

Detection of Toxigenic and Atoxigenic Strains of *Aspergillus flavus* in Telangana and Andhra Pradesh

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ABSTRACT

In groundnut *Aspergillus flavus* causes aflatoxin contamination which is a qualitative problem occurring at both pre-and post-harvest stages. These aflatoxins have carcinogenic, hepatotoxic, teratogenic and immuno-suppressive effects. The *A. flavus* strains which produces aflatoxins are called as toxigenic and which do not produce toxins are called as atoxigenic starins. To detect the toxigenic and atoxigenic starins of *A. flavus* from Telangana and Andhra Pradesh (AP), pod samples were collected from eight selected oil mills/traders' in Mahaboobnagar, Rangareddy, Nizamabad, Karimnagar (Telangana); and Anantapur (AP) districts. A total of 24 *A. flavus* cultures were isolated from the collected pod samples. These isolates were identified as toxigenic/atoxigenic using cultural detection methods on Yeast extract sucrose (YES) media and coconut agar medium (CAM). Based on cultural methods, it was confirmed that there were 18 toxigenic, five atoxigenic and one false positive/negative strain out of the 24 *A. flavus* isolates obtained from surveyed oil mills. Atoxigenic strains were obtained from Karimnagar and Nizamabad districts of Telangana.

Key words: Groundnut, Aflatoxins, *Aspergillus flavus*, Coconut agar medium.

INTRODUCTION

Groundnut is a rich source of protein, dietary fiber, minerals, and vitamins¹⁸. All over the world the production of groundnut is hampered by several biotic stresses that results in severe yield reduction^{25,27}. The important biotic stress in groundnut cultivation is aflatoxin contamination which occurs at both pre-and post-harvest stages of the crop. These aflatoxins are a group of twenty secondary metabolites produced by *Aspergillus flavus*¹⁶

and *Aspergillus parasiticus* (Speare)^{17,24}. In several of the documented cases, there was no direct correlation between the quantum of *A. flavus* infection and kernel aflatoxin contamination⁵. One of the important factors for lack of correlation is the presence of non-aflatoxigenic/atoxigenic *A. flavus* strains in groundnut crop soils and their invasion to pods. It is precisely at this juncture, differentiation of toxigenic and atoxigenic *A. flavus* isolates assumes significance.

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Co-existence of toxigenic and atoxigenic strains in groundnut crop ecosystem is common and so as in different crop soils. Further, both aflatoxigenic and atoxigenic strains are commonly seen infecting the food commodities^{7,13}. Precise differentiation of these toxigenics from atoxigenics will enable to estimate the actual threat due to aflatoxins in a particular food commodity. Differentiation of these *A. flavus* strains can be determined through use of cultural, molecular²⁶ and analytical approaches²². However, in view of the frequent encounters with false positives and false negatives, a polyphasic approach would be an ideal one for precise detection³. In view of the expense and expertise required to maintain molecular and analytical laboratories, it is the cultural methods of detection that is gaining momentum in many areas of the world¹.

These cultural methods rely on either quantification of purified extracts^{10,23} or on qualitative assessments of fluorescence^{4,6,11,16} or UV absorption²⁸. A broad understanding on the ratio of co-existence of toxigenic and atoxigenic strains in a particular crop growing area will help predict the risk and sensitive areas with respect to pre-and post-harvest contamination. Further, the atoxigenic strains

can be used as bioagents in the management of pre-harvest aflatoxin contamination in maize¹² and groundnuts⁸.

MATERIAL AND METHODS

The present investigation was carried out during 2014-15 with the facilities available at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India.

Isolation and confirmation of *A. flavus*

Conidia from the typical *A. flavus* colonies in seed infection assays were picked using a sterile needle and placed in Petri dishes containing PDA. The inoculated plates were kept in the incubator at 28° C. A total of 24 *A. flavus* isolates were picked up representing one isolate from each pod sample collected (Figure 1). The *A. flavus* cultures were confirmed up to species based on standard protocols^{14,19,21}. The *A. flavus* isolates from Telangana were designated as AFT1a; AFT1b; AFT1c; AFT2a; AFT2b; AFT2c; AFT3a; AFT3b; AFT3c; AFT4a; AFT4b; AFT4c; AFT5a; AFT5b; and AFT5c. The isolates from Andhra Pradesh were designated as AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c; AFA8a; AFA8b; and AFA8c.

