Indian Journal of Experimental Biology
Vol. 56, December 2018, pp. 892-898

Differentiation of toxigenic and atoxigenic *Aspergillus flavus*: Polyphasic approach, a new dimension

MK Naik[1], GR Guru Prasad[1], HP Jadhav[2], Hashem A[3], Elsayed F Abd_Allah[4] & RZ Sayyed[2]*

[1]Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India
[2]Department of Microbiology, PSGVP Mandal's Arts, Science & Commerce College, Shahada, Maharashtra-425 409, India
[3]Botany and Microbiology Department, College of Science, King Saud University, P.O. Box. 2460 Riyadh 11451, Saudi Arabia
[4]Department of Plant Production, College of Food and Agriculture Sciences, King Saud University, P.O. Box. 2460 Riyadh 11451, Saudi Arabia

Received 16 April 2018; *revised 25 October 2018*

Contamination of food by aflatoxin of *Aspergillus flavus* is a major global problem affecting trade, quality, utility of food and human health. While all the members of *A. flavus* does not produce aflatoxin, sensitive, cost effective and reproducible methods for large scale screening and differentiation of toxigenic *A. flavus* from atoxigenic ones are scarce. Here, we made one such attempt using coconut milk agar (CMA), yeast extract sucrose agar (YESA), ammonium hydroxide vapour tests, enzyme linked immuno sorbent assay (ELISA) and polymerase chain reaction (PCR) for large scale screening of toxigenic strains of *A. flavus*. Fifty nine isolates of *A. flavus* obtained from major chilli growing regions of India were categorized into toxigenic and atoxigenic strains by using cultural, analytical and molecular methods. Forty two (71.18 %) isolates showed positive response in coconut milk agar (CMA), 17 (28.81%) isolates did not match while 23 (38.98 %) isolates showed red colour and 36 (61.01%) isolates did not produce red colour upon exposure to ammonia vapour in YESA. Out of 59 isolates, isolates CAF43 came out as highly toxigenic, as it produced 3128.20 μg kg^{-1} aflatoxin B1 followed by CAF 42 which produced 3035.10 μg kg^{-1}. Among 59 isolates, eight *A. flavus* isolates were amplified with two regulatory (*aflR* and *aflJ*) and two structural (*norA* and *ver1*) genes at a region of 900, 450, 400 and 450 bp, respectively.

Keywords: Aflatoxin, Coconut milk agar (CMA), Yeast extract sucrose agar (YESA)

Aflatoxin, a mycotoxin produced by filamentous fungus *Aspergillus flavus*, is considered as hazardous to human and animal health. Aflatoxin contamination is a major threat to agriculture, food commodities, trade and human health[1]. It has severely affected the food safety and economic hardship in agriculture sector. While the US Food and Drug Administration (FDA) permits 20 parts per billion (ppb) of aflatoxin as threshold level the European nations allows only 5 ppb[2]. In Indian conditions, particularly in rural areas, instant occurrence of aflatoxin in various commodities goes unnoticed. The mere presence of *A. flavus* on the substrate does not end up with aflatoxin production[3]. Hence, it is important to detect and estimate the amount of aflatoxin in the commodities.

All members of *A. flavus* are not toxigenic, and it mandates differentiation of toxigenic and atoxigenic ones. Although several culture based methods are used for this differentiation, they suffer from sensitivity and reproducibility. Secondly, sensitive methods such as ELISA and molecular techniques *viz*. PCPR are hardly available in many locations. In this context, culture methods that employ use of Coconut milk agar (CMA), ammonium hydroxide vapour tests on Yeast extract sucrose agar (YESA) and ELISA have been regarded as user friendly, cost effective and reproducible methods to differentiate between toxigenic and atoxigenic *A. flavus* isolates. ELISA has emerged as rapid, sensitive, and simpler assay suited for large scale screening and detection of aflatoxins in groundnuts and groundnut products[4,5]. Aflatoxin production involves role of 25 different biosynthesis genes. Of these, presence of two regulatory genes *aflR* and *aflJ* (*norA* and *ver1* are structural genes) is taken as candidate genes for differentiation between toxigenic and atoxigenic *A. flavus*[6]. In the present work, differentiation between toxigenic and atoxigenic *A. flavus* strains was made on the basis of cultural methods, amount of aflatoxin produced by each isolate was detected by ELISA method and genes involved in aflatoxin production were amplified by PCR methods.

