



Studies on seed-borne mycoflora and aflatoxin B₁ contaminations in food based seed samples: Molecular detection of mycotoxigenic *Aspergillus flavus* and their management

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Abstract

In the present study the mould incidence, ergosterol and aflatoxin B₁ (AFB₁) contaminations were evaluated in different food based seed samples viz., chickpea, cowpea, green gram, groundnut, Indian bean, maize, sorghum, soya bean and sunflower collected from different agro-climatic regions of Karnataka (India). The agar plate and standard blotter methods were employed for determination of the fungal incidence, and the ergosterol and AFB₁ contents were estimated qualitatively and quantitatively by TLC and spectrophotometric methods. For detection of aflatoxigenic isolates of *A. flavus*, the target gene specific primer *aflR-F* and *aflR-R* were used in PCR amplification. The antifungal and antiaflatoxigenic activities of some selected edible vegetable extracts were evaluated by measuring mycelial dry weight and AFB₁ content. The results revealed that 15 diverse fungal species belonging to 11 genera were observed. Among the seed samples analyzed, the highest fungal incidence, ergosterol and AFB₁ were observed in sorghum samples followed by maize and chickpea. The PCR amplification showed positive results only for aflatoxigenic isolates of *A. flavus* and no amplification was observed in non-aflatoxigenic isolates and *A. flavus* isolate-2 produced highest AFB₁, showed 99% similarity with an authenticated aflatoxigenic *A. flavus* isolate emb|FN398161.1|. The aqueous extract of *Amorphophallus campanulatus* showed highest antifungal and antiaflatoxigenic activities. The results confirmed that the ergosterol and AFB₁ contents were correlated with the percent mould incidences.

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Introduction

Fungi are significant destroyers of food- and feed-stuffs during storage, rendering them unfit for human consumption by producing mycotoxins along with retarding their nutritive value (Janardhana *et al.*, 1998; Marin *et al.*, 1999). Mycotoxins are well known for their health hazardous effects in human beings (Rocha *et al.*, 2005). The Food and Agriculture Organization (FAO) estimated that more than 25% of the world's foodstuffs are contaminated with mycotoxins (Reddy *et al.*, 2009). Species of *Aspergillus* are common mycotoxigenic fungi predominantly associated with heavy loss of food- and feed-stuffs during storage worldwide (Miller, 1995; Janardhana *et al.*, 1999; Galvano *et al.*, 2001). Among all the mycotoxins, aflatoxin B₁ (AFB₁) is one of the most carcinogenic, teratogenic, hepatotoxic and mutagenic mycotoxin primarily produced by *A. flavus* and *A. parasiticus*. Aflatoxins are considered by the US Food and Drug Administration (FDA) as unavoidable contaminants of food, and in 1993, the International Agency for Research on Cancer (IARC)

classified AFB₁ as Group-1 carcinogens due to its various toxic effects, good thermal stability and wide distribution in all types of food and feedstuffs (IARC, 2002). The molecular mechanism of aflatoxins biosynthesis revealed that the genes involved in the production of aflatoxins are located in a co-regulated gene cluster that encodes two regulatory proteins i.e., *aflR* and *aflJ* (Zhang *et al.*, 2014).

The chemical strategies such as treatment with food preservatives and pesticides are the most commonly used methods to control mould and mycotoxins contamination in food- and feed-stuffs (Passone *et al.*, 2008). However, most of these control strategies are not affordable and cause health hazards to human, animals and plants due to their acute toxicity (Mdee *et al.*, 2009; Shukla *et al.*, 2009; Deng *et al.*, 2011). Considering the serious side effects caused by many synthetic fungicides followed by the continuing resistance exhibited by phytopathogenic fungi to commercially available synthetic fungicides have collectively necessitated to look for alternative strategies. Use of plant derived products provides an alternative opportunity to avoid chemical

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preservatives/fungicides for eco-friendly sustainable management of moulds and mycotoxins in food- and feed-stuffs. Hence, the present investigation evaluated the fungal incidence, ergosterol, AFB₁ content in some food based seed samples, and antimycotic and antiaflatoxic activities of some edible vegetable extracts.

