Journal of Environmental Biology

ISSN : 0254-8704 (Print) ISSN : 2394-0379 (Online) CODEN : JEBIDP

REPRINT

Morphological and toxigenic variability in the *Aspergillus flavus* isolates from peanut (*Arachis hypogaea* L.) production system in Gujarat (India)

Diwakar Singh, Radhakrishnan Thankappan, Vinod Kumar, Naimoddin B. Bagwan, Mukti S. Basu, Jentilal R. Dobaria, Gyan P. Mishra and Sumitra Chanda

Volume 36

Number 2

March 2015

Pages 441-449

JEB
Journal of Environmental Biology

Journal Home page : www.jeb.co.in
E-mail : editor@jeb.co.in

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Morphological and toxigenic variability in the *Aspergillus flavus* isolates from peanut (*Arachis hypogaea* L.) production system in Gujarat (India)

Diwakar Singh[1], Radhakrishnan Thankappan[1]*, Vinod Kumar[1], Naimoddin B. Bagwan[1], Mukti S. Basu[1], Jentilal R. Dobaria[1], Gyan P. Mishra[1] and Sumitra Chanda[2]

[1]Crop Improvement Division, Directorate of Groundnut Research, P.B. No. 5, Junagadh-362 001, India
[2]Department of Biosciences, Saurashtra University, Rajkot-360 005, India
*Corresponding Authors Email : radhakrishnan.nrcg@gmail.com

Publication Info

Paper received:
20 September 2013

Revised received:
25 February 2014

Accepted:
20 June 2014

Abstract

Morphological and toxigenic variability in 187 *Aspergillus flavus* isolates, collected from a major Indian peanut production system, from 10 districts of Gujarat was studied. On the basis of colony characteristics, the isolates were grouped as group A (83%), B (11%) and G (6%). Of all the isolates, 21%, 47% and 32% were found to be fast-growing, moderately-fast and slow-growing respectively, and no-sclerotia and sclerotia production was recorded in 32.1% and 67% isolates respectively. Large, medium and small number of sclerotia production was observed in 55, 38 and 34 isolates respectively. Toxigenic potential based on ammonia vapour test was not found reliable, while ELISA test identified 68.5%, 18.7% and 12.8% isolates as atoxigenic, moderately-toxigenic and highly-toxigenic, respectively. On clustering, the isolates were grouped into 15 distinct clusters, 'A' group of isolates was grouped distinctly in different clusters, while 'B' and 'G' groups of isolates were clustered together. No association was observed between morphological-diversity and toxigenic potential of the isolates. From the present investigation, most virulent isolates were pooled to form a consortium for sick-plot screening of germplasm, against *Aspergillus flavus*. In future, atoxigenic isolates may be evaluated for their potential to be used as bio-control agent against toxigenic isolates.

Key words

Aflatoxin, ELISA, Groundnut, Morphological diversity, Toxigenic potential

Introduction

Peanut (*Arachis hypogaea* L.) also known as groundnut is cultivated in the semi-arid tropical and sub-tropical regions of the world between 40°N and 40°S, mainly in developing countries of Africa and Asia, (Shoba *et al.,* 2012). It is an important oilseed, food and feed crop grown on about 20.88 million ha throughout the world (FAOSTAT, 2012). In India, it occupied an area of 4.20 million ha with a production of 6.9 million tonnes in 2011, which accounted for productivity of 1655 kg ha^{-1} (FAOSTAT, 2012). With the increased awareness of aflatoxin contamination in peanut (Guo *et al.,* 2009), this issue has become a major financial concern to the peanut industry, as more regulatory import measures take effect worldwide (Guo *et al.,* 2011).

Gujarat is one of the major producers and exporters of peanut states of India, where aflatoxin contamination most often becomes a bottleneck. In value terms, the export of peanut reached a level of around 600 million USD during April-November, 2011 from 236 million USD during the previous year (Anonymous, 2012). But the higher aflatoxin load in the exportable commodities like HPS- grade kernels, de-oiled cakes have jeopardized the export earning, thereby depriving our country of valuable foreign exchange (IARC, 2002). The export of peanut from India can be increased to many folds if aflatoxin contamination is reduced to the stringent tolerance limits as imposed by the importing countries.

Aflatoxin contamination of peanut, due to invasion by

Aspergillus flavus and *Aspergillus parasiticus*, is a major problem of rainfed agriculture (Upadhyaya *et al*., 2000) which seriously affects the quality of produce, causing health risks to human and cattle (Abbas *et al.,* 2004). Peanut pods, when come in direct contact with spores of *A. flavus* in soil, are frequently invaded before harvest (Horn *et al.,* 1994). The mode and extent of invasion by the fungus depends on soil population density of *A. flavus*, soil moisture and soil temperature during pod development till maturity. These fungi can invade and produce toxins in peanut kernels before harvest, during drying and in storage (Jiang *et al.,* 2009).

The genetic capacity of *A. flavus* to produce aflatoxin is extremely variable; with many strains being non-toxic. Moreover, soil population of *A. flavus* also differs according to the geographic region and its toxigenic ability (Mishra and Das, 2003). Therefore, there is a need to characterize the isolates for their toxigenic potential *vis a vis* the distribution pattern of these strains in different agro-ecological zones of peanut production systems in the Gujarat state of India. Besides, it would be of help in deploying atoxigenic strains, as candidate bio-control agent for the management of aflatoxin contamination (Das *et al.,* 2008). Moreover, diverse and highly toxigenic isolates are required for screening of resistant peanut genotypes into sick-plot against *A. flavus* infection and aflatoxin contamination. With the objective to develop a consortium of diverse and highly toxigenic isolates of *A. flavus, this* study was carried out to assess the morphological and toxigenic variability in the isolates of *Aspergillus flavus* collected from peanut production system in the Gujarat state (India).

Materials and Methods

Soil samples were collected from peanut fields, shortly after sowing and two weeks before harvesting, from same sites of ten districts *viz*. Amreli, Anand, Bhavnagar, Bhuj, Jamnagar, Junagadh, Porbandar, Rajkot, Sabarkantha, and Surendranagar of Gujarat State, India (Fig. 1). At each sampling, soil samples were collected from five randomly selected spots, at 0-10 cm depth and individual samples pooled for each plot.