*Correspondence:
E-mail: sayyedrz@gmail.com

Material and Methods

Isolation and identification of *A. flavus*

A. flavus isolates were collected from major chilli growing states of India *viz*. Andhra Pradesh, Karnataka, Maharashtra and Tamil Nadu. Chilli fruits infected with *Aspergillus* sp. were cut in to small bits placed on potato dextrose agar (PDA) (1-2 leaf bits per plate). All the plates were incubated at 25±2°C for 3-5 days and observed for growth of fungus. Identification of fungal isolates was carried out based on 18s rDNA sequencing technology, internal transcribed spacer (ITS) regions of 18s rDNA were sequenced as per the method of Sambrook & Russel[7] and Huzefa *et al*.[8]. The amplification reaction was performed as per Adachi *et al*.[9]. The BLAST search program was used to look for nucleotide sequence homology of this fungal isolate. The closely related sequences were aligned by CLUSTAL W using MEGA version 4.0 software, and the neighbor-joining tree was generated using same software.

Detection of toxigenic variability of *A. flavus* isolates

On coconut milk agar (*CMA*)

Coconut milk agar (CMA), was prepared as per Rodrigues *et al*.[10], well was prepared at the centre of solidified CMA followed by spot inoculating 15 µL spore suspension of *A. flavus*, plates were incubated in dark at 28°C for 5 days and observed for presence of fluorescence ring, yellow pigment and beige ring under UV light.

By ammonia hydroxide test on yeast extract sucrose agar (*YESA*)

All isolates of *A. flavus* were grown on YESA for 3 days at 28°C, following the incubation, plates were inverted and 50 µL of ammonium hydroxide solution (25%) was added at the centre of the lower lid of the inverted plate and observed for change in colour of the medium occurring within 30 min[10].

By indirect competitive ELISA method

Healthy chilli fruits were taken and pinpricked with sterile pin and were separately dipped into spore suspension (1×10^6 spores mL^{-1}) of *A. flavus*, incubated for 7 days at 28°C in a perforated trays covered with plastic sheet to retain moisture and placed in plant growth chamber. After incubation period, fruits were shade dried and made to fine powder, 20 g of each chilli powder was mixed with 100 mL of an extracting solvent consisting of 70 mL methanol, 30 mL water and 0.5g KCl and kept under agitation at 250 rpm for 1 h. Extracts were filtered through Whatman No.41 filter paper and diluted to 1:10 with bovine serum albumin (BSA) (0.2%) prepared in 0.05M PBS–Tween (pH–7.4). Amount of aflatoxin produced was estimated by ELISA at 405 nm[4,5].

ELISA procedure

ELSIA plates were coated with 150 µL per well of aflatoxin B1 (AFB1)-BSA prepared in a carbonate buffer (100 ng mL^{-1}). At each step plates were incubated at 37°C followed by three washes with PBS-Tween. In the second step, plates were treated with PBST-BSA. AFB1 standards ranging 25-0.097 ng mL^{-1} were prepared in 10% toxin free extracts. Standards were used in the range of 0.097 to 100 µg mL^{-1}. Hundred µL of standards AFB1 was mixed with 50 µL of antiserum diluted to 1:10000 in 0.2% PBST-BSA. Similarly for test samples 90 µL of 0.2% BSA, 10 µL of extract, 50 µL of antiserum and 150 µL of alkaline phosphatase labeled goat antirabit IgG conjugate diluted to 1:4000 in PBST-BSA was added. Followed by adding p-nitrophenyl phosphate prepared in 10% diethanolamine was used as substrate. Plates were incubated at 37°C and colour development occurring in 20 min was read in ELISA reader at 405 nm[4,5]. Amount of aflatoxin produced was calculated from calibration curve of Log10 values of aflatoxin concentration *vs*. OD values.

Molecular characterization

For molecular characterization, eight isolates (6 toxigenic and 2 atoxigenic) were selected, 6 toxigenic isolates were those that gave positive response and 2 atoxigenic isolates that gave negative response in culture based (CCA and YESA) and ELISA method[3].

DNA extraction and amplification

The genomic DNA was extracted from four days old *A. flavus* cultures grown in potato dextrose broth by following CTAB method. The purified DNA of *A. flavus* was amplified with internal transcribed spacer (ITS) primer set, ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS- 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). PCR amplification reactions were carried out in a 25 µL reaction mixture with PCR conditions (one cycle of 94°C for 4 min, 36 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min and one cycle of final extension at 72°C for 5 min)[7]. Amplified DNA fragments were checked in 1.5% agarose gel and documented using Syngene G-box gel documenting system (Cambridge, England).