Materials and Methods

Chemicals and culture media

Sabouraud dextrose agar/broth (SDA/SDB), sucrose-MgSO₄-KNO₃-yeast extract-broth (SMKYB), dimethyl sulfoxide (DMSO) and all analytical grade solvents were purchased from Hi-Media, Mumbai (India). The standard AFB₁ was obtained from Sigma, Germany. Silica gel 60 F254 coated preparative thin layer chromatography (TLC) plates were obtained from Merck, Germany.

Collection of food based seed samples

A total of 18 food samples viz., 3 varieties each of chickpea (*Cicer arietinum* L.), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench), 2 varieties each of cowpea (*Vigna unguiculata* (L.) Walp.), green gram (*Vigna radiata* (L.) R.Wilczek) and soya bean (*Glycine max* (L.) Merr.), and a variety each of groundnut (*Arachis hypogaea* L.), Indian bean (*Lablab purpureus* (L.) Sweet) and sun flower (*Helianthus annuus* L.) were collected from different agro-climatic regions of Karnataka (India) and subjected to seed-borne mycoflora, ergosterol and AFB₁ estimation.

Mycoflora analysis

The collected seed samples were analyzed for seed-borne mycoflora by standard blotter and agar plate methods following the procedure of ISTA (1996). In brief, 400 seeds from each sample were surface sterilized with 2% sodium hypochlorite solution for 3 min., then 200 seeds were placed on agar plates and another 200 seeds were placed on three layer moistened blotter discs in petri plates. The plates were incubated at 26±2°C under alternating cycle of 12/12 h. of light and darkness up to 7 days. On the 7th day, the fungal colonies expressed on seed samples were isolated by point inoculation method and sub-cultured on SDA. The pure cultures were identified up to genus level, and those belonging to the genus *Aspergillus* were further identified up to the species level with the help of standard fungal key manuals (Booth, 1977; Watanabe, 2002; Nagamani et al., 2006). The percent incidence (PI) and relative preponderance (RP) of the seed-borne fungi were

determined using the following formula

$$PI = \frac{\text{Number of seeds on which the fungus was encountered in each sample}}{\text{Total number of seeds tested in each sample}} \times 100$$

$$\%RP = \frac{\text{Number of seed samples on which the fungus was encountered}}{\text{Total number of seed samples tested}} \times 100$$

Extraction and quantification of AFB₁ from culture filtrate of *A. flavus* and seed-samples

AFB₁ was extracted from the culture filtrate of *A. flavus* following the procedure of Shukla et al. (2008). In brief, 100 µl of spore suspension (10⁴ spores/ml) of aflatoxigenic strain of *A. flavus* isolate-2 was inoculated into a SMKY broth, incubated at 26±2°C for 10 days and filtered through Whatman no.1 filter paper. The obtained filtrate was used for AFB₁ extraction by adding equal volume of CHCl₃, and subjected to evaporation after passing through a bed of anhydrous sodium sulphate. The residue was re-dissolved in 1 ml of chloroform and subjected to TLC separation. AFB₁ was estimated qualitatively by visual comparison with standard AFB₁ on TLC. For quantitative estimation, the fluorescent spots on TLC plates were scrapped out, dissolved in 5 ml of cold CH₃OH, centrifuged at 3000 rpm for 5 min and absorbance of the supernatant was measured at 360 nm using a spectrophotometer (ELICO SL-210, India). The AFB₁ content was calculated using following formula

$$AFB_1 \text{ content } (\mu\text{g/l}) = \frac{D \times M}{E \times L} \times 1000$$

Where, D= absorbance; M= molecular weight of AFB₁ (312); E= molar extinction coefficient of AFB₁ (21,800) and L= path length (1 cm cell).

AFB₁ content from the collected seed samples was extracted and estimated following the procedures of Singh et al. (1991) and Shukla et al. (2008). In brief, 100 g powder of each seed sample was mechanically agitated with 500 ml of methanol-water (60:40, v/v) for 30 min. and filtered through Whatman No. 1 filter paper. The filtrate taken from the separating funnel was mixed with 6 ml of saturated NaCl solution and 10 ml of hexane, then collected the lower aqueous methanol portion. The methanol portion was mixed with 50 ml of chloroform, collected the chloroform portion after passing over a bed of anhydrous sodium sulphate and allowed to evaporate. The residue was re-dissolved in 1 ml of chloroform and subjected to AFB₁ quantification following the procedure of Shukla et al. (2008) as mentioned above.