Isolation of *Aspergillus flavus* : The fungus was isolated using *A. flavus* and *A. parasiticus* agar (AFPA) medium by dilution plating (Horn and Dorner, 1999). Potato Dextrose Agar (PDA) medium was prepared for PDA slants and petri plates; and streptomycin (75 mg l^{-1}) was added before pouring. Typical *Aspergillus* colonies developed on petri dishes were transferred to PDA slants, and cultures were purified using single spore isolation technique. *Aspergillus* spp. were then isolated, purified, characterized and maintained as single spore culture on agar slants, and cultures were given identity, based on their collection details and sampling site. A total of 187 *A. flavus* isolates, collected from different locations across 10 districts of Gujarat state (India), were used for this study (Fig. 1).

Morphological studies : For initial inoculum generation, few spores of *A. flavus* isolates were placed in the centre of a petri dish containing PDA, and incubated for 4 days at 28±2 ºC. Further, 04 mm diameter plugs of inoculums, cut from the periphery of the working culture plates, were seeded in petri dishes containing PDA, in three replicate. The plates were then incubated at 28±2 ºC, and fungal growth was measured 4 days after inoculation. Radial growth of the fungal colony was recorded in millimeter, in two directions at right angles and visual scoring was done using Methuen colour book (Kornerup and Wanscher 1978), the inoculated plates after 7 days of incubation were classified into sclerotia-producers and non-producers. Based on the abundance of sclerotia, the isolates were categorized into high (+++), moderate (++), low (+) and nil (-) classes. The size of sclerotium was measured using micrometry technique and mean sclerotium size was expressed in micrometer.

Toxigenicity studies: Two different methods were used for aflatoxin detection *viz.* ammonia vapor test (qualitative test) and Indirect – competitive ELISA (quantitative test).

Ammonia (NH_4^+) vapor test: This test was done as per Saito and Machida (1999) where, isolates were first inoculated at the center of solidified PDA medium in 9 cm glass petri dishes and further incubated at 28±1ºC. To observe the colour change of colony-reverse after incubation, dishes were placed upside down and a drop (0.2 ml) of 25% ammonia solution was put into the lid of the petri dish. The colony-reverse of aflatoxin producing strains of *A. flavus* turned pink however, no change in colour was observed with plates of atoxigenic strains.

Indirect competitive : Enzyme Linked Immuno Sorbent Assay (ELISA): Indirect competitive ELISA reagents based on polyclonal antibody, was used for qualitative screening of aflatoxin-positive samples. AFB_1-BSA conjugate was prepared in carbonate coating buffer (100 ng ml^{-1}), and added to each well (150 µl). The plates were then incubated (37 ºC; 1 hr), after which the toxin was collected and stored, and plates were washed by PBS-Tween. BSA (0.2%) prepared in PBS-Tween (200 µl $well^{-1}$) was added and incubated (37ºC; 1 hr). Antiserum was diluted in PBS-Tween containing BSA (0.2%) and incubated (37 ºC; 45 min), and after proper blocking the blocked plates were washed by PBS-Tween.

Healthy seed extract of peanut variety J-11, was taken as negative control and for positive control, aflatoxin B_1 standard was diluted (1:10) with peanut extract at concentration ranging from 100 ng to 10 picogram (100 µl). The absorbance was measured at 405 nm in an automatic ELISA reader. Based on the values obtained for aflatoxin B_1 standards, a curve was drawn taking aflatoxin concentrations on x-axis and optical density values on the y-axis.

Diversity analysis : Clustering of isolates was done using

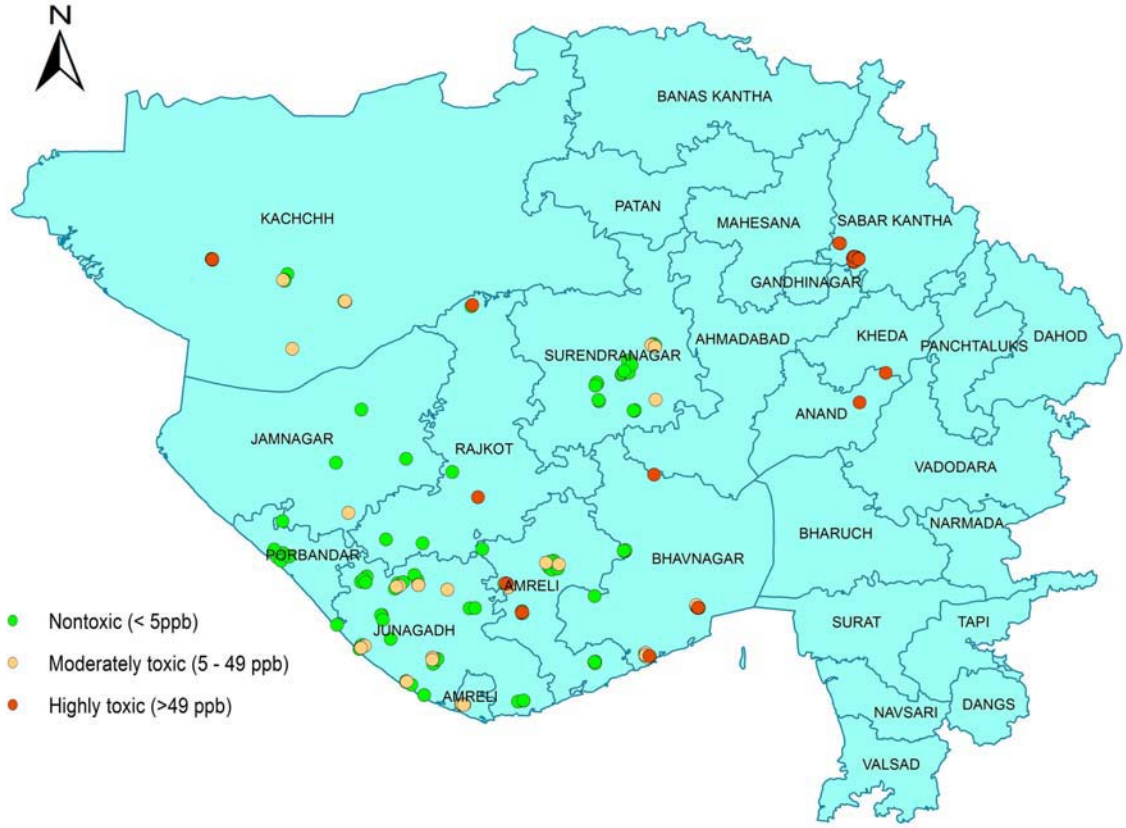

Fig. 1 : GIS map of 187 *Aspergillus flavus* isolates showing their collection sites and toxicity