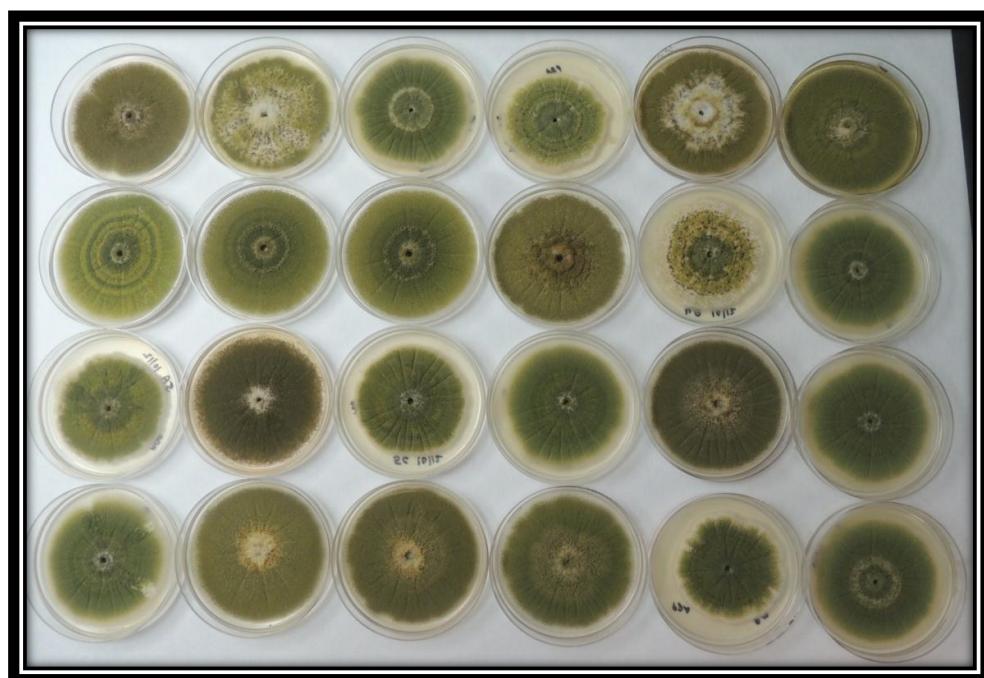


Fig. 1: Twenty four isolates of *A. flavus* isolated from the infected groundnut kernels collected from different groundnut oil mills of Telangana and Andhra Pradesh

To detect the toxigenic *Aspergillus flavus* strains using cultural methods

All the 24 *A. flavus* isolates obtained from the groundnut samples of oil mills/traders' were evaluated for their toxigenicity using cultural methods as follows.

Coconut agar medium

Coconut agar medium (CAM) was prepared based on Dyer and McCammon⁹ with slight modifications. Spore suspension of *Aspergillus flavus* isolates was inoculated at the center of the Petri dishes containing CAM. The Petri dishes were later incubated in dark at 28° C for seven days and then the fluorescence around the fungal colonies was evaluated. Presence of fluorescence surrounding the fungal colonies under UV light indicates the toxigenicity of a strain, whereas absence of fluorescence indicates atoxigenic nature of a strain. Based on the presence or absence of fluorescence, the *A. flavus* isolates were categorized as toxigenics and atoxigenics.

Further, the isolates were observed for the yellow pigmentation on the lower side of the CAM plates. The isolates, that have shown yellow pigmentation were categorized as toxigenics, whereas those without any yellow pigmentation were categorized as atoxigenics.

Yeast extract sucrose (YES) medium and Ammonia vapor test

Single spore cultures of *A. flavus* were inoculated onto Petri dishes containing YES media and incubated in dark at 28° C¹⁵. After 3-4 days, the Petri dishes were kept inverted over 0.5ml ammonium hydroxide (Sigma-Aldrich). Within five minutes, aflatoxigenic isolates turns pink to red in colour. Whereas no colour change was observed for atoxigenic isolates.

RESULTS

Detection of the toxigenic *A. flavus* strains using cultural methods

A total of 24 isolates of *A. flavus* made from the pod/kernel samples collected from groundnut oil mills/traders' of Telangana and Andhra Pradesh were used in the present study. The *A. flavus* strains that have shown both yellow pigmentation and fluorescence or either one of the reactions on CAM were considered as toxigenic strains. In case of YES medium, development of plum red colour was considered as positive for detecting toxigenic strains.

The results obtained from these tests indicate that, out of the 24 strains of *A. flavus*, 18 (AFT1a; AFT1b; AFT1c; AFT2a; AFT2b; AFT2c; AFT4c; AFT5b; AFT5c; AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c; AFA8a; AFA8b; and AFA8c) have shown positive reaction to toxigenicity in both the tests (on CAM and YES media) under study (Table 1 and Table 2). Further, five *A. flavus* strains, AFT3a; AFT3b; AFT3c; AFT4a; and AFT4b were proved to be atoxigenic in both the tests (Fig 2, 3, 4, 5, 6 and 7). The remaining one isolate, AFT5a was categorized into false (+ve/-ve) which may be false +ve with respect to the coconut agar medium tests or may be false -ve with respect to the ammonia vapour test on YES medium. The details of the toxigenic and atoxigenic strains of *A. flavus* in each surveyed district were discussed below (Table 1 and Table 2).

