Food Safety and Risk

RESEARCH

Open Access

Molecular and chemical based characterization of aflatoxin producing aspergillus species obtained from stored grain samples

Kanchan Gopal Choudhary[1], Choudhary Sharfuddin[1] and Arun Kumar[2*]

Abstract

Background *Aspergillus* section *Flavi* is a group of widely distributed multicellular fungi associated with contamination of stored grain. Some species of it secretes aflatoxin contaminants which is a carcinogenic in nature. Due to this it poses a significant global challenge in the health, agriculture and food processing industries. The aim of present study is quantification by using a chemical based detector and molecular identification of aflatoxin producing fungi from stored grain samples of flood prone area of Bihar, India. The samples ($n=61$) were investigated for the presence of aflatoxigenic fungi, which was further confirmed by HPLC-FLD. The aflatoxigenic isolates identified through amplification of the internal transcribed spacer (ITS) region.

Findings A total of $n=119$ fungal isolates from 5 distinct genera were isolated in PDA and CYA. The *Aspergillus* species were the dominant with occurrence of *Aspergillus* section *Flavi* (58.95%), *Aspergillus* section *Nigri* (35.79%), *Aspergillus* section *Fumigati* (3.16%), *Aspergillus* section *Terrei* (1.05%) and *Aspergillus* section *Circumdati* (1.05%). The positive isolates were quantified by HPLC-FLD and highest amount of aflatoxin was determined in W7 isolate of wheat as 6907.47 µg/kg of AFB_1 and 68.88 µg/kg of AFB_2. Further, ITS sequencing of five positive isolates for aflatoxin showed that a significant alignment of 99–100% with the previously isolated fungi *A. aflatoxiformans* DTO 228-G2 and *A. austwickii* DTO 228-F7 which was confirmed by the BLAST and all the sequences were deposited in the NCBI database.

Conclusions To our knowledge, this is the initial report concerning the presence of aflatoxigenic species of *A.* section *Flavi*. Hence, to conclude contamination of food grain samples with aflatoxin producing fungi is alarming in these flood prone areas.

Keywords Aflatoxin, *Aspergillus* section *Flavi*, Grain samples, HPLC-FLD, Mycotoxin, Mycoflora

Introduction

Moulds are widely distributed multicellular fungi associated with food spoilage (Ayofemi 2020). *Aspergillus* is a group of moulds widespread under favorable environment condition which has the potential of contaminating the stored grains (Adejumo and Adejoro 2014; Chen et al. 2023). Their growth on these stored grains can cause off odour, off flavour, discolorations, decrease in nutritional value and loss in weight. All these changes take place before the gross development of these fungi

*Correspondence:
Arun Kumar
drarunk31@gmail.com
[1] Plant Pathology and Microbiology Lab, Department of Botany, Patna University, Patna, Bihar 800005, India
[2] Mahavir Cancer Sansthan & Research Centre, Patna, Bihar 801505, India

(Ayofemi 2020; Girish and Goyal 1986). Mycotoxins responsible for degradation of grains are largely produced by *Aspergillus* spp. of fungi group (Girma et al. 2016; Singh et al. 1983). Some species of *Aspergillus* can produces the aflatoxins, leading to grain contamination at various stages viz. harvesting, processing and storage. Mycotoxins are the low molecular weight secondary metabolite produced by the filamentous fungi (Bennett et al. 2003). Aflatoxins are difuranoumarin derivatives mainly secreted by *Aspergillus* section *Flavi*. These are stable at UHT (Ultra High Temperature) 135–220℃ and decomposes at 237–306℃ (Kumar et al. 2017), therefore boiling or pressure cooking does not assure the complete removal of aflatoxins. They are approximately of 18 types (Okoth et al. 2018), out of which aflatoxin B_1 is the most toxic and class-I carcinogenic agent classified by International Agency for Research on Cancer (IARC 1993; 2016). After consumption the different groups of aflatoxins such as methylene and carbonyl called as polyketides are modified upon consumption and transferred to different body parts (Pandey et al. 2019). The long term aflatoxins exposure leads to aflatoxicosis, a fatal acute poisoning which predominantly causes liver damage (WHO, 2018). Research indicates that children are the most exposed to all food types contaminated with aflatoxins, followed by adolescents, with adults being the group with the lowest exposure (Ezekiel et al. 2021). Current evidence indicates that infants and young children are more likely to experience the harmful effects of mycotoxins because of their underdeveloped metabolic systems, higher intake relative to body weight, faster metabolism, and reduced detoxification capacity compared to adults (Hulin et al. 2014; Milićević et al. 2021; Alameri et al. 2023). Also, it is one of the few mycotoxins that have been developed for use as a biological weapon (Klich 2007). Aflatoxins associated with stored grains are problem of tropical and subtropical region worldwide.

