiophore width ranged from 5.50-12.50 µm. Vesicle diameter measures in between 37.00-68.00 µm and Ascospore ranged 4.00-8.10 µm in diameter (Fig. 3 C-D). Conidial heads on YES media measures 200-2900µm in diameter where as Conidiophore width ranged from 9.00-16.50 µm. Vesicle diameter measures in between 23.00-86.00 µm and Ascospore ranged 5.20-8.60 µm in diameter (Fig. 3 G-H) (Table 2).

A. sojae

A. sojae colonies on PDA showed medium growth rate and attend 80 mm in diameter after seven days at 28^0C. Green colour colonies with white reverse colour were seen on PDA medium (Fig. 4 A-B). Colonies on YES showed fast growth rate and attend 90 mm in diameter after seven days at 28^0C. Colony color on YES brownish green or yellowish green, reverse cream or light orange (Fig. 4 E-F) (Table 1).

A. sojae isolates were subjected to observations under Zeiss Axiocam Imager, model Z 2 microscope. Conidial heads on PDA media measured 350-800 µm in diameter with 5.50-18.00 µm Conidiophore widths. Vesicle diameter measured 35.00-100.00 µm in diameter and Ascospore found spherical in shape with rough walled and ranged 4.10-7.30 µm in diameter (Fig. 4 C-D). *A. sojae* isolates were subjected to observations under Zeiss Axiocam Imager, model Z 2 microscope. Conidial heads on YES media measured 300-930µm in diameter with 9.60-15.00µm Conidiophore widths. Vesicle diameter measured 30.00-60.00µm in diameter and Ascospore found spherical in shape with rough walled and ranged 4.50-7.60µm in diameter (Fig. 4 G-H) (Table 2).

A. parasiticus

A. parasiticus colonies on PDA showed slow growth rate and attend 65 mm in diameter after seven days at 28^0C .Colony color on PDA showed yellow green front colour with cream to pale yellow on reverse (Fig. 5 A-B). *A. parasiticus* colonies on YES showed slow growth rate and attend 90 mm in diameter after seven days at 28^0C. Colony color on YES showed green colour on front with red on reverse side (Fig. 5 E-F) (Table 1).

Microscopy of *A. parasiticus* determined conidial heads, 350-600 µm in diameter on PDA media. The size of Conidiophore width was determined 3.20-4.00 µm and Vesicle diameter of about 45.00-60.00 µm. Ascospore ranged 5.40-8.10 µ in diameter (Fig. 5 C-D).

Microscopy of *A. parasiticus* determined conidial heads, 350-930µm in diameter on YES media. The size of Conidiophore width was determined 11.5-15µm and Vesicle diameter of about 55.00-80.00µm. Ascospore ranged 3.1-6.30µm in diameter (Fig. 5 G-H) (Table 2).

A. niveus

Colonies on PDA showed slow growth rate and attend 55 mm in diameter after seven days at 28^0C. Colony color on PDA showed variation in different strains, brown, reverse yellow (Fig. 6 A-B). Colonies on YES showed slow growth rate and attend 55 mm in diameter after seven days at 28^0C, Colony color on YES White, reverse orange (Fig. 6 E-F) (Table 1). Conidial heads on PDA media were found 300-500µm in diameter and Conidiophore 7.30-10.00 µm in width. Vesicle ranged 45.00-100.00µm in diameter whereas Ascospore measured 4.30-5.00 µm in diameter (Fig. 6 C-D).Conidial heads on YES media were found 230-370µm in diameter and Conidiophore 60.00-80.00 µm in width. Vesicle ranged 60.00-80.00µm in diameter whereas, Ascospore measured 4.5-8.5 µm in diameter (Fig. 6 G-H) (Table 2).

A. fumigates

A. fumigates colonies on PDA showed medium growth rate and attend 65 mm in diameter after seven days at 28^0C. Colony color on PDA showed front gray-green, reverse cream (Fig. 7 A-B). *A. fumigates* colonies on YES showed medium growth rate and attended 90 mm in diameter after seven days at 28^0C. Colony color on YES showed front yellowish green, reverse light orange (Fig. 7 E-F) (Table 1). Microscopy of experimental fungus reveled 250-430 µm diameter of Conidial heads on PDA media. Conidiophore width ranged from 5.00-10.50 µm in and Vesicle 30.00-36.00µm in diameter. Ascospore measured 3.00-4.00 µm in diameter (Fig. 7 C-D). Microscopy of experimental fungus reveled 900-950 µm diameter of Conidial heads on YES media. Conidiophore width ranged from 12.00-21.00 µm in and Vesicle 53.00-60.00 µm in diameter. Ascospore measured 5.60-6.00 µm in diameter (Fig. 7 G-H) (Table 2).

