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RESEARCH ARTICLE

CULTURAL, TOXIGENIC AND MOLECULAR VARIABILITY OF *ASPERGILLUS FLAVUS* ISOLATES OF CHILLI (*CAPSICUM ANNUUM* L.) COLLECTED FROM NORTH EASTERN KARNATAKA

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ABSTRACT

A total of 13 *Aspergillus* isolates were collected from different regions of North Eastern parts of Karnataka. Thirteen isolates varied in radial growth from 80-85 mm. Eight isolates produced sclerotia with minimum number (26 cm^{-2}) produced by AF4 isolate and maximum number (119 cm^{-2}) by AF13. They were inoculated on ripened chilli fruits using pin-prick method. Thirteen isolates varied in aflatoxin production from 99.96 to 1639.10 µg/kg as determined by ELISA. AF10 isolate produced maximum potency of 1639.80 µg/kg followed by AF3 with 1195.80 µg/kg and the least production of 99.96 µg/kg was noticed in AF13. All the 13 isolates were amplified for ITS region and were sequenced. Identity of the isolates was confirmed with sequences of NCBI data base of *Aspergillus flavus*. Further, aflatoxin producing biosynthetic genes were amplified by using two specific primers set AFL and FLA and were confirmed as aflatoxin producing isolates of *A. flavus*.

INTRODUCTION

Chilli (*Capsicum annuum L.*) is an important spice and vegetable crop used all over the world in one form or the other, valued for its pungency which is imparted by an alkaloid capsaicin and red pigment capsanthin, capsourubin and capxanthin. Chillies being the universal spice of India and are grown in almost all states for the domestic, market and export purpose. The major chilli producing states in India are Andhra Pradesh, Karnataka, Maharashtra, Orissa, Rajasthan and Tamil Nadu which contribute to 86 per cent of total area of chilli cultivation in the country and 90 per cent of the total Indian produce. Aflatoxins are the most potent carcinogens of mycotoxins and are the most commonly found toxin in chilli and paprika spice. Asao *et al*. (1963) were the first to identify the toxins as aflatoxin, which were later reported to be potent carcinogens by Hesseltine *et al*. (1966). *Aspergilli* are the predominant contaminant which readily colonize and have potential to produce toxin. The infection occurs on stored fruits and the contamination with aflatoxin deteriorates quality and make the produce unfit for consumption, thereby hitting the export trade in the international market. However, mere presence of *Aspergillus* fungus on chilli need not end up as aflatoxin contamination. Hence it is essential to detect aflatoxin producing isolates of *Aspergillus flavus*. Aflatoxin production is influenced by a number of physical, chemical and biological factors including a suitable substrate and congenial climate conditions apart from toxigenic nature of isolates of *A. flavus* (Naik *et al*., 2007). The present study reveals about the wide variation among the thirteen isolates of *A. flavus* with respect to their cultural and growth characters, their potential to produce aflatoxin and molecular characterization.

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MATERIALS AND METHODS

Isolation of the pathogenic *A. flavus* from chilli fruits

Infected chilli fruits were collected from different fields of North Eastern Karnataka regions comprising Raichur, Bellary and Gulbarga districts. The isolation of the fungus was done by following standard tissue isolation method (Ajith Kumar and Naik, 2006) under aseptic conditions. The infected tissues of the fruits were cut into small bits of size 1-2 mm and surface sterilized in 1:1000 mercuric chloride solution for one min and washed repeatedly thrice in sterile distilled water to remove the traces of mercuric chloride before transferring them to sterile potato dextrose agar (PDA) slants under aseptic condition and incubated at 28±1 °C for growth. The culture, thus obtained was purified by single spore isolation method. The *Aspergillus flavus* isolates were identified based on the morphological features such as conidiophores, conidia, colour of the colony and rate of growth. The conidial heads were bright yellow when young, olive green turning to brown with age, radiate or loosely columnar, conidiophores roughened, vesicles globouse to subglobose, flask shaped in smaller heads, sterimgata

biseriate or uniseriate, conidia globose to subglobose rarely ellipitical, smooth to roughened, sclerotia red-brown to purple-brown or black, globose to elongate (Raper and Fennell, 1965).