India is predominantly an agrarian country with nearly three fourths of the people depending on agricultural or rural economy. The high temperature, humidity, monsoon rain and flash flood causes fungal proliferation and mycotoxin production (GSI – Eastern Region 2014; ICAR 2018). Flood poses a serious threat to food security and is a significant challenge in agriculture. 12.7% of the nation's flood prone land is in Bihar (ICAR 2017; NDMA 2008). Considering the above fact the aflatoxin production is temperature dependent, pre-harvest (plant pathogenic) and storage associated (saprophytic fungi) problem (Duricin et al. 2016; FAO 2017).

Thus, in this study, we sought to achieve an understanding of the different drivers of aflatoxigenic mycoflora contamination in stored grain samples of small scale farmers' food system in Bihar. Also, to determine the presence of aflatoxins in these aflatoxigenic mycoflora by an optimized HPLC-FLD based method coupled with Kobra cell and to construct the maximum likelihood for aflatoxin producing strain based on ITS sequencing. This result will provide the current scenario of aflatoxigenic mycoflora from grain samples in the flood prone areas of Bihar, India.

Materials and methods

Study area

A total of 61 stored grain samples were collected randomly depending on the availability from flood prone areas of Sitamarhi and Madhubani districts of Bihar, India. These samples were collected from small scale farmers' storage sites and kept in a sterile polythene bag (50–150 g/sample) under the storage condition of 4℃. Information regarding (supplementry information S3) the post-handling of the grain storage practices was collected in a questionnaire Proforma. Samples were collected from December 2020 to April 2021.

Chemicals and reagents

Czapek's Yeast Extract Agar (CYA) component and Potato Dextrose Agar component were bought from Sigma-Aldrich, while all other chemicals and solvents system for TLC were bought of HPLC grade. The standards of aflatoxins B_1, B_2, G_1 and G_2 were purchased (R-Biopharm Neugen Pvt. Ltd., Hyderabad, India) in a mixture (concentrations are as follow, AFB_1−1 µg/mL, AFB_2- 0.5 µg/mL, AFG_1- 1 µg/mL and AFG_2- 0.5 µg/mL) of 2 mL. The standard was stored at 4℃ away from light before use.

Mycoflora isolation and mycological identification

The grain samples surface were disinfected before plating. Approximately, 15–30 grains were plated in triplicate onto Potato Dextrose Agar (PDA) medium (Pitt and Hocking 2009; 2022). After incubation of the samples, fungal infections were noted and the incidence of fungal species for each sample was determined in percentage. The morphological characteristic of the strains were studied at 10x, 40×and 100×respectively (Kong et al. 2014). The potential mycotoxigenic fungal colonies were sub cultured on Czapek's Yeast Extract Agar (CYA) medium.

$$\text{Percent incidence} = \left(\text{total number of each isolate in a sample}/\text{total number of all identified isolates in a sample}\right)\times 100$$

Culture condition for extraction of aflatoxins

A total of fifty-six pure culture samples of *Aspergillus* section *Flavi* along with positive strain MTCC2798 were grown on CYA medium. Then with the help of inoculating loop, loopfull of conidia was inserted into the 50 mL SKMY broth (Davis et al. 1966; Ranjan et al. 1991). These flasks were incubated in room temperature for seven days in still condition. Three liquid cultures were produced for each strain tested. In the biosafety cabinet 50 mL fully inoculated cultures were filtered through Whatman filter paper No.1 (Whatman International Ltd., Maidstone, England). Then the equal volume of chloroform was added to the filtrate and then contents were shaken for 30 min. The chloroform fraction was passed through separating funnel followed by sodium sulphate column and collected in the glass tube. Then the chloroform fraction was concentrated at 40℃ (Das et al. 2012; Shotwell et al. 1966; Thomas et al. 1975). These crude extract samples were stored at 4℃ in the dark in 1 mL chloroform for 7–10 days till TLC analysis.

Preliminary detection of aflatoxins

For TLC Silica gel 60 F_{254} plate 20 cm X 20 cm (Merck, Dermstadt, Germany) was taken. About 100 µL of sample's crude extract of all the strains were loaded onto the plate along with positive control strain and mixed standard of B_1, B_2, G_1 & G_2. The plates were developed in mobile phase consisting of toluene, isoamylalcohol and methanol (90:32:2 v/v/v) (Fried 1996; Ranjan et al. 1991; Choudhary et al. 2020). After developing, the plates were visualized under long (366) and short (254) UV wavelength. The bands of standard and positive sample crude extract were scrapped off and mixed with 1 mL methanol and this methanolic extract was subjected to UV Spectrophotometer, at 360 nm for AFB_1 and 362 nm AFB_2, AFG_1 and AFG_2 (with $\mathcal{E}=21800$ for AFB_1, $\mathcal{E}=24000$ for AFB_2, $\mathcal{E}=17700$ for AFG_1 and $\mathcal{E}=19300$ for AFG_2).