Prevalence of toxigenic/atoxigenic *Aspergillus flavus* strains in Telangana

The prevalence of toxigenic and atoxigenic strains of *A. flavus* from the groundnut pods/kernels collected from oil mills/traders of Telangana were as follows (Table 1).

Mahaboobnagar district

All the three *A. flavus* strains, AFT1a; AFT1b; and AFT1c isolated from groundnut mills of Mahaboobnagar district were tested positive (toxigenic) on both YES and CAM media. On CAM, AFT1a and AFT1b isolates have shown

both fluorescence and yellow pigmentation. The third isolate, AFT1c has exhibited fluorescence but with no yellow pigmentation on the reverse side (Table 1).

Rangareddy district

All the three *A. flavus* strains, AFT2a; AFT2b; and AFT2c isolated from groundnut mills of Rangareddy district were tested positive (toxigenic) on both YES and CAM media. On CAM, all the isolates have shown fluorescence, however, only two of three isolates, AFT2b and AFT2c have shown yellow pigmentation. The other isolate, AFT2a has shown fluorescence with no yellow pigmentation (Table 1).

Nizamabad district

All the three *A. flavus* isolates, AFT3a; AFT3b; and AFT3c (Fig 8) isolated from the pod samples collected from Agarwal Agro Industries, Khanapur village of Nizamabad district, were detected as atoxigenics based on their negative reactions on YES and CAM (Table 1).

Karimnagar district

Out of the six *A. flavus* strains isolated from the pod samples collected from Karimnagar district, three strains, AFT4c; AFT5b and AFT5c were toxigenic with positive reaction on both YES and CAM media. Further, two strains, AFT4a and AFT4b were proved to be atoxigenic. The remaining one *A. flavus* strain, AFT5a was categorized as false (+ve/-ve). This strain has shown toxigenic reaction with respect to fluorescence test, but atoxigenic reaction based on fluorescence on CAM. However, on YES media, the strain was atoxigenic with no plum red color development. Hence, the strain AFT5a may be false +ve with respect to fluorescence test on CAM or false -ve with respect to ammonia vapour test on YES medium (Table 1).

Overall, of all the 15 strains of *A. flavus* isolated from pods/kernels from oil

mills from Telangana, there were nine toxigenic strains, five atoxigenic strains and one strain being either a false positive or false negative. Few of the toxigenic strains detected from oil mills of Telangana were shown in Fig 9.

Prevalence of toxigenic/atoxigenic *Aspergillus flavus* strains in Anantapur district of Andhra Pradesh

The prevalence of toxigenic strains of *A. flavus* in the surveyed mandals of Anantapur district of Andhra Pradesh were as follows (Table 2)

Tadipatri mandal of Anantapur district

Out of the six strains isolated from the groundnut pod samples collected from M/S. Sri Buggaramalingeshwara Oil Mills and Sri Sai Ram Oil Mills, Terannapalli village of Tadipatri mandal, all the strains were proved to be toxigenic with respect to both the cultural methods (Table 2). On YES media, all the six strains, AFA6a; AFA6b; AFA6c; AFA7a; AFA7b; AFA7c have shown positive reaction (plum red colour). On CAM, all these six strains have shown fluorescence. However, on CAM, except AFA7a and AFA7b, the remaining four toxigenic strains exhibited yellow pigmentation on the reverse side of colonies.

Tadimarri mandal of Anantapur district

All the three strains isolated from the groundnut pod samples collected from M/S New Sreenivasa Baby decorticators, Tadimarri village of Tadimarri mandal were proved to be toxigenic in both the cultural methods (Table 2). However, the strain AFA8b was the only toxigenic strain without yellow pigmentation on CAM.

Overall, all the six isolates from Tadipatri mandal (Fig 10) and three isolates from Tadimarri mandal (Fig 11) of Anantapur district in Andhra Pradesh were categorized as toxigenic strains.