Euclidean distance parameters (average linkage method) using the software gCLUTOver 1.0 (Rasmussen and Karypis, 2004)

Results and Discussion

Aflatoxin contamination in pre-harvested peanut is caused due to infection of *Aspergillus* species. Currently, peanut cultivars resistant to *A. flavus* and *A. parasiticus* infection are rare, and little is known about the molecular mechanisms conferring resistance (Guo *et al.,* 2011). Therefore, understanding the presence of *Aspergillus* in field, and its toxigenic potential is necessary to develop effective strategies to reduce aflatoxin contamination of pre- and post-harvest peanut crop.

All the 187 isolates, when characterized were found to be *A. flavus* (100%), which is in accordance with that of Jamali *et al.* (2012), where all the 193 *Aspergillus* strains isolated from soil samples of pistachio orchards, were identified as *A. flavus*. This could be due to the fact that, the most abundant species of *Aspergillus* section Flavi existing in that environment was *A. flavus* (Jamali *et al.,* 2012). However, from the corn fields of Iran, Razzaghi-Abyaneh *et al.* (2006) reported *A. flavus* (87.9%) followed by *A. nomius* (9.1%) and *A. parasiticus* (3.0%), which means that under different agro-climatic conditions and cropping systems, different species prevail in nature.

Morphological traits like radial growth of the fungus, colony colour, sclerotia-production, and size of sclerotia were studied in the collected isolates, which varied significantly for all these traits. Of all the isolates, 11 produced maximum colony diameter (8.0 mm), whereas minimum (1.1 mm) was recorded in the isolate NRCG 10007 (Table 1). However, other isolates such as NRCG 06002, 06004, 06009 and 06014 also recorded very poor growth (colony diameter ≤ 2.0 mm). Rinyu *et al.* (1995) studied 61 strains of *A. fumigatus* strains that were highly variable in colony morphology, growth rate and level of pigment production. While characterizing *A. flavus* isolates from

Table 1 : Values of *Aspergillus flavus* isolates in terms of NRCG accession number (AN); colony diameter (CD in cm); number of sclerotia (NOS in plate^{-1}) and size of sclerotia (SS in µm)

AN	CD	NOS	SS	SN	AN	CD	NOS	SS	SN	AN	CD	NOS	SS
01009	6.2	91	1235	64	02040	8.0	0	0	127	08006	4.5	358	589
01012	5.4	112	1112	65	03005	7.3	44	1278	128	08008	6.4	0	0
01016	5.9	67	1269	66	03007	7.1	21	1304	129	08008	4.4	43	1267
01018	6.8	221	948	67	03024	2.2	13	1343	130	08009	5.9	9	1313
01025	6.8	88	1201	68	03026	5.3	0	0	131	08010	4.7	0	0
01026	5.9	254	862	69	03027	6.2	234	878	132	08011	7.5	33	1298
01031	5.4	0	0	70	03028	5.5	8	1349	133	08012	2.4	0	0
01032	5.8	0	0	71	03029	6.3	0	0	134	08013	4.4	15	1323
01035	6.3	0	0	72	03030	6.2	28	1289	135	08014	5.9	369	592
01036	8.0	49	1294	73	03031	2.3	96	1133	136	08015	3.3	0	0
01038	7.6	34	1306	74	03032	2.3	236	860	137	08016	6.8	387	593
01039	4.8	25	1308	75	03037	4.4	244	839	138	08017	2.7	0	0
01040	6.4	0	0	76	04005	6.1	39	1304	139	08018	6.5	11	1343
01041	5.8	113	1114	77	04010	6.3	247	824	140	08019	4.5	23	1288
01043	7.2	154	999	78	05005	7.2	15	1324	141	08020	5.3	0	0
01045	6.5	204	925	79	05010	4.6	14	1320	142	08021	4.4	0	0
01046	7.5	76	1242	80	05011	6.8	9	1341	143	08022	4.3	0	0
01047	6.9	78	1211	81	05016	6.0	7	1345	144	09001	5.1	0	0
01048	7.1	87	1246	82	05017	6.6	9	1335	145	09002	4.4	39	1279
01049	7.9	234	917	83	05018	6.6	12	1329	146	09005	5.8	402	599
01051	7.4	167	981	84	05019	6.6	12	1315	147	10001	6.8	0	0
01052	7.9	198	973	85	05020	6.1	255	801	148	10002	6.4	17	1329
01053	5.0	322	649	86	05021	6.2	143	1107	149	10003	6.3	14	1335
01055	7.6	119	1098	87	05022	6.3	254	774	150	10004	7.4	11	1340
01056	6.4	233	897	88	05023	6.9	0	0	151	10005	6.9	399	608
01057	6.7	206	950	89	05024	6.4	0	0	152	10006	6.3	425	587
01058	3.0	17	1335	90	05025	6.3	0	0	153	10007	1.1	50	1269
01059	6.3	0	0	91	05026	6.6	0	0	154	10008	4.3	34	1297
01060	6.4	0	0	92	05027	6.1	0	0	155	10009	3.9	0	0
01061	3.1	0	0	93	05028	5.8	14	1305	156	01003	6.6	68	1255
01062	6.3	0	0	94	05029	6.6	17	1284	157	01007	7.6	134	1087
01063	4.1	43	1281	95	05030	6.2	254	796	158	01028	6.7	0	0
01064	3.4	38	1301	96	05031	6.9	0	0	159	01037	5.2	0	0
01065	6.4	29	1293	97	05032	6.8	258	767	160	01044	8.0	0	0
01066	5.4	31	1275	98	05033	7.2	10	1336	161	01054	8.0	0	0
01067	7.1	36	1223	99	05034	4.0	46	1297	162	02019	7.3	32	1299
01068	3.2	0	0	100	05035	3.3	9	1345	163	02025	8.0	45	1287
01069	5.0	0	0	101	05036	4.7	11	1322	164	02028	8.0	34	1301
01070	4.5	0	0	102	06001	5.6	127	1071	165	02041	8.0	0	0
01071	3.1	23	1317	103	06002	1.7	19	1310	166	03003	7.8	0	0
01073	3.4	27	1309	104	06003	8.0	20	1312	167	03015	6.9	0	0
01074	3.6	45	1265	105	06004	1.9	27	1295	168	03019	6.7	218	889
01075	4.1	0	0	106	06006	5.1	0	0	169	03020	7.4	125	1103
01076	3.4	0	0	107	06007	4.5	36	1288	170	03025	8.0	227	880
01077	6.4	0	0	108	06008	2.9	13	1317	171	03034	3.5	0	0
02002	6.8	33	1272	109	06009	2.0	304	625	172	06005	4.3	161	1002
02004	6.7	25	1293	110	06011	4.3	11	1337	173	06010	6.7	0	0
02007	6.9	0	0	111	06012	5.2	46	1273	174	06014	2.0	0	0
02008	7.5	0	0	112	06013	4.3	284	775	175	06017	2.4	0	0
02009	6.7	0	0	113	06015	6.2	324	622	176	01042	7.8	0	0
02011	7.1	0	0	114	06016	2.2	354	609	177	02026	7.6	187	962
02013	6.3	0	0	115	06018	3.4	0	0	178	02027	7.9	0	0
02 014	6.4	9	1349	116	06019	3.3	294	754	179	02033	5.1	34	1304
02017	7.1	12	1334	117	06020	3.7	48	1285	180	02036	5.2	22	1311
02021	6.3	19	1307	118	06021	2.4	0	0	181	02042	7.6	27	1323