Quantification by HPLC with Kobra-cell derivatization and fluorescence detector

Aflatoxins extraction and purification

All the fungal isolates which were positive for aflatoxin production were selected for further aflatoxin quantification by HPLC. All the crude chloroform extracts of fungal strains along with the positive strain MTCC 2798 were kept at 0℃ till further processing and this analysis was performed at TUV India food lab, Pune. The AFLARHONE®WIDE Column (IAC, R-Biopharm Rhone Ltd; Glasgow, UK) were used for sample purification and extraction of aflatoxins. Briefly, 1 mL PBS, pH-7.2 was combined with pre-weighed crude extract in a vial and it was passed through the column followed by washing with water (1 mL) five times. Finally, all the waste was collected in SPE (Solid Phase Extraction) chamber. Then column was dried for 10–20 s and aflatoxins were eluted with methanol 1 mL and water 1 mL by back flushing. The test-tube was vortexed and passed through 0.45 µm syringe filter and collected in an autosampler glass vial into HPLC system.

Detection of AFB_1, AFB_2, AFG_1 and AFG_2 by RP-HPLC-FLD-Kobra-cell

A reversed phased (RP)—HPLC protocol was used for determination of aflatoxins in the sample. The method was previously reported by Zhu et al. 2013. A 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump and vacuum degasser (model G1311B), an autosampler (model G1329B, 100 µL total loop volume) and a FLD detector (model G1321B) was used. The separation column connected to guard cartridge was a Zorabax Eclipse XDB C18 (5 µm, 150 mm, 4.6 I.D.) column and the temperature of the column was maintained at 40℃ in a thermostable column compartment (model G1316A). Post-column derivatization was done with a Kobra cell® (R-Biopharm Rhone Ltd., Glasgow, UK). The mobile phase was a mixture of methanol and KBr (119 mg of KBr and 350 µL of 4 M nitric acid were dissolved in 1 L of D/W) in the ratio of water (with KBr): methanol (60:40) (AOAC 2006). The mobile phase was freshly prepared on the day of analysis, as recommended for the post column derivatization with the Kobra cell®. Level of detection (LOD) of AFB_1, AFB_2, AFG_1 and AFG_2 were established by practical experimentation as being 0.3 µg/kg for all four aflatoxins. Limit of quantification (LOQ) is found to be 0.5 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2 (Supplementary information S4). The filtered samples were injected into the system through autosampler and aflatoxins signals were detected with FLD at excitation of 362 nm and emission at 455 nm. The data were acquired and analyzed with the Agilent data handling Chemstation 3 (Agilent Technologies).

Recovery study

To ensure the accuracy of the test aflatoxins free crude extract of fungal strain was spiked with AFB_1, AFB_2, AFG_1 and AFG_2 at level of 5, 7.5 and 10 µg/kg. The spiked samples were examined followed by the estimation of both the recovery and standard deviation.

Molecular characterisation of *Aspergillus* section *Flavi* strain by ITS sequencing

The positive aflatoxin producing strains which were quantified using a HPLC-FLD was subjected to molecular

identification by ITS sequencing at NCIM (National Collection of Industrial Microorganism), Pune. DNA was extracted from the colonies of aflatoxin producers. The spin column kit (Himedia, India) was used to extract the DNA. Exonuclease I-Shrimp Alkaline Phosphatase (ExoSAP) was used to purify the amplified fungal ITS r-RNA gene (600 bp) (White et al. 1990) after it had been amplified using PCR in a thermal cycler (Darby et al. 2005). ABI3500xl genetic analyzer (Life Technologies, USA) used the Sanger technique to sequence purified amplicons. In order to find regions of local similarity between sequences, Basic Local Alignment Search Tool (BLAST) is used to further analyze sequencing files (.ab1) that have been edited using CHROMASLITE (version 1.5) and the closest culture sequence were obtained from the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990).The statistical significance of matches by comparing the nucleotide or protein sequences to sequence database were determined (Gertz 2005). The BLAST algorithms were used to identify members of gene families and infer functional and evolutionary links between sequences (Altschul et al. 1990). Pairwise alignment was used to determine how similar the query sequence was to the sequences found (States et al. 1991). Therefore, each isolate was reported with the hits observed in the said database. Further, multiple sequence alignment and phylogenetic analysis was done for accurate species prediction and Evolutionary analyses were conducted in MEGA6 (Felsenstein 1985; Karlin et al. 1990; Myers et al. 1988; Tamura et al. 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou et al. 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980).