Cultural characters of *A. flavus*

The thirteen isolates of *Aspergillus flavus* were grown on potato dextrose agar (PDA) incubated at 28±1 ^{0}C for growth. At the end of the incubation period the plates were observed for the morphological features such as colony diameter, colour of mycelia, margin colour, texture and production of sclerotia.

Detection of *A. flavus* isolates for toxigenic nature

The chilli (*Capsicum annuum*) fruits harvested at red ripened stage were surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) and then washed in three changes of sterile water. Thereafter, 500g fruits were pricked with pin bundles specially designed for pricking purpose. The pinpricked fruits were then dipped in spore suspension of different isolates (AF1-AF13) separately (1 X 10^6 spores /ml) for 2-3 min. The humid chamber was prepared by keeping water in the tray, which was placed below the perforated tray kept with inoculated fruits. Wet cotton pieces were placed on the tray. The tray was covered with polythene sheet to maintain the relative humidity of over 90 per cent and then incubated at 28±1 ^{0}C. Eight days after inoculation, the fruits were shade dried and made to fine powder, then used for the detection of toxigenic nature of different isolates.

Dried graded chilli fruit samples were collected and samples of approximately 500 g were drawn. About three each of 50 g sub samples were drawn from 500 g samples. Dried fruits were cut into small pieces, thoroughly mixed and a 15 g of analytical sample was drawn from each of 50 g sub samples. The analytical sample were ground in a waring blender and then utilized for analysis. Each 15 g of the chilli powder was extracted with 75 ml of a solvent containing 70 ml methanol + 30 ml water + 0.5 g KCl and blended for 2 min. This was followed by shaking in a rotary shaker for 30 min. at 250 rpm. The extract was filtered through Whatman No. 41 filter paper and diluted to 1: 10 with 0.2% bovine serum albumin (BSA) prepared in 0.05 m PBS – Tween, pH –7.4 (PBST-BSA). Depending on the concentration of aflatoxin, the sample was either used directly or diluted further at 10 fold intervals prior to analysis by ELISA.

ELISA Procedure

An indirect competitive ELISA technique was used, followed by previous workers (Reddy *et al.,* 1988; Devi *et al.,* 1999 and Thirumala Devi *et al.*2000; Ajit Kumar *et al*., 2006). The ELISA was carried out at the Mycotoxin Laboratory, International Crop Research Institute for the Semi Arid Tropics (ICRISAT), Asia Center, Patancheru, Andhra Pradesh. ELSIA plates were coated with 150 µl/ well of AFB_1-BSA at a concentration of 100 ng/ ml prepared in carbonate coating buffer (Hobbs *et al.* 1987). At each step, plates were incubated at 37 ^{0}C followed by three washes with PBS-Tween. In the second step, plates were treated with PBST-BSA. AFB_1 standards ranging from 0.1 to 25 ng/ml were prepared in extracts (diluted to 10 %) from chillies not containing any aflatoxin. Concentrations of the standards used were 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.097 µg/ ml and each concentration was duplicated in two wells. Similarly each test sample was duplicated in two wells. One hundred µl of 10 times diluted test sample extract or standards were mixed with 50 µl of antiserum diluted to 1: 60,000 in 0.2 % PBST-BSA. This step was followed by the addition of alkaline phosphatase labeled goat antirabbit IgG conjugate diluted to 1: 2000 in PBST-BSA. The substrate was p-nitrophenyl phosphate prepared in 10 % diethanolamine. The plates were incubated at room temperature and then read in an ELISA reader. A maximum interval of 30 min was allowed until optical densities from wells not containing any toxin reached 1.5 0 2.0 OD units at 405 nm. Regression curve was drawn using Log_{10} values of concentration for aflatoxin standards and were plotted on the Y-axis and OD values were plotted on the X-axis. Using the regression equation values for the known AFB_1 standards, the aflatoxin concentration in the samples was calculated. Aflatoxin concentration in the sample extract was determined using the following formula (Reddy *et al.* 2001).