Table 1: Cultural methods of detection of toxigenic and atoxigenic strains of *Aspergillus flavus* isolated from groundnut kernels of oil mills/traders' in surveyed districts of Telangana

<i>Aspergillus flavus</i> isolate	Yeast Extract Sucrose Media	Coconut Agar Medium	
		Fluorescence	Yellow Pigmentation
District- Mahaboobnagar			
AFT1a	+	+	✓
AFT1b	+	+*	✓*
AFT1c	+*	+	X
District- Rangareddy			
AFT2a	+	+*	X
AFT2b	+	+	✓*
AFT2c	+	+	✓
District- Nizamabad			
AFT3a	-	-	X
AFT3b	-	-	X
AFT3c	-	-	X
District- Karimnagar			
AFT4a	-	-	X
AFT4b	-	-	X
AFT4c	+*	+	X
AFT5a	-	+	X
AFT5b	+	+	✓
AFT5c	+ *	+	✓*

Green colour denotes atoxigenicity; Red colour denotes toxigenicity of *A. flavus* isolates

Yellow colour denotes false +/- *A. flavus* strains

+ =Toxigenic; - =Atoxigenic, +* = Slightly toxigenic; ✓=positive for pigmentation; x=negative for pigmentation;
✓*=slight pigmentation

Table 2: Cultural methods of detection of toxigenic and atoxigenic strains of *Aspergillus flavus* isolated from groundnut kernels of oil mills/traders' in Anantapur district of Andhra Pradesh

<i>Aspergillus flavus</i> isolate	Yeast Extract Sucrose Media	Coconut Agar Medium	
		Fluorescence	Yellow Pigmentation
Mandal- Tadipatri			
AFA6a	+*	+	✓*
AFA6b	+*	+	✓*
AFA6c	+*	+*	✓*
AFA7a	+*	+	X
AFA7b	+*	+*	X
AFA7c	+*	+*	✓*
Mandal- Tadimarri			
AFA8a	+	+	✓*
AFA8b	+*	+*	X
AFA8c	+*	+	✓

Red colour denotes toxigenicity of *A. flavus* isolates

+ =Toxigenic; - =Atoxigenic, +* = Slightly toxigenic; ✓=positive for pigmentation; x=negative for pigmentation; ✓*=slight pigmentation

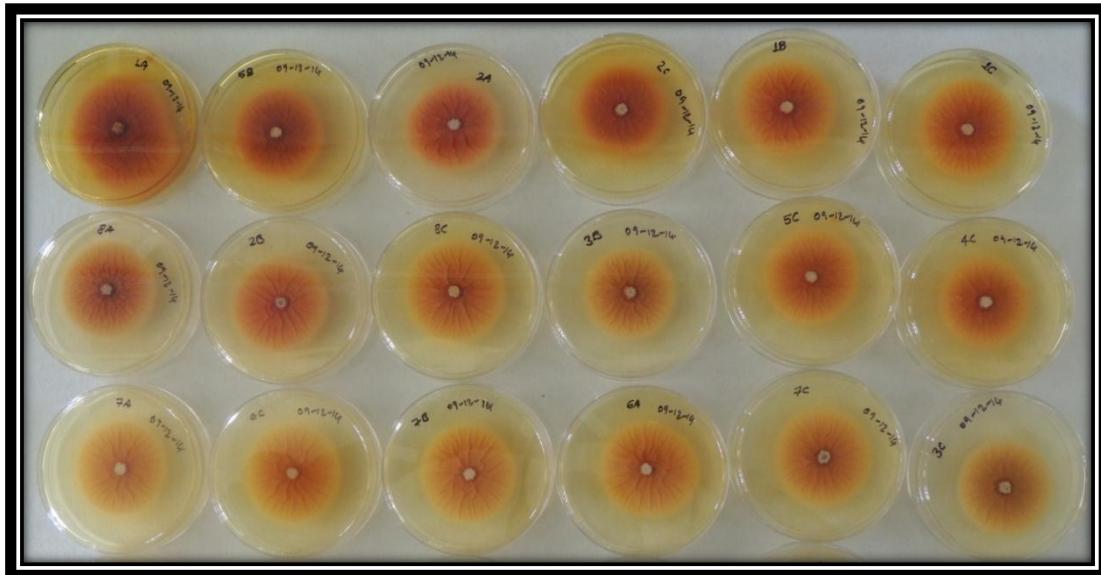


Fig. 2: Development of plum red colour by toxigenic *Aspergillus flavus* strains of groundnut on yeast extract sucrose (YES) media on exposure to ammonium hydroxide vapours