Statistical analysis

The statistical analysis of data was performed by one way ANOVA for grain samples, using Graph Pad Prism (version 8.0). The p-value < 0.05 was considered significant. For rest of the analysis descriptive statistical analysis were done.

Results and discussion

Household survey analyses

A total of 61 samples were collected from small scale farmers in this study. Most of them did not have awareness regarding the toxigenic mouslds and their severe consequences. The Fig. 1B represents 83.33% of farmers disposed the gross mouldy grains and consumed the remaining, unaware of microscopic mycoflora associated with stored grain samples. Remaining 16.66% of farmers used the mouldy grains for animal feeding. The study population included is not dependent on chemical additive during storage of grain samples and mostly dependent only on sun-dry method. The Fig. 1A represents that 8.19% household samples used natural additives such as dried neem leaves and cloves for grain safety along with washing and sun-dry during the storage whereas 91.80% of household samples used traditional methods of washing and sun-dry only. The storage duration of the studied sample is presented in Fig. 1C it shows that 57.38% of samples (includes cereals, pulses and oilseeds) selected were stored for 6 months, while about 40.98% of samples were stored between 9 months to 1 year and 1.63% of the sample were found to be stored for 2 years. Since, farmers were dependent on traditional methods of storage and for long storage there were no proper measures taken leading to contamination of grains with multiple mycoflora, increasing the chances of contamination of grains with potential mycotoxigenic mycoflora. The winter rain and hail storm lead to moistening of grains vulnerable to mycological contamination and these conditions were also observed in previous studies by Kumar et al. 2017 in different regions of India. In this study, Fig. 1D shows that, 11.47% of the total stored samples either experience winter rain or the hail storm during the drying of the grains in the fields, whereas, 88.52% of the samples dried properly by traditional methods and were stored. The Fig. 1E represents that, 8.19% of the total grain samples were stored in the sack such as the rice, wheat and groundnut. The 42.62% of the total grain samples such as pulses, cereals and oilseeds were stored in tin containers. The 11.47% of grain samples mainly oil seeds were stored in plastic containers. About, 37.70% of grain samples mainly cereals were stored in *kothi* (mud warehouse). The type of storage container had no much impact if the moisture content of the grain is properly removed. The above results indicated that the pattern of farming in these flood prone districts is mostly by the traditional means, due to lack of technical and scientific knowledge. The flood during the pre-winter period and moreover, the water logging in the fields play an important role in increasing the moisture content of grains leading to mycological infection and due to low socioeconomic status farmers depends on this contaminated food commodities. This study identified potential toxigenic fungal contamination of grain samples and possible risk factors associated with it. It did not find any differences in terms of mycotoxigenic fungi present on the samples based on different processing or storage system of food grains. Therefore, it was assumed that, during post-harvest management of food grains, moisture content and favorable temperature are more substantial contributors to a mycoflora contamination which was also previously determined by Shashidharan et al. 1992. Also, this may