02024	8.0	14	1322	119	07001	4.2	10	1347	182	02043	7.9	31	1302
02029	8.0	198	975	120	07002	4.3	118	1090	183	03033	3.4	231	877
02031	5.8	0	0	121	07004	4.3	65	1269	184	03035	3.6	46	1285
02034	5.6	14	1319	122	08001	6.3	8	1344	185	03036	2.5	0	0
02035	5.9	204	987	123	08002	4.6	0	0	186	07003	5.4	172	998
02037	3.3	14	1314	124	08003	6.7	0	0	187	09004	5.1	32	1309
02038	5.2	0	0	125	08004	4.4	28	1289					
02039	4.4	219	970	126	08005	7.3	16	1309					

groundnut production system of India (Gujarat), for radial growth and colony diameter, around 50% of total isolates recorded more than 7.0 mm colony diameter (Table 1). Bigger colony diameter in general reflects better ability of the fungus to utilize the available nutrition and conversion of the same into effective biomass. Variability in growth among isolates of fungi due to various edaphic and nutritional factors was also reported in previous studies (Amaike and Keller, 2011; Desai *et al.,* 2003).

In this study, based on the colony colour, isolates were grouped into three distinct classes *viz*. parrot-green (Group 'A'; 155 isolates), white-fluffy with yellow sporulation (Group 'B'; 20 isolates) and olive-green (Group 'G'; 12 isolates; Table 2). Variation in the colony colour of *A. flavus*, *A. parasiticus* and *A. tamarii* was also reported by Horn *et al.* (1996).

Sclerotia are generally produced by many fungi including *Aspergillus* to overcome adverse climatic conditions (Hesseltine *et al.,* 1970). Lisker *et al.* (1993) while working with 200 isolates of *A. flavus* isolated from peanut field, found that about 30% and 70% as sclerotia-producing and non-producing types. In the present investigation, it was also recorded that 68% (127 numbers) and 32% (60 numbers) of the isolates were sclerotia producing and non-producing types (Table 1). Jamali *et al.* (2012) observed approximately 61% of the isolates (of 193 *Aspergillus* isolates studied) were capable of producing sclerotia.

Out of 127 sclerotia-producing isolates, 34 (18%), 38 (20%) and 55 (29%) belonged to low, moderate and high sclerotia-producing types (Table 1). Among the low sclerotia-producing isolates, NRCG 05016 produced the lowest, 07 sclerotia per plate, whereas 33 other isolates produced sclerotia ranging from 8-19 per plate. Razzaghi-Abyaneh *et al.* (2006) reported sclerotia production in only 4 isolates of *A. flavus* among the 66 isolates studied, from the corn fields of Iran. These studies clearly indicated that in different parts of the world, under different agro-climatic conditions, the ratio of sclerotia producers and non-producers maintain different equilibrium.

The size of sclerotia ranged from 587 to 1349 µm (Table 1), and the largest was observed for two isolates *viz*. NRCG 03015 and 07001 (1349 µm) whereas, NRCG 01051 recorded the smallest sclerotia (587 µm). It was also observed that the isolates which produced relatively less numbers of sclerotia usually produced large sclerotium and *vice-versa* (Data not shown).

Previous studies have revealed that *A. flavus* could be grouped into small (S-type) and large (L-type) sclerotia producer types based on size of sclerotia, and S-types are generally less-toxigenic, compared to L-type isolates (Geiser *et al.,* 2000). In the present investigation, significant variation in the size of sclerotia was observed, which ranged from 587 to 1349 µm (Table 1). When these isolates were characterized for sclerotia producing ability *vis-à-vis* toxigenicity, no significant correlation was observed (data not shown) which is similar to the results obtained by Amani *et al*. (2012). It means, there is significant natural variability exists in *A. flavus* isolates for traits like formation of sclerotia and size of sclerotia.