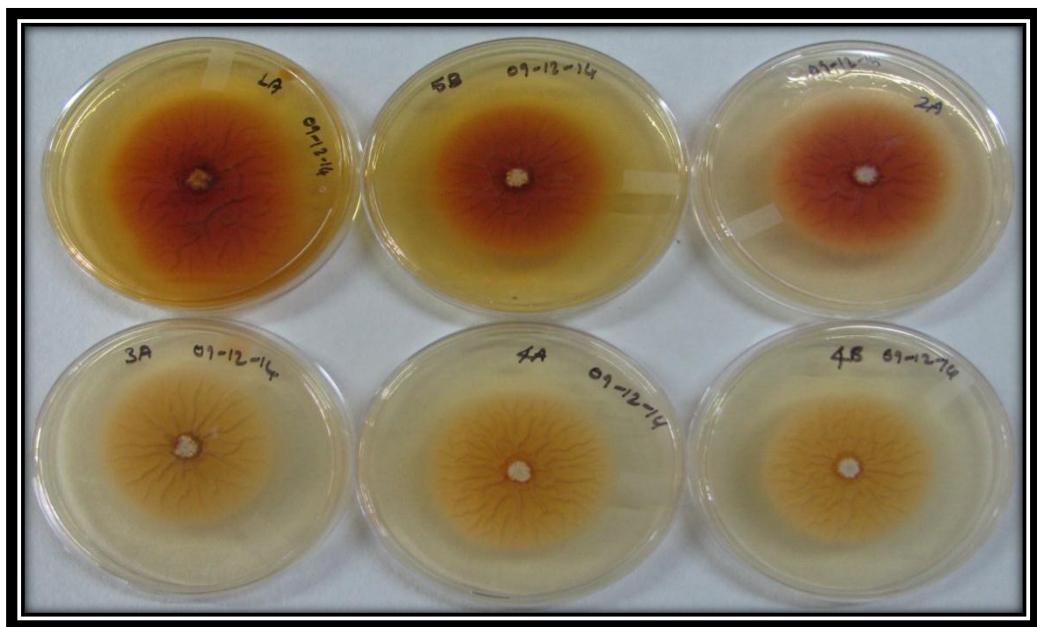


Fig. 3. Differentiation of toxigenic (plum red) and atoxigenic (absence of plum red) *Aspergillus flavus* strains on yeast extract sucrose (YES) media on exposure to ammonium hydroxide vapours

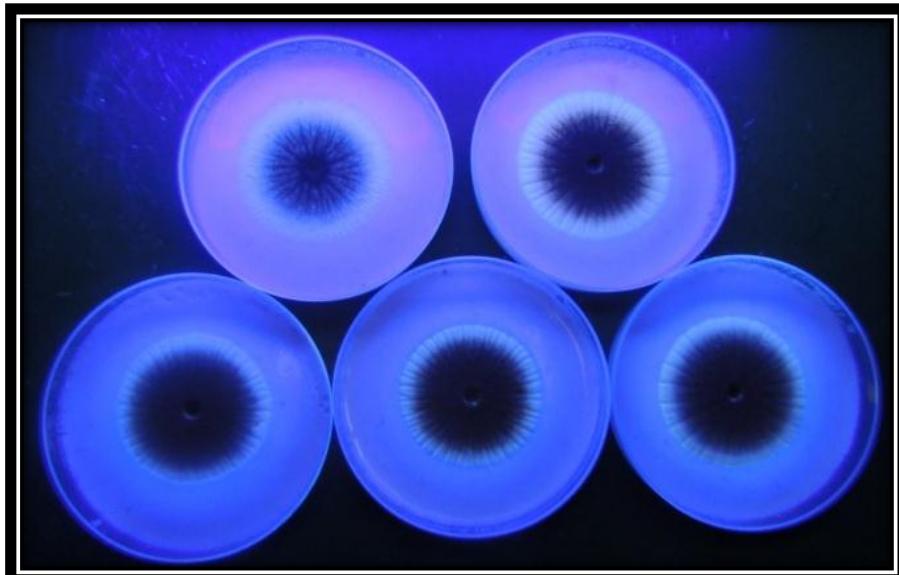


Fig. 4: Development of fluorescence around toxigenic *Aspergillus flavus* strains on coconut agar medium (CAM) under UV light