Table.1 Colony morphology of Aspergillus strains on PDA and YES matrix

Iso-late No.	Isolate code	Type of Media	Colony colour	Colony reverse colour	Colony edge	Mycelial colour	Conidiation	Shape of conidia	Conidial wall	Mycelium Growth		(mm) Growth rate
										3 day	7 day	
1	JND-VAD-VAD-GG45	PDA	Yellowish Green	Orange	Smooth	White	Circular	Round	Smooth	35	79	Medium
		YES	Creamiest Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
2	RJD-UPA-KUN-G2	PDA	gray-Green	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
		YES	Yellowish Green	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	75	90	Fast
3	JAM-JKB-BHA-GG20	PDA	Green	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	50	90	Medium
		YES	Yellowish Green	Light Orange	Smooth	White	Spot ring Like flat	Round	Smooth	65	90	Fast
4	JND-MEN-MEN-GG41	PDA	Greenish brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	35	90	Medium
		YES	Greenish Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
5	DWK-DWK-GG20	PDA	Green	White	Smooth	White	Spot ring Like flat	Round	Smooth	45	80	Medium
		YES	Yellowish Green	Light Orange	Smooth	White	Spot ring Like flat	Round	Smooth	40	80	Medium
6	JND-MEN-MEN-GG41	PDA	Greenish Brown	White	Smooth	White	Spot ring Like flat	Round	Smooth	65	90	Medium
		YES	Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	45	90	Medium
7	JND-MISC-3	PDA	Green	White	Smooth	White	Spot ring Like flat	Round	Smooth	40	90	Medium

		YES	Yellowish Green	Cream	Smooth	White	Circular	Round	Smooth	60	90	Fast
8	JND-MISC-2	PDA	Greenish Brown	White	Smooth	White	Spot ring Like flat	Round	Smooth	60	90	Medium
		YES	Yellowish Green	Yelloe	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
9	JND-MISC-1-GG20	PDA	Green	White	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
		YES	Brownish Green	Light Orange	Smooth	White	Spot ring Like flat	Round	Smooth	55	90	Fast
10	DWK-DWK-GG20	PDA	Brown	White	Smooth	White	Spot ring Like flat	Round	Smooth	55	90	Medium
		YES	Brown	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	50	90	Medium
11	JND-MEN-GUN-GG41	PDA	Brown	White	Smooth	White	Spot ring Like flat	Round	Smooth	45	75	Medium
		YES	Brown	Cream	Smooth	White	Circular	Round	Smooth	60	90	Fast
12	RJD-UPA-KUN-TJ37A	PDA	Green	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
		YES	Yellowish Green	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	70	90	Fast
13	RJD-DHO-PAR-GG37	PDA	Dark Green	Yellow	Smooth	White	Circular	Round	Smooth	55	90	Slow
		YES	Yellowish Green	Yellow	Smooth	White	Circular	Round	Smooth	55	90	Slow
14	RJD-DHO-KAN-GG45	PDA	Green	Yellow	Smooth	White	Spot ring Like flat	Round	Smooth	50	90	Medium
		YES	Green	Whitish Orange	Smooth	White	Spot ring Like flat	Round	Smooth	55	90	Fast
15	JND-MAN-LIM-GG20	PDA	Dark Green	Yellow	Smooth	White	Circular	Round	Smooth	50	90	Slow
		YES	Green	Red	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast

16	JND-MAN-LIM-GG20	PDA	Dark Green	Yellow	Smooth	White	Circular	Round	Smooth	65	90	Slow
		YES	White	Orange	Smooth	White	Circular	Round	Smooth	40	55	Slow
17	DAT-DAT-PAT-GG37	PDA	Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	55	75	Medium
		YES	White	Orange	Smooth	White	Circular	Round	Smooth	45	55	Slow
18	JND-MEN-MEN-TJ45	PDA	Green	White	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
		YES	Greenish Yellow	Orange	Smooth	White	Spot ring Like flat	Round	Smooth	50	90	Medium
19	JND- JND-Cak-1	PDA	Green	White	Smooth	White	Spot ring Like flat	Round	Smooth	30	85	Medium
		YES	Greenish Yellow	Orange	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
20	JND- JAU –HSS-1	PDA	Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	30	70	Medium
		YES	Brown	Cream	Smooth	White	Spot ring Like flat	Round	Smooth	90	90	Fast
21	JND- JAU –ISS-1	PDA	Green	Light Gre	Smooth	White	Spot ring Like flat	Round	Smooth	50	90	Medium
		YES	Yellow	Light Brown	Smooth	White	Spot ring Like flat	Round	Smooth	40	90	Medium