$$\text{Aflatoxins concentration (µg/kg)} = \frac{AFB_1 \text{ (ng/ml)} \times \text{dilution factor} \times \text{Extract solvent (ml)}}{\text{Sample weight (g)}}$$

The aflatoxin is chemically highly substituted coumarins and contained a fused dihydrofurofuron configuration, naturally occurring compounds. They are known to occur in two series aflatoxin B_1 and its derivatives and aflatoxin G_1 and its derivatives. Of all the aflatoxins, aflatoxin B_1 has the highest potency as toxin and as a carcinogen (Wogan, 1975).

DNA extraction

The isolates of *A. flavus* were grown in 25ml Czapeck broth for 3-4 days. The cultures of Czapeck broth were filtered and dried completely with pre-autoclaved filter paper. The DNA was extracted using fungal DNA isolation kit of CTAB (Cetyltrimethyl ammonium bromide) method.

PCR amplification

The purified DNA of *A. flavus* was amplified with ITS primer set, ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification reactions were carried out in a 25 µl reaction mixture with PCR conditions (one cycle of 94 ^{0}C for 4 min, 36 cycles of denaturation 94 ^{0}C for 1 min, annnealing 55 ^{0}C for 1 min, extension 72 ^{0}C for 1.5 min and one cycle of final extension 72 ^{0}C for 5 min) as described by Jimenez *et al*. (2011). Amplified DNA fragments were checked in 1.5 % agarose gel and documented using Syngene G-box gel documenting system.

Cloning, sequencing and phylogenetic analysis

PCR products were cloned into pGEM®-T Easy Vector Systems (Promega) and transformed into competent *E. coli* strain DH5α by following 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. Online multiple sequence alignment tools (www.genome.jp/tools/clustalw) were used to construct the phylogenetic tree using UPGMA-NJ method.

Aflatoxin gene detection

The aflatoxin producing genes were amplified with specific primers sets *viz*., AFL-r (5'-CTTGTTCCCCGAGATGACCA-3'), AFL-f (5'-CGCGCTCCCAGTCCCCTTGATT-3') and FLA-1 (5'-GTAGGGTTCCTAGCGAGCC-3') FLA-2 (5'-GGAAAAAGATTGATTTGCGTTC-3').

RESULTS

Diversity in cultural characteristics of *A. flavus* isolates

Thirteen *A. flavus* isolates were collected from major chilli growing regions of North Eastern parts of Karnataka. Among 13 isolates, ten isolates (AF1, AF2, AF4, AF5, AF6, AF8, AF9, AF10, AF12 and AF14) produced olive green and four isolates (AF3, AF7 and AF11) produced parrot green mycelia with a diameter ranging from 80-85mm. Four isolates (AF2, AF5, AF10 and AF14) showed green and nine isolates (AF1, AF3, AF4, AF6, AF7, AF8, AF9, AF11 and AF12) were white in margin. Eight isolates of *A. flavus* (AF1, AF2, AF3, AF4, AF5, AF6, AF8 and AF10) showed course texture and five isolates (AF7, AF9, AF11, AF12 and AF14) had smooth texture. Sclerotial production was observed after a week of inoculation. Only eight isolates (AF3, AF4, AF6, AF9, AF10, AF11, AF12 and AF13) produced sclerotia and the colour of sclerotia was dark brown with minimum number of sclerotia (26/9cm^2 Petri dish) was produced by AF4 isolate and maximum number of sclerotia (119 /9cm^2 Petri dish) was produced by AF13 isolate (Table 1).