Extraction and quantification of ergosterol from seed samples

The ergosterol content in each seed sample was estimated quantitatively by spectrophotometric method following the procedure of Bankole *et al.* (2010). In brief, 20 g powder of each seed sample was mixed with 75 ml of methanolic KOH (133 g of KOH in 1000 ml of methanol) and 50 ml of ethanol, and refluxed for 30 min. at 80°C. The filtrate was collected, separated with 50 ml of hexane and 10 ml of water in a separating funnel, and collected the upper hexane layer. The hexane fraction was subjected to ergosterol estimation using spectrophotometer at 230 and 300 nm. The amount of ergosterol was calculated using the following formula.

$$\% \text{ergosterol} + \%24(28) \text{ dehydroergosterol} = \frac{(A_{282}/290)}{\text{pellet weight}}$$

$$\%24(28) \text{ dehydroergosterol} = \frac{(A_{230}/518)}{\text{pellet weight}}$$

$$\% \text{ergosterol} = (\% \text{ergosterol} + \%24(28) \text{ dehydroergosterol}) - \%24(28) \text{ dehydroergosterol}$$

Where, A_{282} and A_{230} are absorbance values of ergosterol, 290 and 518 are the E values (in percentages per cm) determined for crystalline ergosterol and 24(28) dehydroergosterol, respectively, and pellet weight is the net dry weight of sample (g).

PCR based detection of aflatoxigenic strain of *A. flavus*

The genomic DNA was isolated from 14 different strains of *A. flavus* by phenol:chloroform (1:1) method and subjected to PCR amplification using aflatoxin gene specific primers *aflR-F* and *aflR-R* following the procedure of Rahimi *et al.* (2008). In brief, 500 µg of mycelia was ground in lysis buffer, heated at 65°C for 30 min. and 500 µl of phenol-chloroform (1:1, v/v) was added. The upper layer was collected after centrifugation at 10,000 rpm for 10 min. and mixed with 500 µl of chloroform-isoamyl alcohol solution (48:2, v/v) and 25 µl of 3 M sodium acetate and again centrifuged at 10,000 rpm for 10 min. The DNA was precipitated by adding equal amount of chilled ethanol. The DNA pellet was collected after centrifugation at 12,000 rpm for 10 min., re-suspended with 50 µl sterile distilled water and subjected to PCR amplification using aflatoxin gene specific primers *aflR-F* (5'-CGCGCTCCCAGTCCCCTTGATT-3'), *aflR-R* (5'-CTTGTTCCCCGAGATGACCA-3') obtained from Bioserve Biotechnology Pvt. Ltd, Hyderabad (India). The amplification was carried out

in 25 µl of a mixture containing 50 ng of genomic DNA, 2.5 µl of 10X buffer, 1 µl of 25 mM MgCl₂, 3 µl of 2 mM dNTPs, 0.5 µl of 1.5 U DNA Taq polymerase, 1.5 µl of 10 pM each primer and 14.5 µl of sterile distilled water. The PCR products were analyzed by electrophoresis in 1% agarose gel following the procedure of Sambrook *et al.* (1989) and the obtained bands were subjected to gene sequencing in Genombio Technologies, Pune (India).

Inhibitory activities of some vegetable extracts on growth of *A. flavus* and AFB₁ production

The aqueous extracts of some selected edible vegetables viz., *Raphanus raphanistrum* subsp. *sativus* (L.) Domin (radish), *Ipomoea batatas* (L.) Lam. (sweet potato), *Spondias pinnata* (L. f.) Kurz (Indian hog plum), *Amorphophallus campanulatus* Decne. (elephant foot yam), *Amorphophallus paeoniifolius* (Dennst.) Nicolson (Stink lily) and *Momordica charantia* L. (bitter gourd) were prepared and subjected to evaluation of antifungal and antiaflatoxigenic activities at 10% concentration following the procedure of Shukla *et al.* (2008). After 10 days incubation, the culture was filtered and the obtained mycelial mat was used for estimation of mycelial dry weight (MDW) losses and the filtrate was used for quantification of AFB₁ using spectrophotometric method as mentioned above.