Table.2 Microscopic descriptions of Aspergillus strains on PDA and YES matrix

	PDA Medium (µm)				YES Medium (µm)					
Isolate No.	**Vesicle diameter**	**Conidiophore width**	**Conidial Head (length)**	**Ascospore Diameter**	**Vesicle diameter**	**Conidiophore width**	**Conidial Head (length)**	**Ascospore Diameter**	**Identified as**	**Afla-toxigenic**
1.	54.44 ± 16.12	9.32 ± 2.59	833.36 ± 87.45	4.06 ± 0.05	34.33 ± 0.62	12.26 ± 3.58	527.95 ± 167.97	5.83 ± 2.09	*A.tamarii*	**- ve**
2.	43.81 ± 8.26	6.47 ± 2.03	**713.36 ± 183.08**	5.14 ± 0.26	27.57 ± 3.67	11.59 ± 0.45	221.29 ± 32.75	6.53 ± 0.88	*A.fumigatus*	**+ ve**
3.	34.37 ± 3.38	7.75 ± 2.60	**346.23 ± 92.26**	3.66 ± 0.54	56.48 ± 3.05	16.00 ± 4.61	937.24 ± 25.21	7.01 ± 1.69	*A.flavus*	**+ ve**
4.	38.51 ± 6.05	10.95 ± 0.64	548.64 ± 220.21	5.07 ± 1.42	52.12 ± 15.06	12.38 ± 3.85	833.71 ± 259.82	6.88 ± 0.64	*A.flavus var. columnaris*	**- ve**
5.	57.24 ± 2.07	8.33 ± 2.32	830.17 ± 139.65	6.25 ± 1.41	69.70 ± 11.57	12.56 ± 3.36	1528.21 ± 758.10	6.88 ± 0.64	*A.tamarii*	**- ve**
6.	40.39 ± 4.35	9.58 ± 3.69	650.05 ± 217.70	5.17 ± 0.89	64.84 ± 25.92	12.70 ± 1.00	1461.29 ± 1297.91	7.81 ± 0.92	*A.flavus var. columnaris*	**- ve**
7.	43.46 ± 12.37	7.44 ± 1.86	528.54 ± 180.31	6.00 ± 1.68	30.78 ± 0.54	11.23 ± 1.47	347.00 ± 26.31	6.22 ± 1.54	*A.sojae*	**- ve**
8.	51.09 ± 14.73	8.02 ± 2.09	674.53 ± 213.62	7.36 ± 0.87	35.03 ± 2.38	9.01 ± 0.33	385.80 ± 35.62	8.12 ± 0.73	*A.flavus var. columnaris*	**- ve**
9.	67.54 ± 31.49	13.32 ± 6.05	576.48 ± 225.00	5.78 ± 0.35	56.95 ± 4.95	14.37 ± 0.56	905.29 ± 39.09	6.53 ± 1.00	*A.sojae*	**- ve**
10.	43.93 ± 4.52	11.36 ± 2.98	707.26 ± 163.07	4.60 ± 0.38	36.40 ± 6.08	10.85 ± 1.45	411.08 ± 155.85	8.19 ± 0.96	*A.tamarii*	**- ve**
11.	54.69 ± 9.77	8.82 ± 1.39	674.98 ± 162.24	6.64 ± 0.46	49.83 ± 4.94	12.05 ± 4.72	815.33 ± 28.32	7.77 ± 1.02	*A.flavus var. columnaris*	**- ve**
12.	66.37 ± 23.55	9.80 ± 3.26	**785.57 ± 245.34**	4.92 ± 0.59	77.50 ± 3.16	12.48 ± 0.82	1108.08 ± 84.43	7.48 ± 0.76	*A.flavus var. columnaris*	**+ ve**
13.	57.45 ± 7.43	5.10 ± 1.43	**301.81 ± 45.73**	2.69 ± 0.55	47.96 ± 13.50	12.77 ± 3.07	578.52 ± 244.66	9.05 ± 1.37	*A.flavus*	**+ ve**
14.	43.06 ± 3.63	10.19 ± 1.71	**450.59 ± 90.98**	7.05 ± 1.65	31.34 ± 6.80	9.18 ± 1.18	345.23 ± 139.36	5.04 ± 1.23	*A.flavus*	**+ ve**
15.	52.60 ± 7.30	3.63 ± 0.49	**619.40 ± 286.47**	4.74 ± 0.39	67.02 ± 14.52	13.55 ± 1.76	555.53 ± 327.28	4.96 ± 1.66	*A. parasiticus*	**+ ve**
16.	70.84 ± 26.49	8.58 ± 1.39	**719.25 ± 206.58**	6.70 ± 1.35	68.61 ± 12.65	10.14 ± 2.71	291.82 ± 68.31	6.57 ± 2.10	*A.niveus*	**+ ve**
17.	66.20 ± 8.72	10.06 ± 0.99	730.21 ± 49.52	6.64 ± 0.46	45.63 ± 6.92	12.62 ± 2.33	617.24 ± 207.70	7.02 ± 1.64	*A.tamarii*	**- ve**
18.	46.18 ± 4.34	8.09 ± 2.83	585.20 ± 114.19	5.52 ± 0.31	36.33 ± 9.95	13.86 ± 3.47	595.86 ± 244.88	7.45 ± 1.29	*A.flavus*	**- ve**
19.	35.62 ± 4.41	9.21 ± 2.09	536.70 ± 86.74	4.59 ±0.80	78.95 ± 36.05	15.43 ± 8.14	1191.28 ± 709.73	7.60 ± 1.97	*A.flavus*	**- ve**
20.	37.61 ± 3.61	10.14 ± 1.65	514.83 ± 99.38	7.37 ± 0.75	46.86 ± 2.60	11.72 ± 0.23	581.62 ± 174.03	7.74 ± 0.24	*A.tamarii*	**- ve**
21.	50.99 ± 10.25	6.64 ± 2.12	**753.57 ± 120.44**	4.95 ± 1.12	23.09 ± 3.06	6.79 ± 1.63	183.58 ± 17.76	7.11 ± 0.29	*A.flavus*	**+ ve**