Toxigenic diversity of *Aspergillus flavus* isolates

Thirteen isolates varied in their ability to produce toxin. There was a wide range of aflatoxin production by different isolates. The range of toxin production varied from 99.96 - 1639.10 µg/kg. All isolates were virulent for aflatoxin production but AF10 isolate showed the maximum potency of 1639.10 µg/kg followed by AF3 with 1195.80 µg/kg and least production of 99.96 µg/kg was noticed in AF13. However, the remaining isolates were in the range of 500-900 µg/kg (Table 1). Two isolates produced above 1000µg/kg which includes isolates AF10 with 1639.10 µg/kg and AF3 with 1195.80 µg/kg and the least production of 99.96 µg/kg was noticed in AF13.

Molecular characterization of *A. flavus* isolates and aflatoxin gene detection

For Molecular diversity analyses, 13 *A. flavus* isolates were amplified at a size of around 600 bp by using ITS primer pair (Fig. 1).

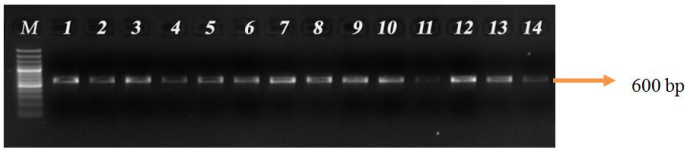

Fig.1.

The amplified products were checked on 1.5 per cent agarose gel and then the sequences were obtained. The BLAST data results revealed that the *A. flavus* isolates matched with the reference strains of NCBI results and identified as *Aspergillus flavus*. Out of 13 isolates, all the species were distinct from one another in the cluster but identical to each other in homology and divergence with different nodes (Fig. 2).

The aflatoxin producing gene of *A. flavus* was successfully amplified and confirmed by using AFL and FLA primers. All the isolates amplified at 750 and 500 bp using AFL and FLA primer (Fig. 3) which confirmed the presence of aflatoxin production gene on the cluster except AF11 isolate which was not amplified at FLA primer. Whereas, the PCR amplicon from FLA primer was successfully amplified in all the 13 isolates of *A. flavus*.

Table 1. Cultural and toxigenic diversity of thirteen isolates of *Aspergillus flavus* infecting chilli along with their accession numbers

Isolates	Description	Location	Colony diameter (mm)	Aflatoxin B_1 (µg/kg)	Colour of the mycelium	Colour of the margin	Texture	No. of sclerotia	NCBI, USA Accession number
AF1	*Aspergillus flavus-KAR-1*	Raichur	85	965.90	Olive green	White	Course	-	KF433939
AF2	*Aspergillus flavus-KAR-2*	Raichur	85	730.40	Olive green	Green	Course	-	KF433940
AF3	*Aspergillus flavus-KAR-3*	Gulbarga	80	1195.80	Parrot green	White	Course	34	KF433941
AF4	*Aspergillus flavus-KAR-4*	Gulbarga	80	716.63	Olive green	White	Course	26	KF433942
AF5	*Aspergillus flavus-KAR-5*	Gulbarga	80	991.63	Olive green	Green	Course	-	KF433943
AF6	*Aspergillus flavus-KAR-6*	Bellary	80	692.96	Olive green	White	Course	67	KF433944
AF7	*Aspergillus flavus-KAR-7*	Bellary	85	542.26	Parrot green	White	Smooth	-	KF433945
AF8	*Aspergillus flavus-KAR-8*	Bellary	85	163.13	Olive green	White	Course	-	KF433946
AF9	*Aspergillus flavus-KAR-9*	Bellary	85	531.43	Olive green	White	Smooth	96	KF433947
AF10	*Aspergillus flavus-KAR-10*	Bellary	80	1639.10	Olive green	Green	Course	62	KF433948
AF11	*Aspergillus flavus-KAR-11*	Bellary	85	670.00	Parrot green	White	Smooth	90	KF433949
AF12	*Aspergillus flavus-KAR-12*	Bellary	80	625.93	Olive green	White	Smooth	99	KF433950
AF13	*Aspergillus flavus-KAR-13*	Bellary	85	99.96	Olive green	Green	Smooth	119	KF433951

Nucleotide sequence accession number

All the sequences of *Aspergillus flavus* isolates were deposited in NCBI Gene Bank, USA along with location of the isolates. Accession numbers are: KF433939, KF433940, KF433941, KF433942, KF433943, KF433944, KF433945, KF433946, KF433947, KF433948, KF433949, KF433950 and KF433951.