Detection and amplification of aflatoxin biosynthetic genes

Two regulatory genes of *A. flavus*, *aflR* and *aflJ* and two structural genes, *norA* and *ver1* were amplified using specific primers described as follows. *aflR*- F

(5'-TGA GAA AGG GGA CGC TGG AT-3') and aflR (5'-CAA TCG AAT CAA CCA CCA CA-3'), *aflJ*- F (5'-CTT CAA CAA CGA CCA AGG TT-3') and *aflJ- R* (5'-AGA TGA GAT ACA CTG CCG CA-3'), *nor A*- F (5'-CCT TAT GCC TGG GAA CGA T-3') and nor A-R (5'-TTC GCA TCA CTT CCT CCA CA-3'), *Ver1*-F (5'-ACC ACC GTT TAG ATG GCA AA-3') and *Ver1-R* (5'-AGA GCT GGT CAG GAT AATCCG-3'). The 25 µL reaction mixture includes 6 µL of DNA, 2.5 µL of 10× PCR buffer, 0.5 µL of 5 mM dNTPs, 0.3 µL Taq polymerase, 14 µL water and 1.0 µL (10 ng μL^{-1}) of each primer. The PCR reactions were performed with a DNA thermo cycler (My Cycler, Bio-Rad, Canada) using PCR conditions: 5 min at 95°C followed by 35 cycles at 95°C for 30 s, 55°C for 20 s, 72°C for 30 s. A final extension was conducted for 10 min at 72°C. Annealing temperature was optimized for each primer set with the result that the annealing temperature varied from 49-59°C. Electrophoresis of PCR products was performed in 1.5% agarose gel containing ethidium bromide solution (10 mg mL^{-1}) along with 100 bp DNA ladder and amplicon products were measured[7] using gel documentation unit (Gel Doc, Cambridge, England).

Cloning, sequencing and phylogenetic analysis

PCR products were cloned into pGEMR-T Easy Vector Systems (Promega Corporation, Madison, USA) and transformed into competent *E. coli* strain DH5α as per manufacturer's instructions. Plasmid DNA was isolated and the presence of the insert was confirmed by restriction digestion of plasmid DNA with *Eco*RI restriction enzymes (Fermentas Life Sciences, Canada). Clones of interest were sequenced commercially (GeNei, Bengaluru, India). All the sequences were confirmed with NCBI BLAST database for the identity of the isolates based on previously published database sequences[8,9].

Results and Discussion

Isolation and identification of *A. flavus*

A total of 59 isolates of *Aspergillus* were obtained from chilli samples. Among these 8 isolates were found to be potent producers of aflatoxin. All these 8 isolates showed 100% homology with *A. flavus* (Fig. 1) and hence were identified as *A. flavus*. Gene sequences of these isolates were deposited in Genebank under particular accession number (Table 1).

Toxigenic variability of *A. flavus* isolates

On coconut milk agar (*CMA*)

In CMA, out of 59 isolates, 42 isolates exhibited beige ring on upper surface of the plate, yellow pigmentation and blue fluorescence on underside of the plate. These isolates were taken as aflatoxin producers (toxigenic), while 17 isolates that did not produce, beige ring and pigmentation on CMA were considered atoxigenic. Among the 42 isolates which showed positive response in CMA, 40 isolates produced toxin as determined by ELISA while two isolates did not produce toxin. Four isolates did not produce aflatoxin in CMA as well as in ELISA (Table 2 and Fig. 2).

The underside of Petri dish of the colony reflecting bright-yellow pigmentation in the medium are aflatoxin producers as absorption of yellow light is the characteristic feature of aflatoxin[10]. Gupta and Gupta[11] have also reported similar absorption of yellow fluorescence by atoxigenic strains of *A. flavus*. Many researchers have reported CMA and YESA as useful media for experimental production of aflatoxin[12-15]. In many cases a bright yellow pigmentation was found consistently associated with the yellow fluorescence (Aflatoxin producing ability) by *A. flavus* isolates on all media[11] while none of the aflatoxin negative (AT negative) isolates produced yellow pigmentation[13,14] indicating yellow pigment production was not a reliable predictor of the amount of aflatoxin in all media.