Values after ± indicates standard deviations (SD) of five replications

Fig. 1 Macroscopic and microscopic characteristics of *A. flavus*. A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YESmedium, front and reverse surface ;G-H: conidial heads and ascospore

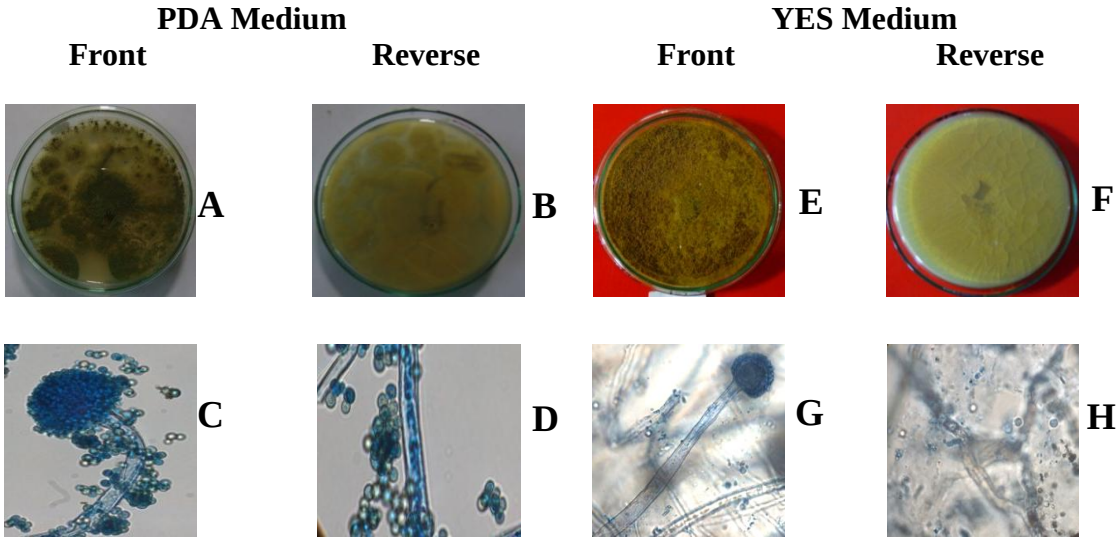


Fig. 2: Macroscopic and microscopic characteristics of *A. tamarii* . A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YESmedium, front and reverse surface ;G-H: conidial heads and ascospore