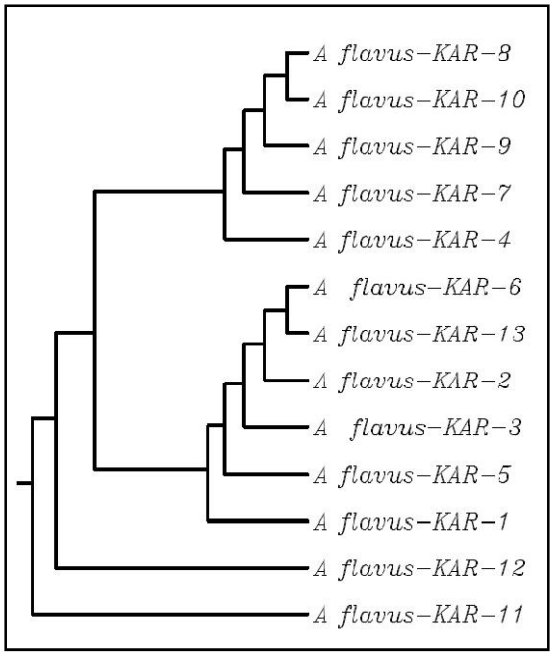

Fig. 2.

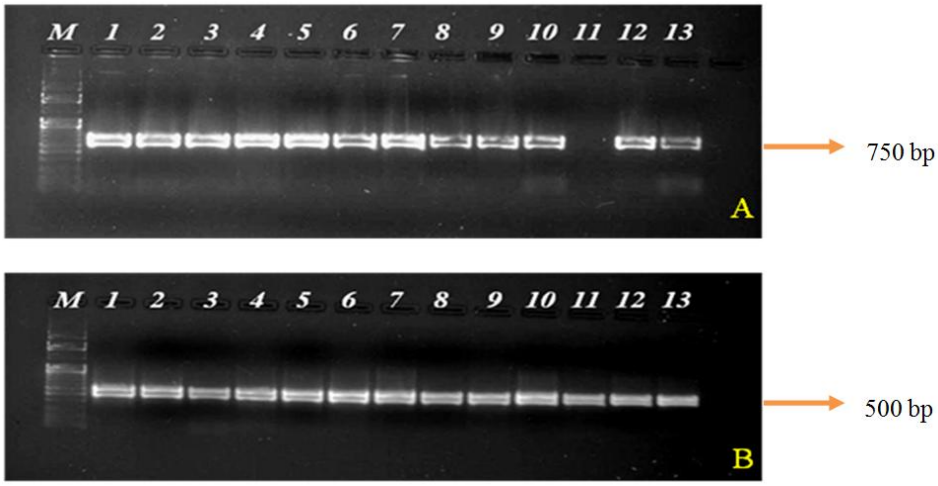

Fig. 3.

