International Food Research Journal 24(1): 422-427 (February 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Studies on seed-borne mycoflora and aflatoxin B_1 contaminations in food based seed samples: Molecular detection of mycotoxigenic *Aspergillus flavus* and their management

*Mohana, D. C., Thippeswamy, S., Abhishek, R. U., Shobha, B. and Mamatha, M. G.

Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi, Bangalore-560 056, India

Article history

Received: 9 December 2015
Received in revised form: 16 March 2016
Accepted: 23 March 2016

Keywords

Aspergillus flavus
Food grains
Aflatoxin B_1
Seed-borne mycoflora
Vegetable extracts

Abstract

In the present study the mould incidence, ergosterol and aflatoxin B_1 (AFB_1) 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 AFB_1 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 AFB_1 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 AFB_1 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 AFB_1, 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 AFB_1 contents were correlated with the percent mould incidences.

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 B_1 (AFB_1) 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 phyto-pathogenic 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 preservatives/fungicides for eco-friendly sustainable management of moulds and mycotoxins in food- and

*Corresponding author.
Email: *mohanadc@gmail.com*
Tel: 080 22961461 (Office); Cell: +91 9972065064

feed-stuffs. Hence, the present investigation evaluated the fungal incidence, ergosterol, AFB_1 content in some food based seed samples, and antimycotic and antiaflatoxigenic activities of some edible vegetable extracts.

Materials and Methods

Chemicals and culture media

Sabouraud dextrose agar/broth (SDA/SDB), sucrose- $MgSO_4$ - KNO_3 - yeast extract - broth (SMKYB), dimethyl sulfoxide (DMSO) and all analytical grade solvents were purchased from Hi-Media, Mumbai (India). The standard AFB_1 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

Extraction and quantification of AFB_1 from culture filtrate of A. flavus *and seed-samples*

AFB_1 was extracted from the culture filtrate of *A. flavus* following the procedure of Shukla *et al*. (2008). In brief, 100 µl of spore suspension (10^4 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 AFB_1 extraction by adding equal volume of $CHCl_3$, 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. AFB_1 was estimated qualitatively by visual comparison with standard AFB_1 on TLC. For quantitative estimation, the fluorescent spots on TLC plates were scrapped out, dissolved in 5 ml of cold CH_3OH, 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 AFB_1 content was calculated using following formula

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

Where, D= absorbance; M= molecular weight of AFB_1 (312); E= molar extinction coefficient of AFB_1 (21,800) and L= path length (1 cm cell).

AFB_1 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 AFB_1 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 $MgCl_2$, 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* AFB_1 *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 AFB_1 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 AFB_1 analysis and the obtained results are presented in Table 1 and Figure 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%) 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*

Table 1. AFB_1 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) $p < 0.001$

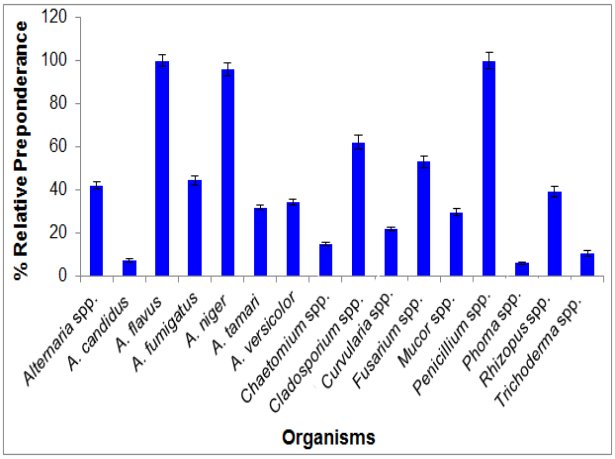

Figure 1. Percent relative preponderances of seed-borne fungi in food samples

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

(RP 7.4%). *A. flavus*, a predominant producer of aflatoxins, was observed in all seed samples. The AFB_1 and ergosterol contents in the collected 18 seed samples were analyzed and the results revealed that the highest AFB_1 (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 AFB_1 (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 AFB_1 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 AFB_1 biosynthesis was estimated qualitatively by comparison with standard AFB_1 and quantitatively by spectrophotometric method. Among the aflatoxigenic isolates, the highest AFB_1 production was observed in isolate-2 (905.0 µg/kg) 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 AFB_1, 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 of *A. flavus* (isolate-2) and AFB_1 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 AFB_1 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*

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
A. campanulatus	96.2±0.03	139.0±0.19
A. paeonifolius	296.4±0.57	776.5±1.11
I. batatas	277.6±0.47	646.0±0.80
M. charantia	215.1±0.50	475.5±0.58
R. raphanistrum	298.2±0.40	902.0±0.85
S. pinnata	120.2±0.36	311.2±0.57

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

against *Aspergillus flavus, Aspergillus niger, Candida albicans, Colletotrichum lindemuthianum, Colletotrichum dematium, Fusarium oxysporum, Rhizopus aryzae* 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.

Acknowledgement

The authors are grateful to the DST-SERB, Government of India (Grant No. SB/EMEQ-044/2013), New Delhi, India, for the financial support.

References

Bankole, S.A., Schollenberger, M. and Drochner, W. 2010. Survey of ergosterol, zearalenone and trichothecene contamination in maize from Nigeria. Journal of Food Composition and Analysis 23: 837-842.

Booth, C. 1977. The Genus Fusarium: Laboratory guide to the identification of the major species. Commonwealth Mycological Institute, Ferry Lane. Kew, Surrey, England, 6-133.