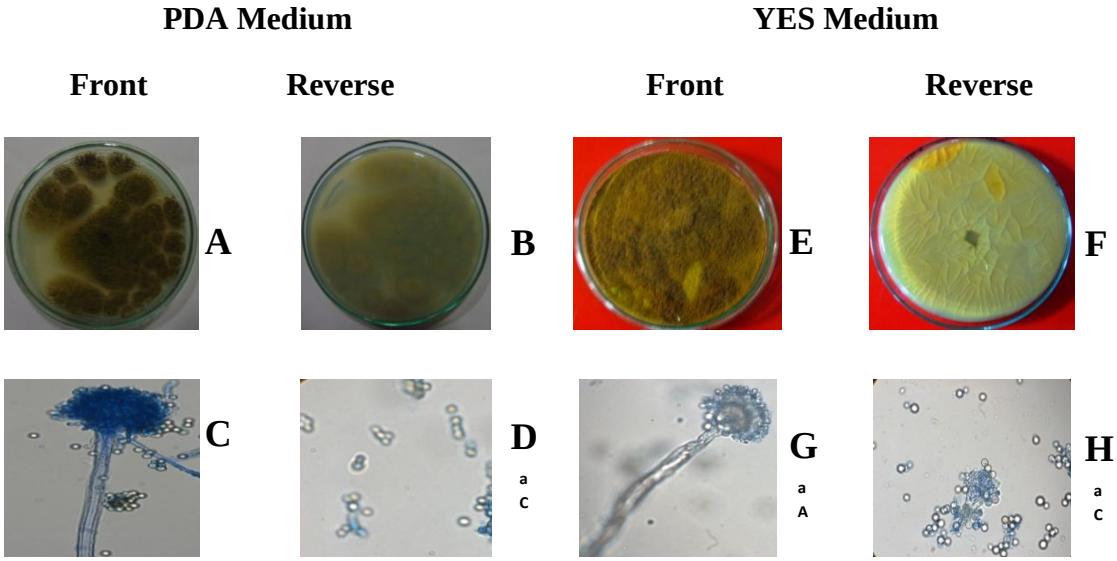


Fig.3 Macroscopic and microscopic characteristics of *A. flavus var. columnaris.* A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YES medium, front and reverse surface; G-H: conidial heads and ascospore

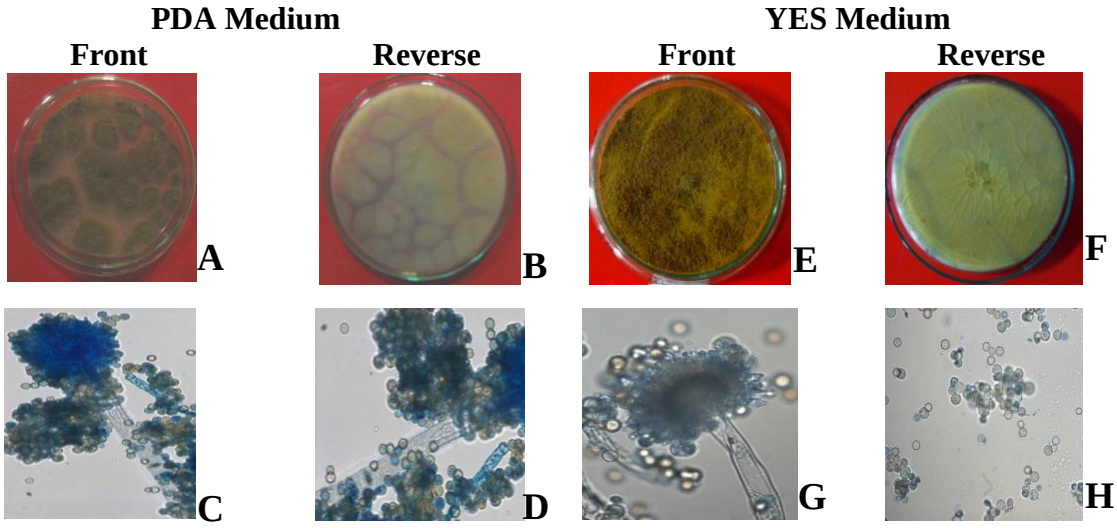


Fig.4 Macroscopic and microscopic characteristics of *A. sojae.* A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YES medium, front and reverse surface; G-H: conidial heads and ascospore