Statistical analysis

Values were expressed as Mean ± standard error. Analysis of variance (ANOVA) was performed, and the differences between values were tested for significance by Tukey's multiple comparison tests employing the SPSS 20 (IBM, USA) programme. Differences at $p \leq 0.05$ were considered as statistically significant.

Results and Discussion

Cereals, grains, pulses and oilseeds are more susceptible to fungal infestations during storage, which are a good substrate for mycotoxins production. In the present investigation, a total of 18 different seed samples comprising of 6 cereal samples, 10 pulse samples and 2 oil seed samples collected from different agro-climatic regions of Karnataka (India), were subjected to mycological, ergosterol and AFB₁ analysis and the obtained results are presented in Table 1 and Graph 1. The results revealed that 15 diverse fungal species belonging to 11 genera were observed. Among the fungi isolated, species of *Aspergillus* (RP 7.4 to 100.0%) followed by *Penicillium* (RP 100.0%), *Cladosporium* (RP 62.2%), *Fusarium* (RP 53.1%)

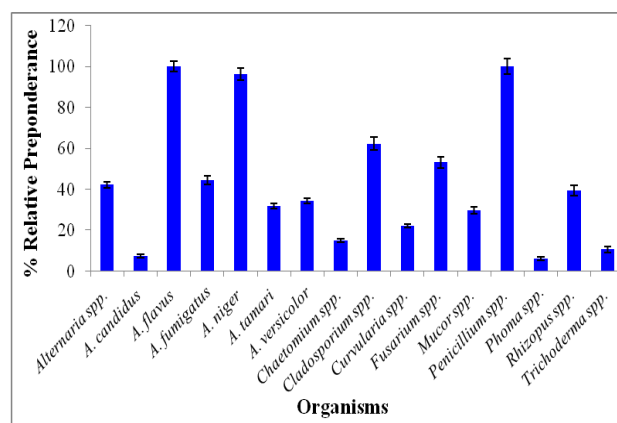
Table 1. AFB₁ and ergosterol contaminations, and percent fungal incidences the collected 18 different food samples

Seed samples	AFB ₁ content (µg/kg)	Ergosterol (µg/g)	% Fungal incidence (PI)
Chick pea variety 1	62.2±4.5	1.3±0.57	39.3±1.5
Chick pea variety 2	190.0±10.4	2.5±0.68	70.2±3.2
Chick pea variety 3	250.0±16.3	3.7±0.44	77.6±4.2
Cow pea variety 1	0.0±0.0	0.9±0.72	28.5±1.4
Cow pea variety 2	0.0±0.0	1.3±0.98	36.7±2.5
Green gram variety 1	0.0±0.0	0.8±0.36	26.3±1.8
Green gram variety 2	0.0±0.0	1.4±0.44	37.7±3.4
Ground nut	510.7±22.3	6.1±1.15	89.4±4.6
Indian bean	0.0±0.0	0.6±0.24	11.6±2.3
Maize variety 1	200.5±12.8	4.3±0.57	80.0±3.4
Maize variety 2	714.0±20.3	8.2±0.98	100.0±0.0
Maize variety 3	390.3±18.3	7.9±0.86	95.1±3.4
Sorghum variety 1	814.3±22.3	9.7±0.76	100.0±0.0
Sorghum variety 2	582.4±24.1	4.5±0.57	88.2±4.1
Sorghum variety 3	1250.2±28.3	10.6±1.21	100.0±0.0
Soyabean variety 1	96.4±4.2	3.4±0.68	46.2±3.7
Soyabean variety 2	50.0±2.4	4.4±0.57	54.7±2.5
Sunflower	130.6±4.4	3.6±0.44	69.5±3.8

Data given are mean of four replicates ±SE; analysis of variance (ANOVA) $p < 0.001$

and *Alternaria* (RP 42.2%) were found to be more dominant fungi, whereas least %RP values were observed in species of *Phoma* (RP 6.0%). Among the seed samples analyzed, the highest mould incidence was observed in sorghum samples followed by maize, whereas the least incidence was observed in Indian bean. Among the *Aspergillus* species, *A. flavus* recorded highest relative preponderance (RP 100.0%) followed by *A. niger* (RP 96.2%), whereas least relative preponderance was observed in *A. candidus* (RP 7.4%). *A. flavus*, a predominant producer of aflatoxins, was observed in all seed samples. The AFB₁ and ergosterol contents in the collected 18 seed samples were analyzed and the results revealed that the highest AFB₁ (582.4 to 1250 µg/kg) and ergosterol (4.5 to 10.6 µg/g) contaminations, and mould incidence (PI 88.2-100%) were observed in sorghum samples followed by maize samples AFB₁ (200.5 to 714 µg/kg) and ergosterol (4.3 to 8.2 µg/g) contaminations and mould incidence (PI 80-100%). The obtained results clearly confirm that ergosterol content correlated with percent mould incidence and AFB₁ correlated with PI of *A. flavus*. Such data have an immense value for assessing the possible health hazards upon consumption of these contaminated food grains.