Out of ten surveyed districts, group 'A' isolates were most prevailing (82.89%) followed by group 'B' (10.69%) and group 'G' isolates (6.42%). Four districts namely, Surendranagar, Bhavnagar, Anand and Porbandar showed the spread of only group 'A' isolates, whereas all the three groups *i.e.,* 'A', 'B' and 'G' were prevailing in Junagadh, Amreli and Bhuj districts (Table 2, Fig. 1). This may be due to the close proximity of Junagadh and Amreli districts or it could also be due to ecological factors in those districts which helped in the survival of all these groups of isolates, which needs further investigation.

It was observed that warm dry climates favoured 'B' and 'G' isolates over other members of section Flavi, and were most prevalent in the agroecological zones bordering the Sahara desert (Cardwell and Cotty, 2002). However, it was observed that 'B' and 'G' group of isolates as least prevalent under Gujarat conditions, which could be due to the fact that in India groundnut is mostly taken as rainfed crop during *kharif*, when average mean temperature is not so high. In North America, the similarly adapted S-strain of *A. flavus* is also most common in dry and hot regions (Cotty 1989, 1997; Jaime-Garcia and Cotty, 2006). Relative virulence of different *A. flavus* isolates on maize, peanut, and other susceptible crops needs to be examined in order to fully evaluate the risk posed by this potent aflatoxin producer (Donner *et al.,* 2009).

Variation in aflatoxin content was observed, when aflatoxin content was assessed using Indirect competitive ELISA and NH_4^+ vapour test. In ELISA test, out of 187 isolates, 128 (68.5%) were

Table 2 : Values of *Aspergillus flavus* isolates toxicity in terms of ELISA and NH_4^+ vapor

Districts	Groups	Total No.	Toxigenicity by ELISA (µg kg^{-1})		Toxigenicity by NH_4^+ vapor method*			
			Nontoxic (<5)	Moderate(5-49)	High(>49)	Nontoxic	Moderate	High
Junagadh	A	45	35	10	0	21	21	3
	B	6	4	2	0	4	2	0
	G	1	0	1	0	0	1	0
Amreli	A	19	10	7	2	11	6	2
	B	4	4	0	0	3	1	0
	G	6	6	0	0	4	2	0
Bhuj	A	11	7	3	1	4	7	0
	B	6	5	1	0	4	2	0
	G	3	3	0	0	3	0	0
Anand	A	2	0	0	2	0	0	2
Bhavnagar	A	24	14	6	4	10	11	3
Sabarkantha	A	17	6	1	10	3	5	9
	B	4	1	0	3	0	1	3
Jamnagar	A	3	2	1	0	1	2	0
	G	1	1	0	0	0	1	0
Surendra Nagar	A	22	19	3	0	8	14	0
Rajkot	A	3	3	0	0	0	3	0
	G	1	0	0	1	0	0	1
Porbandar	A	9	8	0	1	7	1	1
	Total	187	128(68.5)	35(18.7)	24(12.8)	83(44.4)	80(42.8)	24(12.8)
	A	155(82.89)**	104(67.10)	31(20.00)	20(12.90)	65(41.94)	70(45.16)	20(12.90)
	B	20(10.69)	14(70.00)	3(15.00)	3(15.00)	11(55.00)	6(30.00)	3(15.00)
	G	12(6.42)	10(83.33)	1(8.33)	1(8.33)	7(58.33)	4(33.33)	1(8.33)

*Based on development of colour upon NH_4^+ treatment; ** Figures in parenthesis are % of total isolates

found to be atoxigenic, 35 (18.7%) as moderately toxigenic and 24 (12.8%) as highly toxigenic (Table 2). However, with Ammonia vapor test, 83 (44.4%) isolates were found atoxigenic, 80 (42.8%) as moderately toxigenic and only 24 (12.8%) isolates highly toxigenic (Table 2). When data was compared for these methods 12.9% isolates were found to be highly toxigenic.

Some of the isolates, which were identified as atoxigenic by ammonia vapour test, were found to be moderately toxigenic by ELISA test. Ammonium vapour test is relatively not so reliable test for detection of aflatoxins, as false negatives and positives were produced (Yazdani *et al.,* 2010). Variation in result might be due to low aflatoxin content in the isolate, which could not be detected by NH_4^+ vapor test (no colour development). However, these isolates when analyzed by ELISA, having aflatoxin content >5 ppb were included under moderately toxigenic isolate, e.g. NRCG 01035, 01056, 01068, 01073, 01075, 02040, etc. (Table 2). Kumar *et al.* (2007) observed 92% efficacy for ammonium vapour test having 8% false negatives. Abbas *et al.* (2004) reported that using yellow pigmentation combined with ammonium hydroxide vapour test, reduced false negatives to 7%. Thus, it is clear that NH_4^+ vapour test may be useful for preliminary screening of large number of samples, but ELISA should be used for accurate quantification of the aflatoxin content in samples.

Aflatoxins are secondary metabolite produced mainly by *A. flavus* and *A. parasiticus*, however, not all strains are toxigenic. Moreover, among toxigenic strains also, the capability to produce aflatoxins varies across the strains (Amani *et al.*, 2012). Vaamonde *et al*. (2003) showed that 29% of *A. flavus* strains isolated from different substrates in Argentina were able to produce aflatoxin whereas, distribution of toxigenic isolates in feeds (at Spain) revealed that of 115 isolates, only 65 (56.5%) were toxigenic (Moreno *et al.,* 1988). Approximately 59% of 193 *A. flavus* isolates studied by Jamali *et al.* (2012) were found to be capable of producing aflatoxins.

In the present study, it was also observed that 68.5% of isolates did not produce aflatoxin, as tested by ELISA which is in correspondence with the report of Amaike and Keller (2011). More than 50 atoxigenic strains of *A. flavus* from peanut fields were isolated in China, and the incidence of atoxigenic strain AF051 was found 12% higher than that of other atoxigenic strains (Jiang *et al.,* 2009). Zanon *et al*. (2013) have utilized such atoxigenic strains of *A. parasiticus* for successful management of aflatoxin in peanut soils.