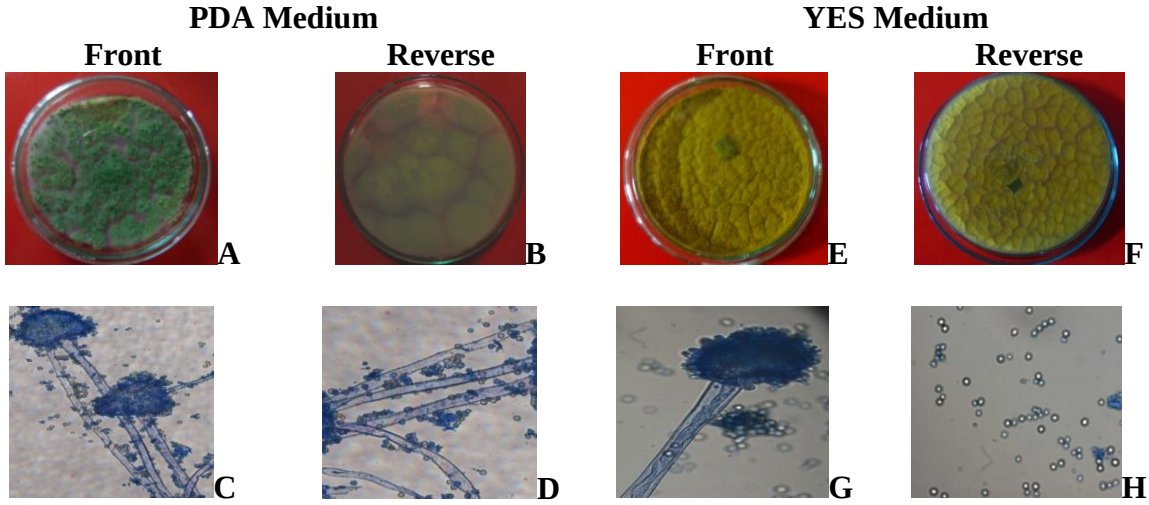


Fig.5 Macroscopic and microscopic characteristics of *A. parasiticus* A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YES medium, front and reverse surface ;G-H: conidial heads and ascospore

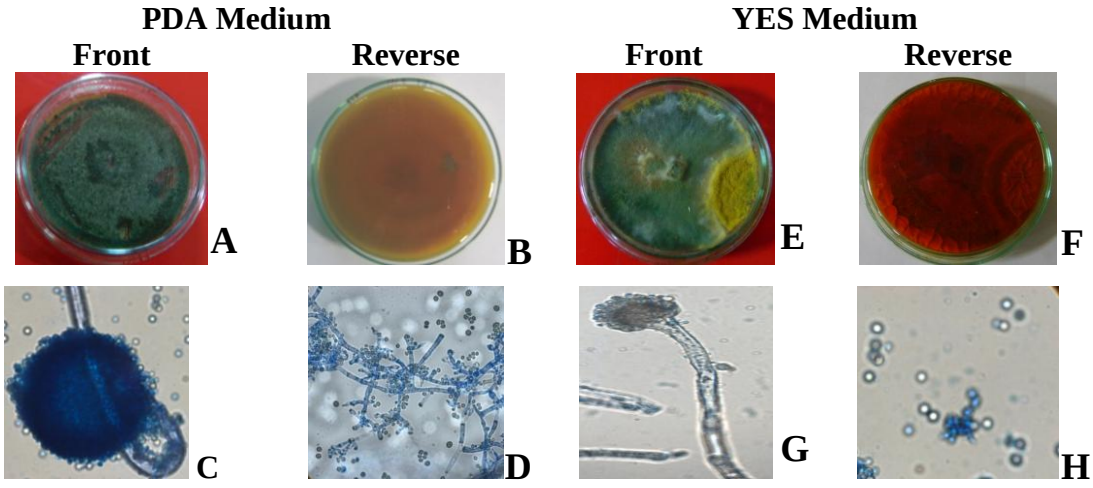


Fig.6 Macroscopic and microscopic characteristics of *A.niveus*. A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YES medium, front and reverse surface ;G-H: conidial heads and ascospore.

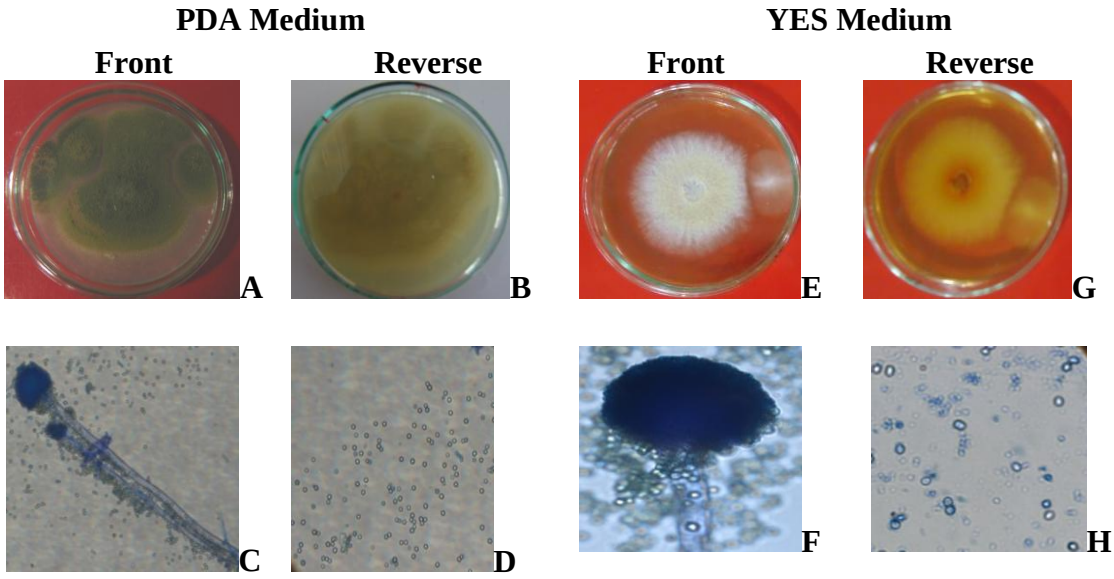


Fig. 7: Macroscopic and microscopic characteristics of *A. fumigates.* A-B: colony features on PDA medium, front and reverse surface; C-D: conidial heads and conidia; E-F: colony features on YES medium, front and reverse surface ;G-H: conidial heads and ascospore