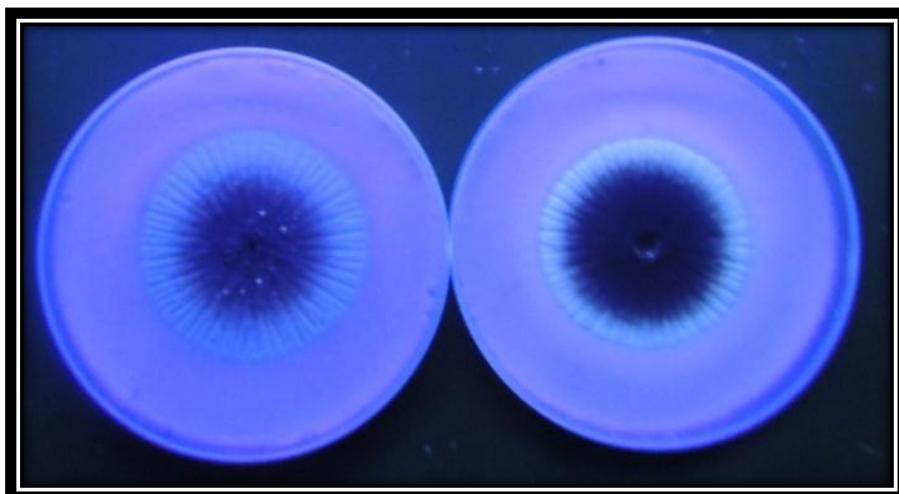


Fig. 5: Differentiation of toxigenic and atoxigenic *Aspergillus flavus* strains of groundnut on coconut agar medium (CAM) based on fluorescence (right) around the colonies

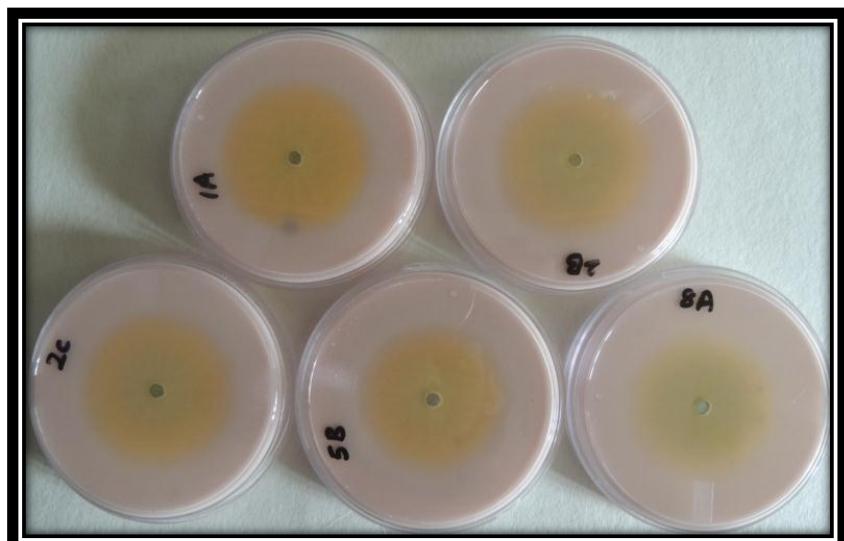


Fig. 6: Yellow pigment production by toxigenic *Aspergillus flavus* isolates on coconut agar medium (CAM)

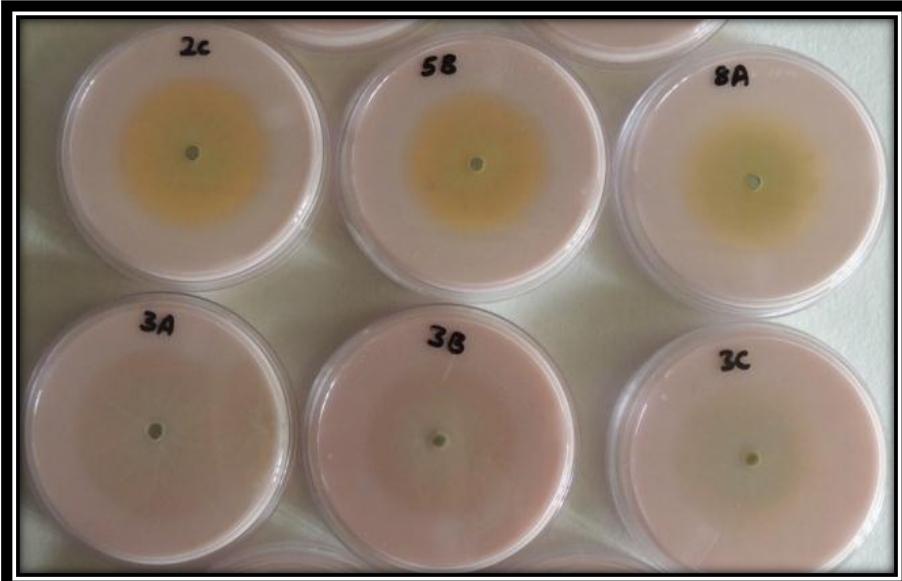


Fig. 7: Differentiation of toxigenic (yellow pigmentation) and atoxigenic *Aspergillus flavus* strains of groundnut on coconut agar medium (CAM)