Sourabie *et al.* (2012) while studying the isolates from Burkina Faso reported toxigenic potential of *Aspergillus* spp. present in peanut field. On the similar lines Razzaghi-Abyaneh *et al.* (2006), while studying the soil isolates of *Aspergillus* section Flavi from different provinces of Iran, found that only 27.5% of the isolates were toxigenic. Moreover, relatively larger proportion of toxigenic *A. flavus* strains were isolated from corn field soils of Mazandaran province (Iran), which indicate a possible

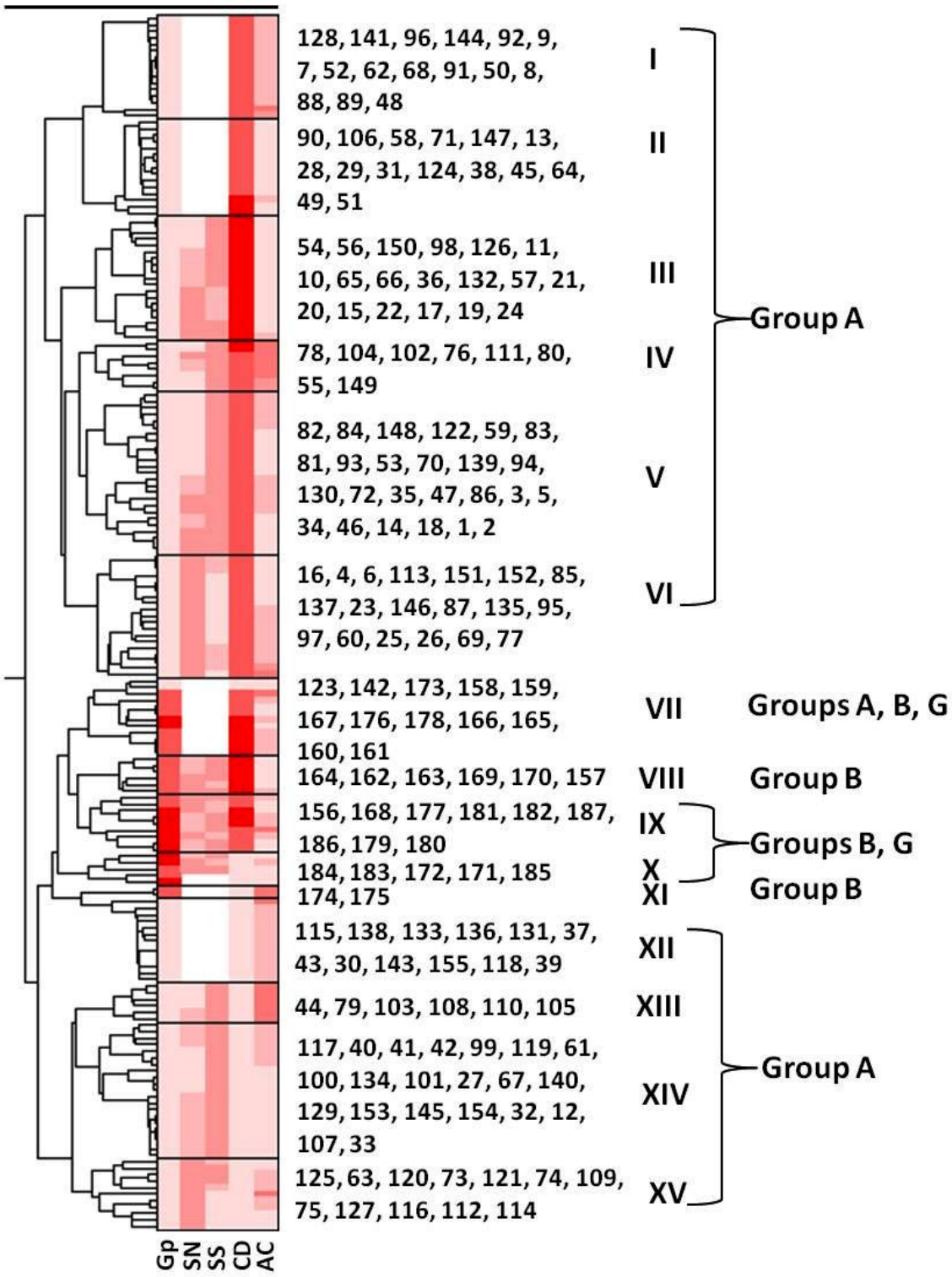

Fig. 2 : Dendrogram based on standardized Euclidean distance of morphological characters of 187 *A. flavus* isolates collected from groundnut fields of Gujarat, India (Gp= Group; SN= sclerotia number; SS= sclerotia size; CD= colony diameter; AC= aflatoxin content)

relationship between high level of relative humidity, and incidence of aflatoxin-producing fungi (Razzaghi-Abyaneh *et al.,* 2006).

Dorner and Lamb (2006) applied a commercial product, named AFLA-GUARD® as a bio-pesticide to approximately 2000 ha of peanut field in Georgia and Alabama (USA), and noticed a significant reduction in the composition of *A. flavus* soil population, from 71.1% toxigenic strains (untreated fields) to 4.0% (treated soils), and aflatoxin content measured was 78.9 ng g^{-1} in untreated and 11.7 ng g^{-1} in treated groundnuts. In order to manage aflatoxin contamination in cotton, over 50,000 ha area of commercial cotton in USA have been treated with atoxigenic strain AF36 since 2000 (Das *et al.,* 2008) and it was noted that this strain produced large number of conidia than toxigenic isolates. Since both occupy same niches, as the naturally occurring toxigenic populations, and subsequently reduce aflatoxin contamination in crops (Dorner, 2004).

Characterization of identified atoxigenic strains would be useful in identifying the regions that have atoxigenic native populations, which could be used as potential regions for the production of export purpose peanut. Further, identification of atoxigenic strains with better competitive saprophytic ability and rhizosphere competence would help in deploying these strains as biological agents to tackle aflatoxin contamination.