The amount of AFB₁ biosynthesis was estimated qualitatively by comparison with standard AFB₁ and quantitatively by spectrophotometric method. Among the aflatoxigenic isolates, the highest AFB₁ production was observed in isolate-2 (905.0 µg/kg)



Graph 1. Percent relative preponderances of seed-borne fungi in food samples

Data given are mean of four replicates ±SE; analysis of variance (ANOVA) $p < 0.001$

followed by isolate-9 (730.6 µg/kg) and isolate-13 (508.6 µg/kg), whereas least toxin production was observed in isolate-5 (220.8 µg/kg). The results of PCR amplification followed by sequence analysis by BLAST (Basic Local Alignment Search Tool, NCBI) revealed that, among the 14 isolates of *A. flavus*, the aflatoxin gene specific DNA amplification was observed only for four isolates (isolate-2, 5, 9 and 13) with expected size of 629 bp. The isolate-2, which produced highest amount of AFB₁, showed 99% similarity with an authenticated aflatoxigenic *A. flavus* isolate (emb|FN398161.1|) in BLAST analysis.

The inhibitory activities of aqueous extracts of some vegetables on growth of aflatoxigenic strain

Table 2. Antifungal and antiaflatoxigenic efficacies of aqueous extracts of some selected vegetables against *A. flavus*

Test extracts	Mycelial dry weight (mg)	AFB ₁ Content (µg/l)
Control	315.2±0.53	905.0±1.34
<i>A. campanulatus</i>	96.2±0.03	139.0±0.19
<i>A. paeoniifolius</i>	296.4±0.57	776.5±1.11
<i>I. batatas</i>	277.6±0.47	646.0±0.80
<i>M. charantia</i>	215.1±0.50	475.5±0.58
<i>R. raphanistrum</i>	298.2±0.40	902.0±0.85
<i>S. pinnata</i>	120.2±0.36	311.2±0.57

Data given are mean of four replicates ±SE; analysis of variance (ANOVA) $p < 0.001$

of *A. flavus* (isolate-2) and AFB₁ biosynthesis were evaluated and presented in Table 2. The results revealed that the extracts of *A. campanulatus*, *S. pinnata* and *M. charantia* showed significant antifungal as well as antiaflatoxigenic properties. The MDW and AFB₁ concentration in control set was 315.2 mg and 905.0 µg/l, whereas in *A. campanulatus*, *S. pinnata* and *M. charantia* were ranged from 96.2 to 215.1 mg and 139.0 to 475.5 µg/l, respectively. The perusal of literature revealed that, only a few reports are available on the antifungal activities of *R. raphanistrum* subsp. *sativus*, *I. batatas*, *S. pinnata*, *A. campanulatus*, *A. paeoniifolius* and *M. charantia* against *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Colletotrichum lindemuthianum*, *Colletotrichum dematium*, *Fusarium oxysporum*, *Rhizopus arryzae* and *Ton mentagrophyte* (Khan et al., 2008; Keawsa-ard and Liawruangrath, 2009; Kalita et al., 2012; Masangwa et al., 2013) and no reports are available on antiaflatoxigenic activities of these plants. To the best of our knowledge this is the first kind of report on antiaflatoxigenic activities of these plants.

Conclusions

The present investigation showed that aqueous extract of *A. campanulatus*, *S. pinnata* and *M. charantia* significantly inhibited the growth of aflatoxigenic *A. flavus* and its toxin compared to control. Hence, the present findings indicate the possible use of extracts of *A. campanulatus*, *S. pinnata* and *M. charantia* as alternative agents for developing plant based preservatives against post-harvest fungal infestation of food commodities and aflatoxin contaminations.

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