On Yeast extract sucrose agar (*YESA*)

A total of 59 isolates of chilli were examined to know the relation between aflatoxin producing ability

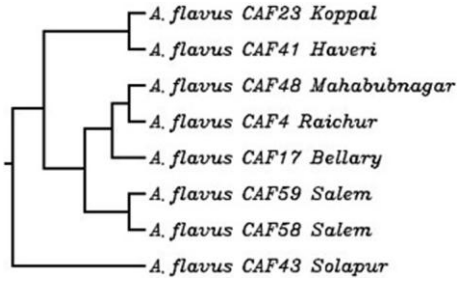

Fig. 1—Dendrogram based on UPGMA cluster analyses obtained from multiple sequences alignment tools for *A. flavus* isolated from chilli.

Table 1—Comparison and identity of *A.flavus* isolates of chilli sequenced and deposited in Gene Bank, Mary land, USA

Isolates designation	Identified as	Gene Bank accession No.
A. flavus-CAF1	*Aspergillus flavus*	KJ882391
A. flavus-CAF2	*Aspergillus flavus*	KJ882392
A. flavus-CAF3	*Aspergillus flavus*	KJ882394
A. flavus-CAF4	*Aspergillus flavus*	KJ882384
A. flavus-CAF5	*Aspergillus flavus*	KJ882382
A. flavus-CAF6	*Aspergillus flavus*	KJ882396
A. flavus-CAF7	*Aspergillus flavus*	KJ882386
A. flavus-CAF8	*Aspergillus flavus*	KJ882387

Table 2—Comparison between cultures based detections methods with that ELISA of *A. flavus* isolates of chilli for aflatoxin contamination

No. of *A. flavus* isolates of chilli	No. of isolates showing +ve &-ve response on culture media	Isolates showing +ve & -ve response on culture media (%)	No of isolates showing positive response with ELISA	Isolates showing positive response with ELISA (%)	No of isolates showing negative response with ELISA	Isolates showing negative response with ELISA (%)
			CCA media			
59	42 +ve	71.18	40	67.79	2	3.38
	17 –ve	28.81	13	22.03	4	6.77
			YESA media			
59	23 +ve	38.98	22	37.28	1	1.69
	36 –ve	61.01	31	52.54	5	8.47

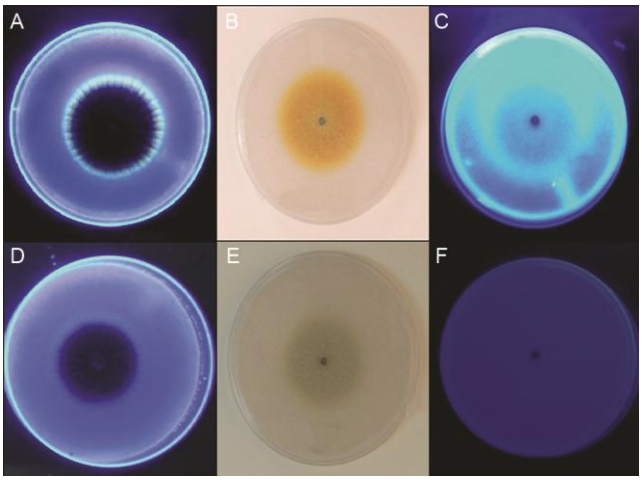

Fig. 2—Isolates of *A. flavus* grown on CMA after 5 d of incubation. A, B and C with beige ring, yellowish pigment and bluish fluorescence, D, E and F without beige ring, yellowish pigment and bluish fluorescence.

and the colour change by ammonia vapor method (Table 2). Immediately after addition of ammonia solution into the Petri dishes, the toxin producing strains turned to red colour. No colour change was observed with the plates of atoxigenic isolates and surrounding agar did not show any colour change. The agreement between cultural based methods of ammonia hydroxide vapor test on YESA with that of the aflatoxin production as determined by ELISA was noted (Table 2 and Fig. 2). Among 59 isolates of chilli, 23 (38.98%) showed red colour and 36 (61.01%) isolates did not produce red colour when exposed to ammonia hydroxide vapor. Among 23 isolates, 22 were aflatoxin producing and one isolate was atoxigenic. Five isolates did not show red colour change in the media and also did not produce any afltoxin. Ammonia vapour test on YESA assay has been recognized as rapid cultural method to detect toxigenic and atoxigenic *Aspergillus* sp. A total of 14 yellow pigments were purified from the methanol