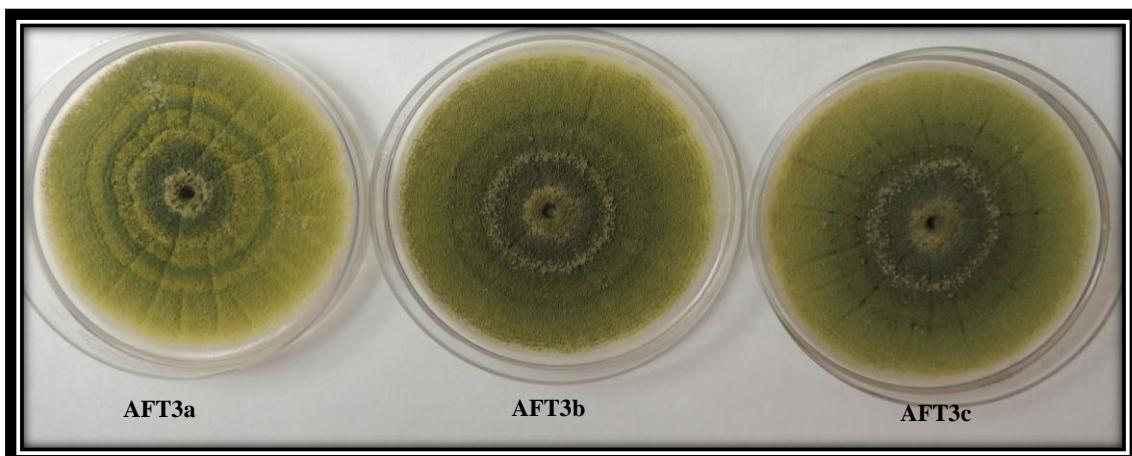


Fig. 8: Atoxigenic *Aspergillus flavus* strains isolated from groundnut pod samples collected from Oil mills in Nizamabad district of Telangana

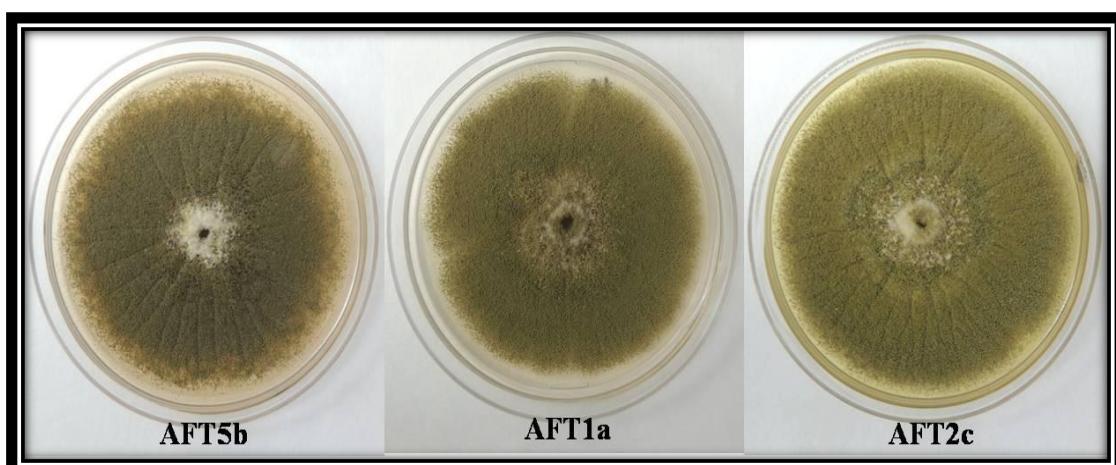


Fig. 9: Toxigenic *Aspergillus flavus* strains isolated from groundnut pod samples collected from Oil mills of Telangana