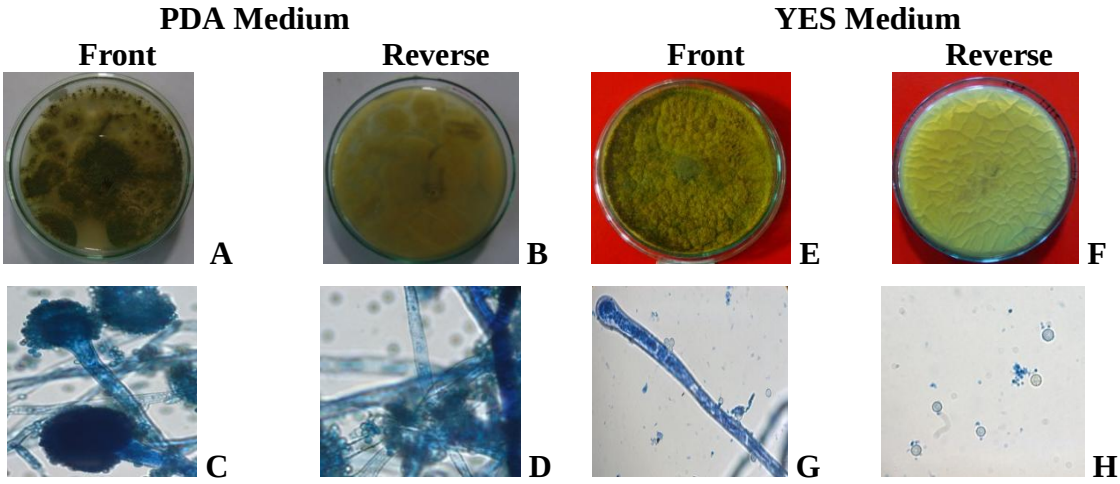
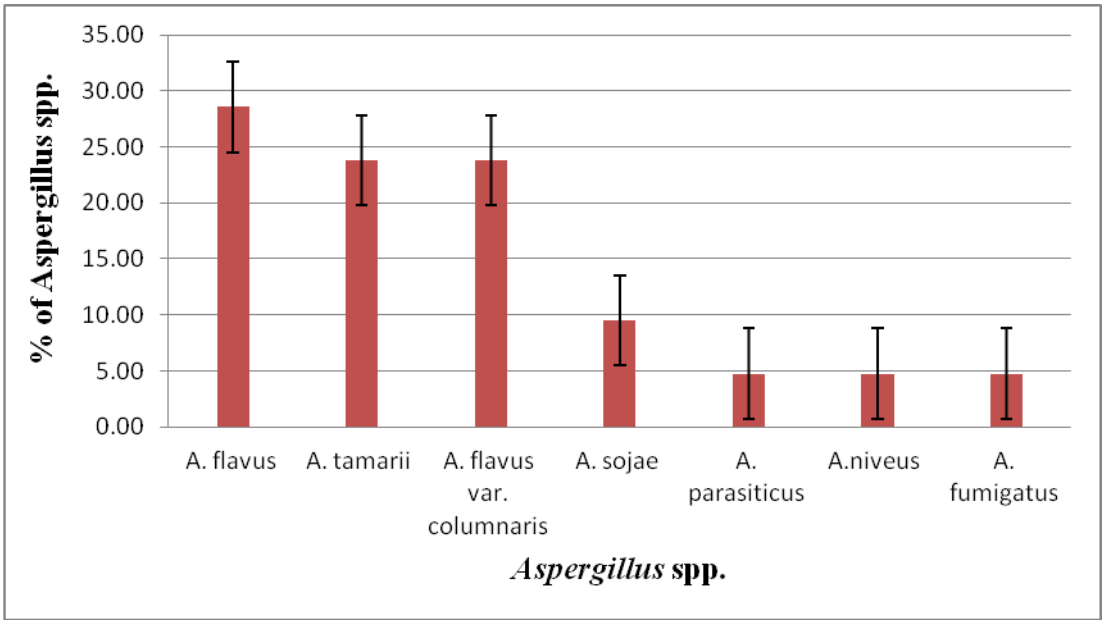