Table 3—Comparison between PCR and ELISA based methods of aflatoxin detection

Crop	Isolate Number	PCR Results				Aflatoxin production by ELISA (µg/Kg)
		aflR	aflJ	norA	ver1	
Chilli	CAF17	+	+	+	+	>2248.40
	CAF23	-	+	+	-	>2828.60
	CAF43	+	+	+	-	>3128.20
	CAF48	-	-	-	-	>2055.80
	CAF58	+	+	+	+	>2404.40
	CAF59	+	+	+	+	>2136.00
	CAF4	+	+	+	+	0.0
	CAF41	+	+	+	+	0.0

extract of aflatoxigenic *A. flavus* cultures[16-18]. All of these pigments are anthraquinone intermediates in the aflatoxin biosynthetic pathway[18].

Large numbers (8%) of false negative results along with positive results were reported. Occurrence of false negative results reflected the unreliability of ammonium hydroxide test[18-20]. Ninety two per cent efficacies for the ammonium vapour test having 8% false negatives were obtained. Using yellow pigmentation, combined with the ammonium hydroxide vapour test, false negatives were reduced from 8-7%[21].

By indirect competitive ELISA method

The potentiality of isolates to produce aflatoxin collected from different places was detected by using indirect competitive ELISA technique. The isolates exhibited wide range of variability in toxin production from 0 to 3128.20 µg kg^{-1}. Among 59 isolates, isolate, CAF43 was highly toxigenic as it produced maximum i.e. 3128.20 µg kg^{-1} of aflatoxin B_1 indicating the most potent nature, whereas isolate CAF48 produced lowest (3035.10 µg kg^{-1}) amount of aflatoxin while six isolates did not produce aflatoxin (Table 3).

Mere presence of *A. flavus* on the crop need not end up in aflatoxin contamination. It is therefore

essential to know the toxigenic nature of the isolate. For this purpose, ELISA was used for detection of aflatoxin contamination caused due to exposure of Aflatoxin B1 and *A. flavus*[21,22].

The isolates of chilli when inoculated in the chilli fruits, exhibited more aflatoxin production because of availability of chilli as their natural medium/substrate. It becomes imperative to know the genes involved in toxin biosynthesis when such occurrences of highly toxigenic and low toxigenic isolates are existing[18,19,22]. The aflatoxin production from 16 isolates of *A. flavus* from chilli was elaborated using an indirect competitive ELISA. Amount of aflatoxin produced by isolates ranged between 99.96 and 1639.10 µg kg^{-1}, highest being 1652.2 µg kg^{-1} produced by isolate AFL-16 followed by isolate AFL-8 (1081.90 µg kg^{-1}). The isolates variability in potency of aflatoxin production might be attributed to various factors[23] and mechanisms such as asexual and parasexual cycle[24] operating in *A. flavus* fungus in agroecosystem.

Molecular characterization

A. flavus CAF4 showed presence of four sets of primer for different genes indicating the presence of all the four genes. Bands of the fragments of *aflR*, *aflJ*, *norA* and *ver1*genes were observed at 900, 450, 400 and 450 bp, respectively (Fig. 3 A-D). Genes *aflR* and *aflJ* are well known for their role in biosynthesis of aflatoxin[2,23-26] and have been considered as a good candidate for diagnostic purposes in differentiation of aflatoxin producing (toxigenic) strains from atoxigenic isolates. While two genes, *norA* and *ver1* which are known to encode for enzymes of aflatoxin biosynthesis pathway. In ELISA, only aflatoxin B_1 type of toxin was detected, other types of aflatoxins, such as B, etc. produced by *A. flavus* were not detected. Five isolates (CAF17, CAF58, CAF59, CAF4 and CAF41) showed a similar pattern in electrophoresis indicating the presence of all the four genes in them and remaining isolates presented varying patterns. Of these, six isolates matched with the PCR and aflatoxin production as determined by ELISA (Table 2). Among eight isolates of chilli, three isolates (CAF17, CAF58 and CAF59) matched with aflatoxin production as well. Isolates (CAF4 and CAF41) showed negative result for toxigenicity in ELISA, while it showed positive in PCR. Hence, the isolates showing negative for toxigenicity in ELISA may not be considered as atoxigenic, as they have only showed presence of aflatoxin producing genes in PCR based method (Table 3). CAF48 isolate did not show amplification of all the four genes but produced aflatoxin in ELISA indicating the role of other regulatory genes in production of aflatoxin.