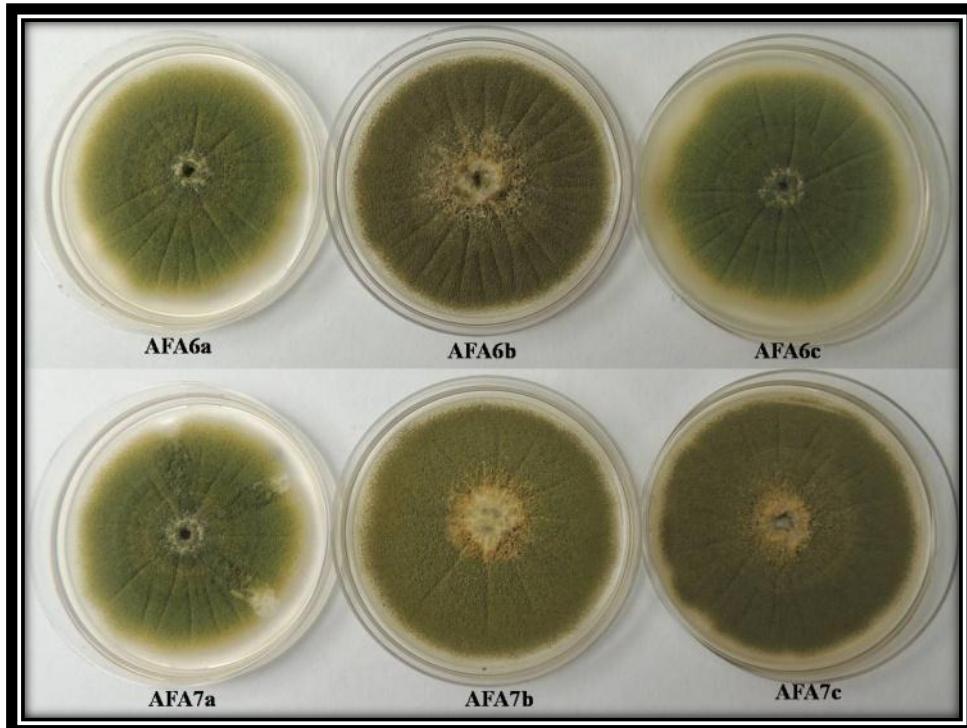


Fig. 10: Toxigenic *Aspergillus flavus* strains isolated from groundnut oil mills of Tadipatrimandal of Anantapur district

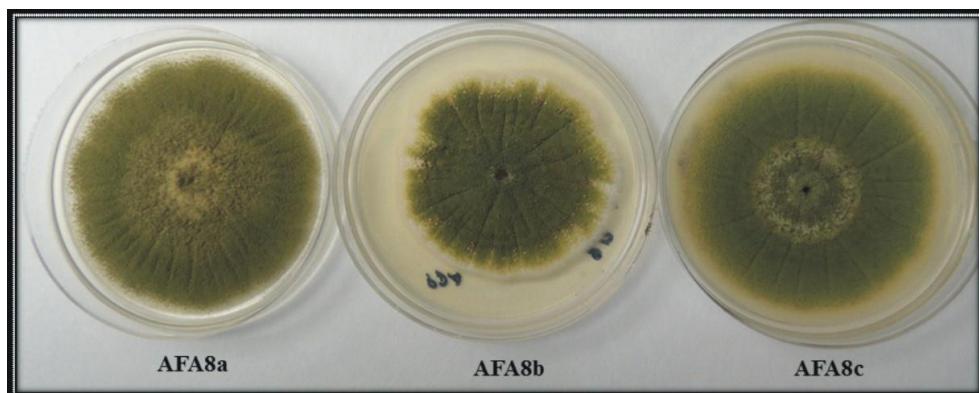


Fig. 11: Toxigenic *Aspergillus flavus* strains isolated from groundnut oil mills of Tadimari mandal of Anantapur district

DISCUSSION

In our present study, we have differentiated toxigenic *A. flavus* strains from atoxigenics using cultural methods such as on YES media and CAM. Based on our results, it was evident that oil mills from Mahaboobnagar, Rangareddy (districts from Telangana) and Tadipatri, Tadimari mandals of Anantapur district (Andhra Pradesh) had incidence of toxigenic strains alone. However, in Karimnagar district, there were co-existence of mold infections from both toxigenic and atoxigenic strains (Table 5). Co-existence of atoxigenic strains of *A. flavus* along with

toxigenic strains is a common phenomenon in crop soils²⁰. Further, a false positive/false negative *A. flavus* strain (AF5a) was noticed in kernel samples from oil mills of Karimnagar. This AF5a strain might be a false negative strain according to YES; and false positive according to CAM (Table 4.3). In view of the frequent false positives/false negatives in detection through a single method, polyphasic detection is an ideal approach to differentiate *A. flavus* strains. Hence, further investigations on the aflatoxicogenicity of this AF5a strain needs to be reconfirmed based on other

cultural, analytical and molecular techniques using a polyphasic approach.

In our studies, atoxigenic strains were detected in groundnut samples from oil mills Nizamabad besides Karimnagar district (Table 5). These atoxigenic strains of *A. flavus* in the present study as contaminants at oil mills can be originated from groundnut soils in the respective districts and adjoining areas as pre-harvest contaminants or during storage at farmers' end. Antagonistic activities of atoxigenic *A. flavus* strains in mitigating pre-harvest aflatoxin contamination in maize² and groundnut⁸ are reported. However, before exploring the antagonistic potential of these atoxigenic *A. flavus* strains, their atoxigenicity need to be reconfirmed using polyphasic studies. Further, there are metabolites produced by *A. flavus* other than aflatoxins that are toxic in nature^{17,24}. Screening of these atoxigenic strains in the present study for production of these other toxic metabolites is a pre-requisite before exploring their antagonistic activities on toxigenic *A. flavus* at fields.

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