Fig. 8: Species distribution of *Aspergillus* isolates in percentage identified from groundnut Rhizosphere



Screening of aflatoxin producing and non producing *Aspergillus* strains

To differentiate aflatoxin producing and aflatoxin nonproducing strains ammonium hydroxide vapor test was conducted. Results revealed that 08 fungal isolates *viz.* 2. RJD-UPA-KUN-G-2, 3. JAM-JKB-BHA-GG20, 12. RJD-UPA-KUN-TJ37A, 13. RJD-DHO-PAR-GG37, 14. RJD-DHO-KAN-GG45, 15. JND-MAN-LIM-GG20 A, 16. JND-MAN-LIM-GG20 B, 21. JND- JAU –ISS-1 when

exposed to ammonium hydroxide vapors, the reverse side of colonies turn from yellow to pink indicating the isolates produced toxic compound called aflatoxin. The remaining fungal isolates remain unchanged for reverse colony color (Table 2**)**.

The *A. flavus, A. tamarii, A. flavus var. columnaris, A. sojae, A. parasiticus, A.niveus,* and *A. fumigatus* isolated in the present study are reported to be the most common fungal contaminant of groundnut rhizosphere. The fungal colonization was fast in most of the isolates and the mold presence on groundnut seed was also distinct. Among the different *Aspergillus species* obtained, the *flavus* species was found to be predominating in the study. Though the soil is a habitat for many types of fungal strains, only selected fungi have the ability to establish infection in the post harvest groundnut. The fungal contamination occurs not only from the field soil but also from field instruments, insects, pests, birds and storage methods. The wide spread occurrence of *Aspergillus flavus* showed the extent of pre-harvest and post-harvest contamination occurring naturally (Kabir *et al.,* 2013). Among the *Aspergillus species* obtained in the present study, the predominating fungal species found to be *A. flavus* (28.57%) followed by, *A. tamari* (23.81%), *A. flavus var. columnaris* (23.81%), *A. sojae* (9.52%), *A. parasiticus* (4.76%), *A. niveus* (4.76%) and *A. fumigatus* (4.76%).

The results have shown that PDA and YES media are easy, simple and reliable as also recorded by Raper and Fennell (1965). No any increase or decrease trade was observed in microscopic character on both media. In most of isolates when vesicle diameter increase on YES medium , all other microscopic characters were found to be increase but it was not in all the isolates . Various reports have been published that used morphological characters as key identifying factors (Morya *et al.,* 2009; Bandh *et al.,* 2012). *A. flavus and A. tamari* were dominating species isolated from all obtained isolates. Identifying character of *Aspergillus flavus* were yellow to green or dark green colony color and radiate heads with smooth conidiophore wall. Maximum isolates showed spot ring like flat conidiation on YES medium, while mix (circular or spot ring like flat) condition was seen among isolates on PDA medium. The YES medium is easy to prepare, relatively inexpensive, and is suitable for production of higher levels of aflatoxin than those reported for other media. For these reasons, YES medium appears to be suitable for both production of aflatoxin and for screening fungi for their ability to produce aflatoxins (Davis *et al.,* 1966).

Saito and Machida (1999) developed a rapid method for identifying aflatoxin producing and non-producing strains of *A. flavus* and *A. parasiticus*, which may provide a useful pre-screen for identifying non-toxigenic strains. In this method, the reverse side of colonies of aflatoxin producing *Aspergillus* strains on potato dextrose agar (PDA) medium turn from yellow to pink immediately after exposure to ammonium hydroxide vapor. In present study more than 60% of *Aspergillus* isolates belongs to either *Aspergillus flavus* or *Aspergillus parasiticus sp.* producing aflatoxin in present investigation. Besides that, *A. fumigates* and *A. niveus* also showed positive biochemical test for aflatoxin production. Further the level of quantification of aflatoxin production need to be conformed for newly identified *Aspergillus* spp. The percentage of aflatoxigenic strains of *Aspergillus flavus* has been shown to vary with the nature of substrate and environmental factors. The incidence of aflatoxigenic *Aspergillus flavus* strains was higher in peanuts (69%) than in wheat (13%) (Vaamonde *et al.,* 2003). Abdi *et al* (2014) had found *Aspergillus flavus* contamination in peanut kernels ranging from 20% to 48% varying with the region and place of sample

collection.

In conclusion, characterization of *Aspergillus* associated with aflatoxic and non aflatoxic isolates from groundnut seed, cake and soil showed diversity in groundnut rhizosphere. The study showed that earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques. Their earlier detection may help to adopt physical management practices and to initiate some biocontrol methods to avoid afla-toxinogenic contamination in groundnut and maintain seed quality for export.

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