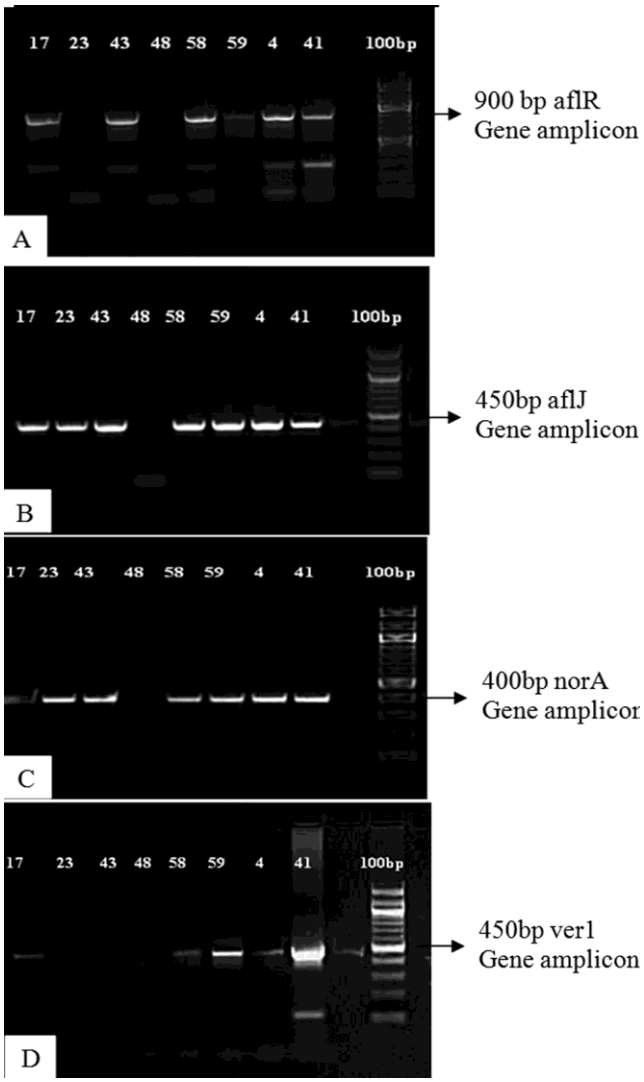

Fig. 3—Gel electrophoresis analysis of PCR products using specific aflatoxin gene specific primers from 8 isolates of *A. flavus* from chilli (17 to 41). [Lane A: amplified product of aflR at 900 bp; Lane B: amplified product of aflJ at 450 bp; Lane C: amplified product of norA at 400 bp and Lane D: DNA of *A. flavus* isolates using VER1 primer with 450 bp]

Studies on the expression and regulation of the aflatoxin biosynthetic and regulatory pathway genes involving *afl*R, *nor*1 and *ver*1 showed that the transcription of *nor*1 and *ver*1 is activated by the *afl*R gene product, AFLR. In other words, the transcription of the structural genes is dependent on the transcription of the *afl*R gene[27,28]. The expression of certain

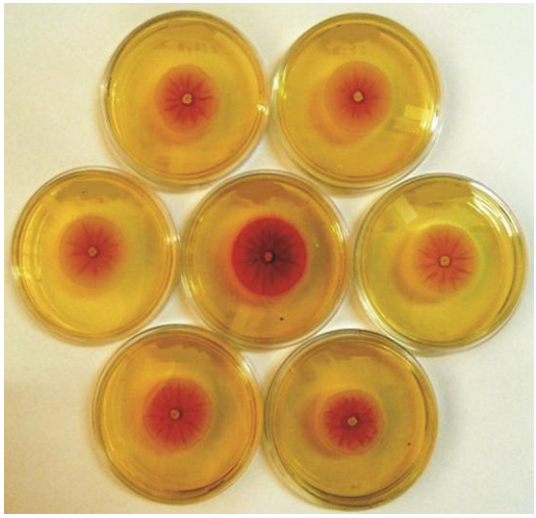

Fig. 4—Isolate of *A. flavus* CAF4 showing red colour (Center plate) and without red colour (peripheral plates) grown on YESA after 3 days of incubation.

regulatory (aflatoxin regulatory [*aflR*] and aflatoxin J [*aflJ*]) and norsolonic acid-1 [*nor-1*]) genes in four corneal *A. flavus* isolates was evaluated by reverse transcription PCR[29,30]. All the isolates were distinct from one another in the cluster but identical to each other in homology and divergence with different nodes (Fig. 4). In particular, *aflR* and *aflJ* gene integrity has been considered a good candidate for diagnostic purposes so it is concluded that presence of *AflR* and /or *AflJ* genes considered for differentiation of toxigenic and atoxigenic isolates still the gene expression is varied by different factors[30-32]. Moreover, the molecular mechanisms responsible for loss of toxigenicity of *A. flavus* are not clearly understood[32].