Deng, Y., Yu, Y., Luo, H., Zhang, M., Qin, X. and Li, L. 2011. Antimicrobial activity of extract and two alkaloids from traditional Chinese medicinal plant *Stephania dielsiana*. Food Chemistry 124: 1556-1560.

Galvano, F., Piva A., Ritieni A. and Galvano G. 2001. Dietary strategies to counteract the effect of mycotoxins: A review. Journal of Food Protection 64(1): 120-131.

IARC. 2002. Aflatoxins: In traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC monographs on the evaluation of carcinogenic risks to humans, Lyon, France. International Agency for Research on Cancer 82: 171-366.

ISTA. 1996. International rules for seed testing. Seed Science and Technology 21(1): 25-30.

Janardhana, G.R., Raveesha, K.A. and Shetty, H.S. 1998. Modified atmosphere storage to prevent mould-induced nutritional loss in maize. Journal of Science Food and Agriculture 76: 573-78.

Janardhana, G.R., Raveesha, K.A., Shetty, H.S. 1999. Mycotoxin contamination of maize grains grown in Karnataka (India). Food and Chemical Toxicology 37: 863-868.

Kalita, D., Saha, J. and Bharthi, S. 2012. Ethno botanical, antibacterial and antifungal study of three medicinal plants of Morigaon district of Assam, India. Asian Journal of Pharmaceutical and Biological Research 2(4): 234-237.

Keawsaard, S. and Liawruangrath, B. 2009. Antimicrobial Activity of *Spondias pinnata* Kurz. Pure and Applied Chemistry International Conference 428-429.

Khan, A., Rahman, M. and Islam, M.S. 2008. Antibacterial, antifungal and cytotoxic activities of amblyone isolated from *Amorphophallus campanulatus*. Indian Journal of Pharmacology 40(1): 41-44.

Marin, S., Homedes, V., Sanchis, V., Ramos, A.J. and Magan, N. 1999. Impact of *Fusarium moniliforme* and *F. proliferatum* colonization of maize on calorific losses and furnonisin production under different environmental conditions. Journal of Stored Product Research 35: l5-26.

Masangwa, J.I.G., Aveling, T.A.S. and Kritzinger, Q. 2013. Screening of plant extracts for antifungal activities against *Colletotrichum* species of common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* (L.) Walp). Journal of Agricultural Science 151: 482–491.

Mdee, L.K., Masoko, P. and Eloff, J.N. 2009. The activity of extracts of seven common invasive plant species on fungal phytopathogens. South African Journal of Botany 75: 375-379.

Miller, J.D. 1995. Fungi and mycotoxins in grain implications for stored product research. Journal of Stored Product Research 31(1): 1-16.

Nagamani, A., Kunwar, I.K. and Manoharachary, C. 2006.

Handbook of soil fungi (1st ed.). I. K International Pvt. Ltd., New Delhi.

Passone, M.A., Resnik, S. and Etcheverry, M.G. 2008. The potential of food grade antioxidants in the control of *Aspergillus* section *flavi*, interrelated mycoflora and aflatoxin B1 accumulation on peanut grains. Food Control 19: 364-371.

Rahimi, P., Sharifnabi, B. and Bahar, M. 2008. Detection of aflatoxin in *Aspergillus* species isolated from Pistachio in Iran. Journal of Phytopathology 156: 15-20.

Reddy, K.R.N., Reddy, C.S. and Muralidharan, K. 2009. Detection of *Aspergillus* spp. and aflatoxin B_1 in rice in India. Food Microbiology 26: 27-31.

Rocha, O., Ansari, K. and Doohan, F.M. 2005. Effect of trichothecene mycotoxins on eukaryotic cells: A review. Food Additives and Contaminants 22: 369-318.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Gel electrophoresis of DNA. In: Sambrook, J., Fritsch, E.F. and Maniatis, T. (Eds.) Molecular Cloning: a Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, chapter 6.

Shukla, R., Kumar, A., Prasad, C.S., Srivastava, B. and Dubey, N.K. 2008. Antimycotic and antiaflatoxigenic potency of *Adenocalymma alliaceum* Miers. on fungi causing biodeterioration of food commodities and raw herbal drugs. International Biodeterioration and Biodegradation 62: 348–351.

Shukla, R., Kumar, A., Singh. P. and Dubey, N.S. 2009. Efficacy of Lippa alba (Mill.) N. E. Brown essential oil and its monoterpene aldehyde constituents against fungi isolated from some edible legume seeds and aflatoxin B_1 production. International Journal of Food Microbiology 135: 165-170.

Singh, K., Frisvad, J.C., Thrane, U. and Mathur, S.B. 1991. An illustrated manual on identification of some seed-borne *Aspergilli, Fusaria, Penicillia* and their mycotoxins (1st ed.). Danish Government Institute of Seed Pathology for Developing Countries, Hellerup, Denmark, (Chapter 4).

Watanabe, T. 2002. Pictorial Atlas of soil and seed fungi: morphologies of cultured fungi and key to species. 2ndedn. CRC Press LLC, 2000 N. W. Corporate Blvd., Boca Raton, Florida 33431, 21-183.

Zhang, J.D., Han, L., Yan, S. and Liu, C.M. 2014. The non-metabolizable glucose analog D-glucal inhibits aflatoxin biosynthesis and promotes kojic acid production in Aspergillus flavus. BMC Microbiology 14(95): 1-9.