Diversity analysis of isolates was done on the basis of morphological characters (group of isolate, colony diameter, number and size of sclerotia) and toxigenic ability of the isolates. Clustering of isolates was done using Euclidean distance parameter (average linkage method) using Graphical Clustering Tool Kit *ver*. 1.0. On the basis of diversity analysis, the isolates could be grouped into fifteen distinct clusters (I-XV; Fig. 2). Of these, cluster V consisted of maximum 20 isolates whereas, cluster XI consisted of minimum 02 isolates (*i.e.,* NRCG 06014 and 06017) which are from group 'B'. It was observed that all the group 'B' and 'G' isolates, grouped into five separate clusters (VII-XI), and all group 'A' isolates, pooled independently in 10 distinct clusters (*i.e.* I-VI and XII-XV) except two isolates (*i.e.,* NRCG 08002 and 08021) which fell in cluster VII.

From the clustering pattern, it is very clear that group 'A' isolates were morphologically quite distinct than 'B' and 'G' group isolates. On the other hand, group 'B' and 'G' isolates showed some morphological similarities, hence were grouped together (Fig. 2). All 'B' group isolates were grouped together in 05 distinct clusters (VII-XI) while, all 'G' group isolates were grouped together in 03 clusters i.e. VII, IX and X. Three clusters *viz*., VII IX and X consisted of both group 'B' and group 'G' types of isolates. It was observed that in cluster VII all the three group isolates clustered together. This could be due to morphological similarity of these isolates with each other.

When toxigenicity was compared with morphological diversity, no association was observed. McAlpin *et al.* (1998) also reached to similar conclusion, while studying the genotypic diversity of *A. parasiticus* and *A. flavus* soil isolates from peanut field in Illinois, where both sclerotia producing and non-producing types were aflatoxin producers. Bayman and Cotty (1993) observed that morphological characters significantly contributed towards diversity analysis while, toxigenicity did not in *A. flavus*. Thus, it is evident from this result, that morphological character of *A. flavus* was good for diversity analysis, while toxigenic potential was not associated.

A district wise GIS map was also constructed, showing distribution pattern of different morphological groups of isolates of *A. flavus* that may be taken into consideration, for the development of location specific aflatoxin management programme (Fig. 1). The location comprising atoxigenic native *A. flavus* isolates could be safely used for the production of aflatoxin free peanut (Table 2, Fig. 1). Diversity analysis revealed that both within district and within group, lot of genetic variability was present.

From the present study, a consortium of highly toxigenic and diverse *A. flavus* isolates were identified, which can be used for the sick-plot screening of peanut genotypes at our institute. A repository of these isolates were also maintained at our directorate, which may be tested for its potential to be used as biocontrol agent (Dorner and Lamb, 2006). In order to have aflatoxin free peanut production, this information could be of great help, so as to avoid procurement of seed material from those locations, where toxigenic isolates of *Aspergillus flavus* naturally prevails.

Acknowledgment

Authors are thankful to the ICAR, New Delhi for funding of the project under the "Network project on Prevention and management of mycotoxin contamination in commercially important agricultural commodities".

References

Abbas, H.K., W.T. Shier, B.W. Horn and M.A. Weaver: Cultural methods for aflatoxin detection. *J. Toxicol. Toxin Rev.*, **23**, 295–315 (2004).

Amaike, S. and N.P. Keller: *Aspergillus flavus. Annu. Rev. Phytopathol.*, **49**, 107–33 (2011).

Amani, S., M.S. Ghahfarokhi, M. Banasaz and M. Razzaghi-Abyaneh: Mycotoxin-producing ability and chemotype diversity of *Aspergillus* section *Flavi* from soils of peanut-growing regions in Iran. *Indian J. Microbiol.*, **52**, 551–556 (2012).

Anonymous: Annual Report 2011-2012. Directorate of Groundnut Research, Junagadh- 362 001, Gujarat, India (2012).

Bayman, P. and P.J. Cotty: Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. *Canadian J. Bot.*, **71**, 23–31 (1993).

Cardwell, K.F. and P.J. Cotty: Distribution of *Aspergillus flavus* section Flavi among soils from the four agroecological zones of the Republic of Bénin, West Africa. *Plant Dis.*, **86**, 434–439 (2002).

Cotty, P.J.: Aflatoxin producing potential of communities of *Aspergillus* section Flavi from cotton producing areas in the United States. *Mycological Res.*, **101**, 698–704 (1997).

Cotty, P.J.: Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathol.*, **79**, 808–814 (1989).

Das, M.K., K.C. Ehrlich and P.J. Cotty: Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. *Phytopathology*, **98**, 282–288 (2008).

Desai, A.G., S.R.S. Dange, D.S. Patel and D.B. Patel: Variability in *Fusarium oxysporum* f. sp. *ricini* causing wilt of castor. *J. Mycol. Plant Pathol.*, **33**, 37–41 (2003).

Donner, M., J. Atehnkeng, R.A. Sikora, R. Bandyopadhyay and P.J. Cotty: Distribution of *Aspergillus* section Flavi in soils of maize fields in three agroecological zones of Nigeria. *Soil Biol. Biochem.*, **41**, 37–44 (2009).

Dorner, J.W. and M.C. Lamb: Development and commercial use of aflaguard®, an aflatoxin biocontrol agent. *Mycotoxin Res.*, **21**, 33–38 (2006).

Dorner, J.W.: Biological control of aflatoxin contamination of crops. *J. Toxicol. Toxin Rev.*, **23**, 425–450 (2004).

FAOSTAT: http://faostat.fao.org/ (Accessed: August 20, 2013) (2012)

Geiser, D.M., J.W. Dorner, B.W. Horn and J.W. Taylor: The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genet. Biol.*, **31**, 169–179 (2000).

Guo, B., N.D. Fedorova, X. Chen, C.H. Wan, W. Wang, W.C. Nierman, D. Bhatnagar and Y.J. Jiujiang: Gene expression profiling and identification of resistance genes to *Aspergillus flavus* infection in peanut through EST and microarray strategies. *Toxins*, **3**, 737–753 (2011).