Aflatoxin produced by different isolates as determined by ELISA did not correlate with the presence or absence of aflatoxin producing gene specific primers. Okoth *et al*.[31] reported the lack of correlation between presence of aflatoxin synthesizing genes and aflatoxin synthesis. Hence, it should be confirmed on a wider basis using all the aflatoxin producing gene specific primer[32]. Polyphasic approach involving cultural, molecular, and analytical methods have been used to identify toxigenic and atoxigenic *A. flavus* by Mamo *et al*.[32].

Conclusion

Differentiation between toxigenic and atoxigenic strains of *A. flavus* isolates of chilli was made on the basis of cultural methods (CMA and YESA added with ammonia vapour), ELISA and PCR methods. While cultural methods yielded positive results for toxin production, some of these cultures came negative in ELISA method where as the presence of four genes *viz*. *aflR*, *aflJ*, *norA* and *ver1* as detected by the PCR-electrophoresis could not be correlated with aflatoxin production in ELISA.

Of the total of 59 isolates of *A. flavus* obtained from chilli, six isolates came out as toxigenic while two isolates were found atoxigenic. Growth on CMA, use of ammonia hydroxide vapor test on YESA can be used for preliminary detection and isolation of aflatoxin producing strains and ELISA can be used for rapid and large scale screening of aflatoxin producing strains and for estimation of amount of aflatoxin produced by *A. flavus*.

Acknowledgment

The first author (MKN) acknowledges ICRISAT, Patancheru, Hyderabad, India for financial support provided through CGIAR Research Program-Agriculture for Nutrition and Health (CRP-A4NH). Authors AH and EFAA extend their appreciation to the Deanship of Scientific Research at King Saud University for its funding to the Research Group number (RG-1435-014).

References

1 Yu J, Bhatnagar D & Ehrlich KC, Aflatoxin biosynthesis. *Rev Iberoam and Micol*, 19 (2002) 191.

2 Mangal M, Khan F, Bansal S & Oberoi HS, Validitation of PCR based systems for aflatoxin producing molds. *Indian J Exp Biol*, 54 (2016) 472.

3 Aiko V & Mehta A, Occurrence, detection and detoxification of mycotoxins. *J Biosci*, 40 (2015) 943.

4 El-Nakib O, Pestka J & Chu F, Determination of aflatoxin B_1 in corn wheat and peanut butter by enzyme-linked immunosorbent assay and solid phase radioimmunoassay. *J Assoc Off Anal Chem*, 64 (1981) 1077.

5 Morgan M, Kang A & Chan H, Aflatoxin determination in peanut butter by enzyme-linked immunosorbent assay. *J Sci Food Agric*, 37 (1986) 908.

6 Yu J, Chang P, Ehrlich K, Cary J, Bhatnagar D, Cleveland T, Payne G, Linz J, Woloshuk C & Bennett J, Clustered pathway genes in aflatoxin biosynthesis. *Appl Environ Microbiol*, 70 (2004) 1253.

7 Sambrook J & Russel DW, Molecular Cloning, A Laboratory Manual, 3rd ed. (Cold Spring Harbor Laboratory Press, New York, USA), 2001, 2100.

8 Huzefa AR, Miller AN, Pearce CJ & Oberlies NH, Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *J Nat Prod*, 24 (2017) 756. doi: 10.1021/acs.jnatprod.6b01085

9 Adachi A, Kiyono T, Hayashi Y, Ohashi M & Ishibashi M, Detection of human papillomavirus (HPV) type 47 DNA in malignant lesions from epidermo dysplasia verruciformis by protocols for precise typing of related HPV DNAs. *J Clin Microbiol*, 34 (1996) 369.

10 Rodrigues P, Soares C, Kozakiewicz Z, Paterson R, Lima N & Venancia A, Identification and characterization of *Aspergillus flavus* and Aflatoxins, In : *Communication in Current Research and Education Topics and Trends in Applied Microbiology*, (Eds. A Mendez Vilas & Badajoz; Formatex, Spain), 2007, 527.