DISCUSSION

Mehan and Chohan (1973) categorized different isolates of *A. flavus* on the basis of cultural characters. Bedi and Ratan (2003) characterized *A. flavus* isolates based on the colour of conidial heads, texture and production of sclerotia. The reverse colony colour of all the isolates was found to be orange which was the indication of production of toxin. Sclerotia are commonly produced by strains of *A. flavus* in culture and likely to serve as resistant structures for surviving adverse environmental conditions. (Cotty, 1989; Horn *et. al.,* 1996; Mc Alpin *et. al.,* 1998; Shearer *et. al.,* 1992; Coley–Smith and Cooke 1971). Sclerotia of *A. flavus* can germinate sporogenically and could be a potential source of primary inoculums (Wicklow and Donahue, 1984; Wicklow and Wilson, 1986). The present study revealed a wide variation among the thirteen isolates of *A. flavus* in their ability to produce toxin. Four of the isolates produced above 1000µg/kg of aflatoxin. All the thirteen isolates were toxigenic as the isolates were showing more than 20µg/kg which is above permissible limit (Vasanthi and Bhat, 1998 and Reddy *et al*., 2001). The detection of aflatoxin by an isolate is important because a mere occurrence of *A. flavus* fungus on chilli fruits could be misleading to call it as aflatoxin contamination unless it is proved for elaboration of aflatoxin production at above permissible limit (Naik *et al*., 2004). The present investigation indicates the availability of highly potent (AF10, AF3), low potent (AF13, AF8) and moderately potent (AF5, AF1, AF2, AF4, AF6, AF11, AF12, AF7, AF9) isolates of aflatoxin production within the provinces. The availability of high and low potent strains in the same geographical region of North Eastern Karnataka suggests the existence of natural variation among the population of *A. flavus*. It would be interesting to see the genes responsible for producing toxin when such occurrence of highly toxigenic and low toxigenic isolates are existing (Naik *et al*., 2003; Naik *et al*., 2007 and Ajith Kumar and Naik, 2006). The variability in potency of aflatoxin production might be attributed to several mechanisms such as asexual and parasexual cycle (Pontecorvo, 1956) operating in *A. flavus* fungus in agro-ecosystem. Aflatoxin production is influenced by a number of physical, chemical and biological factors including a suitable substrate and congenial climate conditions apart from toxigenic nature of isolates of *A. flavus* (Naik *et al*., 2007). However, relation between cultural characters and aflatoxin production has not been reported so far.

PCR amplification of the ITS regions of the *A. flavus* isolates (AFM1, AFM3 and AFM5) by using ITS1 and ITS4 primers gave products of approximately 580 base pairs in length. The nucleotide sequences were compared with ITS sequences of *A. flavus* from Genbank database and were in the range of 12 and 99%, respectively (Karthikeyan *et al*., 2009). Henry *et al*. (2000) reported sequence variation in several areas in the ITS regions among referenced and clinical isolates of *Aspergillus* species. RFLP analysis of the ITS regions of nuclear rDNA has been used to study the genetic diversity among the isolates of different groups of fungi (Appiah *et al*., 2004). DNA was extracted from *A. flavus* isolates of maize and their molecular

variability was investigated by using restriction fragment length polymorphism. Analysis of the PCR-amplified internal transcribed spacer (ITS) regions of ribosomal DNA with ITS1 and ITS4 primers resulted in the amplification of a product of approximately 600 bp (Mohankumar *et al*., 2010). Lee *et al*. (2006) amplified and sequenced 23 strains of *Aspergillus* section *flavi* by using aflR gene. No aflR PCR products were found in five *A. sojae* strains or six strains of *A. oryzae*. The sequenced aflR genes from the 23 positive strains had greater than 96.6% similarity. The *A. flavus* strains were analyzed for the presence of seven aflatoxin biosynthesis genes targeting the regulatory genes aflR and aflS, and the structural genes aflD, aflM, aflO, aflP, and aflQ. The results revealed that *A. flavus* was the only species responsible for aflatoxin contamination (Gallo *et al*., 2012). An unusual mutation at the afl-1 locus, affecting aflatoxin biosynthesis in *A. flavus* 649 was investigated by (Woloshuk *et al*., 1995). *A. flavus* and *A. parasiticus* were distinguished based on gene-specific primers that targeted the intergenic spacer (IGS) for the AF biosynthesis genes, aflJ and aflR. Both the species displayed different PCR-based RFLP (PCR-RFLP) profile. PCR products from *A. flavus* cleaved into three fragments of 362, 210, and 102 bp. However, *A. parasiticus* produced only 2 fragments of 363 and 311 bp (Khoury *et al*., 2011).

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