Guo, B.Z., J. Yu, C.C. Holbrook, T.E. Cleveland, W.C. Niermanand and B.T. Scully: Strategy in prevention of prehravest aflatoxin contamination in peanuts: Aflatoxin biosynthesis, genetics and genomics. *Peanut Sci.*, **36**, 11–20 (2009).

Hesseltine, A.C., W.C. Sorenson and M. Smith: Taxonomic studies of the aflatoxin producing strains in the *Aspergillus flavus* group. *Mycologia*, **62**, 123–132 (1970).

Horn, B.W. and J.W. Dorner: Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl. Environ. Microbiol.*, **65**, 1444–1449 (1999).

Horn, B.W., J.W. Dorner, R.L. Greene, P.D. Blankenship and R.J. Cole: Effect of *Aspergillus parasiticus* soil inoculum on invasion of peanut seeds. *Mycopathologia*, **125**, 179–191 (1994).

Horn, B.W., R.L. Greene, V.S. Sobolev, J.W. Dorner, J.H. Powell and R.C. Layton: Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus, A. parasiticus and A. tamarii*. *Mycologia*, **88**, 574–587 (1996).

IARC (International Agency for Research on Cancer): Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monograph, 82, Lyon, France, (2002).

Jaime-Garcia, R. and P.J. Cotty: Spatial distribution of *Aspergillus flavus* and its toxigenic strains on commercial cotton seed from South Texas and its relationship to aflatoxin contamination. *Plant Pathol.*, **55**, 358–366 (2006).

Jamali, M., M.A. Ebrahimi, M. Karimipour, M. Shams-Ghahfarokhi, N. Dinparast-Djadid, S. Kalantari, Y. Pilehvar-Soltanahmadi, A. Amani and M. Razzaghi-Abyaneh: An insight into the distribution, genetic diversity, and mycotoxin production of *Aspergillus* section Flavi in soils of pistachio orchards. *Folia Microbiol.* (*Praha*), **57**, 27–36 (2012).

Jiang, J., L. Yan and L. Ma: Molecular characterization of an atoxigenic *Aspergillus flavus* strain AF051. *Appl. Microbiol. Biotechnol.*, **83,** 501-501 (2009).

Kornerup, A. and J.H. Wanscher: Metheun hand book of colour. 3rd edition. Metheun London Ltd., London (1978).

Kumar, S., M. Shekhar, K.A. Ali and P. Sharma: A rapid technique for detection of toxigenic and non-toxigenic strain of *Aspergillus flavus* from maize grain. *Ind. Phytopathol.*, **1**, 31–34 (2007).

Lisker, N., R. Michaeli and Z.R. Frank: Mycotoxigenic potential of *Aspergillus flavus* strains isolated from groundnuts growing in Israel. *Mycopathologia*, **122**, 177–183 (1993).

Mcalpin, C.E., D.T. Wicklow and C.E. Platis: Genotypic diversity of *Aspergillus parasiticus* in an Illinois corn field. *Plant Dis.*, **82**, 1132–1136 (1998).

Mishra, H.N. and C. Das: A review on biological control and metabolism of aflatoxin. *Crit. Rev. Food Sci. Nutr.*, **43**, 245–264 (2003).

Moreno, M.A., M.J. Pro, A. Olivares and G. Suarez: Considerations on the distribution of aflatoxigenic *Aspergillus flavus* in feeds. *Mycopathologia*, **104**, 149–151 (1988).

Rasmussen, M. and G. Karypis: gCLUTO– An interactive clustering, visualization, and analysis system. Department of Computer Science and Engineering, University of Minnesota, Minneapolis, MN, USA (CSE/UMN),Technical Report No. 04–021. http://glaros.dtc.umn.edu/gkhome/cluto/gcluto/download (2004).

Razzaghi-Abyaneh, M., M. Shams-Ghahfarokhi, A. Allameh, A. Kazeroon-Shiri, S. Ranjbar-Bahadori, H. Mirzahoseini and M.B. Rezaee: A survey on distribution of *Aspergillus* section Flavi in corn field soils in Iran: population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. *Mycopathologia*, **161**, 183–92 (2006).

Rinyu, E., J. Varga and L. Ferenczy: Phenotypic and genotypic analysis of variability in *Aspergillus fumigatus*. *J. Clinical Microbiol.*, **33**, 2567–2575 (1995).

Saito, M. and S. Machida: A rapid identification method for aflatoxin producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. *Mycosci.*, **40**, 205–208 (1999).

Shoba, D., N. Manivannan, P. Vindhiyavarman and S.N. Nigam: SSR markers associated for late leaf spot disease resistance by bulked segregant analysis in groundnut (*Arachis hypogaea* L.). *Euphytica*, **188**, 265-272 (2012).

Sourabie, P.B.S., P.A. Nikiema, N. Barro, A. Savadogo and A.S. Traore: Aflatoxigenic potential of *Aspergillus* spp. isolated from groundnut seeds, in Burkina Faso, West Africa. *Afr. J. Microbiol. Res.*, **6**, 2603–2609 (2012).

Upadhyaya, H.D., S.N. Nigam and R.P. Thakur: Genetic enhancement for resistance to aflatoxin contamination in groundnut. In: Summary proceedings of the Seventh ICRISAT Regional Groundnut Meeting for Western and Central Africa, 6-8 Dec. 2000, Cotonu, Benin (2000).

Vaamonde G., A. Patriarca, V.F. Pinto, R. Comerio and C. Degrossi: Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. *Int. J. Food Microbiol.*, **88**, 79-84, (2003).

Yazdani, D., M.A. Zainal-Abidin, Y.H. Tan and S. Kamaruzaman: Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. *Afr. J. Biotech.*, **9**, 7654–7659 (2010).

Zanon, M.S.A., M.L. Chiotta, G. Giaj-Merlera, G. Barros and S. Chulze: Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. *Int. J. Food Microbiol.* **162**, 220-225 (2013).

Published by :

Triveni Enterprises

1/206, Vikas Nagar, Lucknow - 226 022 (India)
Tel.: +91-522-4017359 (O)
Website: www.jeb.co.in; **e-mail:** editor@jeb.co.in