11 Gupta A & Gopal M, Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. *World J Microbiol Biotechnol*, 18 (2002) 325.

12 Arseculeratne S, De Silva L, Wijesundera S & Bandunatha C, Coconut as a medium for the experimental production of aflatoxin. *Appl Microbiol*, 18 (1969) 88.

13 Lin M & Dianese J, A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* sp. *Phytopathology*, 66 (1976) 1466.

14 Davis N, Iyer S & Diener U, Improved method of screening for aflatoxin with a coconut agar medium. *Appl Environ Microbiol*, 53 (1987) 1593.

15 Cole R, Versicolorin group. In: *Handbook of Toxic Fungal Metabolites* (Eds. Cole RJ & Cox RH; Academic Press, New York), 1981, 94.

16 Heathcote J & Dutton M, New metabolites of *Aspergillus flavus. Tetrahedron*, 25 (1969) 1497.

17 Bhatnagar D, Ehrlich K & Cleveland T, Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl Microbiol Biotechnol*, 61 (2003) 83.

18 Kumar S, Shekar M, Khan AA & Sharma P, A rapid technique for detection of toxigenic and non toxigenic strains of *Aspergillus flavus* from maize grains. *Indian Phytopathol*, 60 (2007) 31.

19 Abbas H, Shier W, Horn B & Weaver M, Cultural Methods for Aflatoxin Detection. *J Toxicol*, 23 (2004) 295.

20 Naik M, Aflatoxin and post harvest disease problems in exportable horticulture produce. In: *Microbial diversity for Asian prosperity*, 2nd Asian Congress of Mycology and Plant Pathology. Osmania University Hyderabad, India, 19-22 December, 2007.

21 Ajith Kumar K & Naik MK, Detection of aflatoxin producing *Aspergillus flavus* isolates from chilli and their management by post harvest treatments. *J Food Sci Technol*, 43 (2006) 200.

22 Naik M & Sudha S, Current issues and future strategies on aflatoxin contamination and management in chilli. In: *National Workshop on Current Trends and Future Prospects in Production and Export of Spice with Special Reference to Chilli*. Dharwad Karnataka, India, 27-28 February, 2009.

23 Pontecorvo G, The parasexual cycle in fungi. *Ann Rev Microbiol*, 128 (1956) 162.

24 Payne G, Nystrom G, Bhatnagar D, Cleveland T & Woloshuk C, Cloning of the afl-2 gene involved in aflatoxin biosynthesis from *A. flavus. Appl Environ Microbiol*, 59 (1993) 156.

25 Woloshuk C, Yousibova G, Rollins J, Bhatnagar D & Payne G, Molecular Characterization of the afl-1 Locus in *Aspergillus flavus. Appl Environ Microbiol*, 61 (1995) 3019.

26 Meyers D, Obrian G, Du W, Bhatnagar D & Payne G, Characterization of aflJ a gene required for conversion of pathway intermediates to aflatoxin. *Appl Environ Microbiol*, 64 (1998) 3713.

27 Chang P, The *Aspergillus parasiticus* protein aflJ interacts with the aflatoxin pathway-specific regulator *aflR*. *Mol Genet Genomics*, 268 (2003) 711.

28 Yu J, Chang P, Cary J, Wrigth M, Bhatnagar D, Clevel T, Payne G & Linz J, Comparative mapping of aflatoxin pathway gene clusters in *A. parasiticus* and *A. flavus. Appl Environ Microbiol*, 61 (1995) 2365.

29 Leema G, Chou D, Jesudasan C, Geraldine P & Thomas P, Expression of genes of the aflatoxin biosynthetic pathway in *Aspergillus flavus* isolates from keratitis. *Mol Vis*, 17 (2011) 2889.

30 Schmidt-Heydt M, Megan N & Geisen R, Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiol Lett*, 284 (2008) 142.

31 Okoth S, De Boevre M, Vidal A, Di Mavungu JD, Landschoot S, Kyallo M & Saeger SD, Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya. *Front Microbiol*, 9 (2018) 57 https://doi.org/10.3389/fmicb.2018.00057

32 Mamo FT, Selvaraj JN, Wang Y & Liu Y, Recent Developments in the Screening of Atoxigenic *Aspergillus flavus* towards Aflatoxin Biocontrol. *J App Environ Microbiol*, 5 (2017) 20. doi: 10.12691/jaem-5-1-3