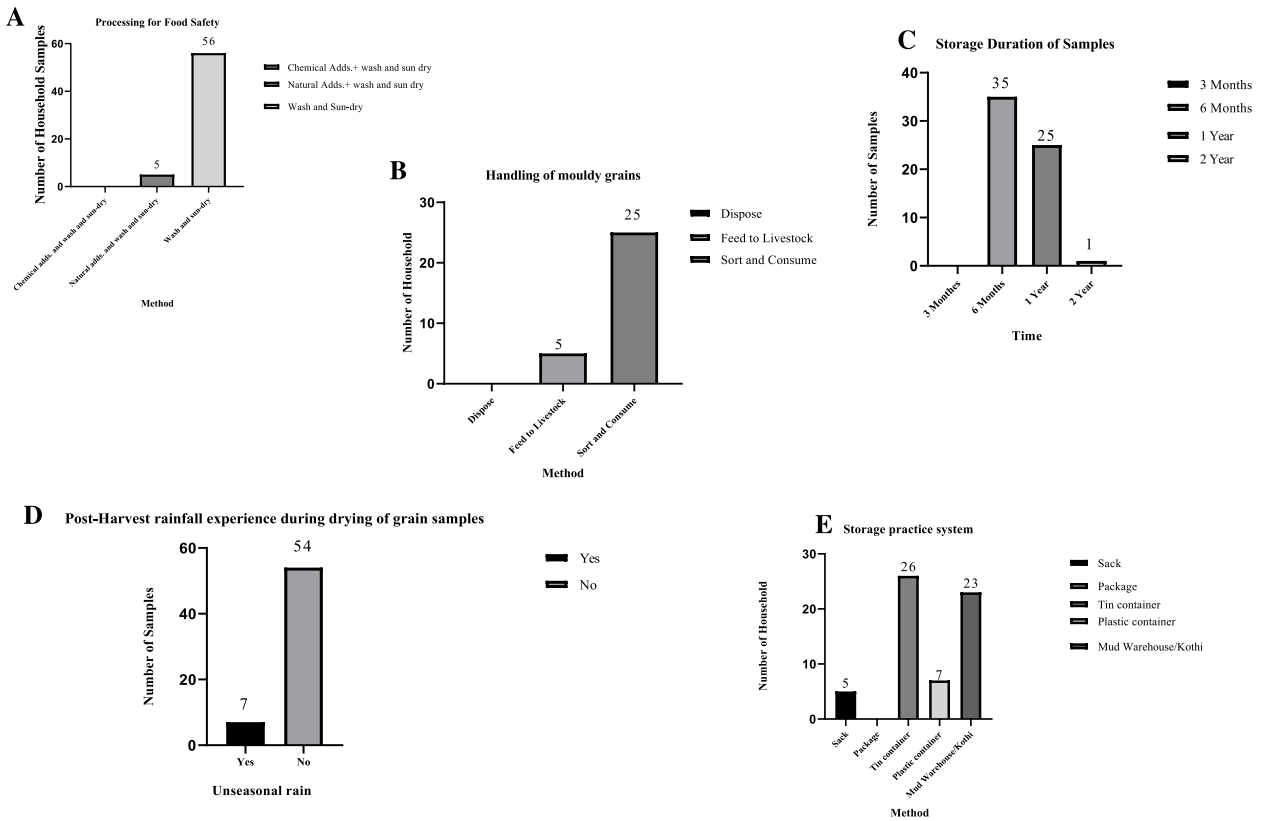


Fig. 1 Summary of (**A**) house-hold crop protection practice (**B**) house-hold handling of mouldy food grain samples (**C**) storage duration of different samples (**D**) post-harvest unseasonal rain experience of food grain samples (**E**) house-hold level storage practice by small scale farmers

be due to increase in humidity level of flood prone area of sample collection (Kansal et al. 2017; Pitt and Hocking 2022).

Mould isolates of tested grain samples

The stored grain samples were analyzed on the PDA medium followed by CYA medium, after 7–10 days incubation of the samples at room temperature. Mostly, the grain samples became mouldy in seven days to a different extent Fig. 2D and 2E. About 119 distinct genera of fungal isolates represented in Fig. 2A such as, *Aspergillus* (95), *Penicillium* (16), *Alternaria* (7) and *Fusarium* (1) were identified and recorded from the 61 food grain samples of different varieties. *Aspergillus* and *Penicillium* prevalence in the samples were noticeably higher in the 119 fungal isolates. About, 95 isolates belonged to *Aspergillus* spp. and 16 belonged to *Penicillium* spp. with the incidence of 79.83% and 13.44% respectively. The isolates number of *Alternaria* and *Fusarium* were 7 and 1 respectively, representing 5.88% and 0.84% of the total number. Among 95 isolates, 58.95% belonged to *Aspergillus* section *Flavi,* 35.79% belonged to *Aspergillus* section *Nigri*, 3.16% belonged to *Aspergillus* section *Fumigati*, 1.05% belonged to *Aspergillus* section *Circumdati* and 1.05% belonged to *Aspergillus* section *Terrei* Fig. 2B. Out of 61 samples isolates of different groups (cereals, pulses and oil seeds) represented in Fig. 2C cereals, were found to be significantly contaminated ($p < 0.05$) with 91.70%, in comparison with oilseeds (89.63%) and pulses (88.39%). These results indicate that all types of grain samples, vulnerable to contamination by the discovered fungal mycoflora notably those from the genera of *Aspergillus* and *Penicillium.* These findings were found to be similar with Reddy et al. 2009 which also shows contamination of stored grain samples mostly with *Aspergillus* in different state of India. Table 1: represents the infection (in percent) of different grain samples with potential mycotoxigenic mycoflora. All the stored grain samples were infected by *Aspergillus* except the rice (R5) and flaxseed (T4) which did not show contamination by mycotoxigenic mycoflora. With regard to contamination of stored grain samples with mycotoxigenic mycoflora cereal samples (R3) and (R10) of rice, were found

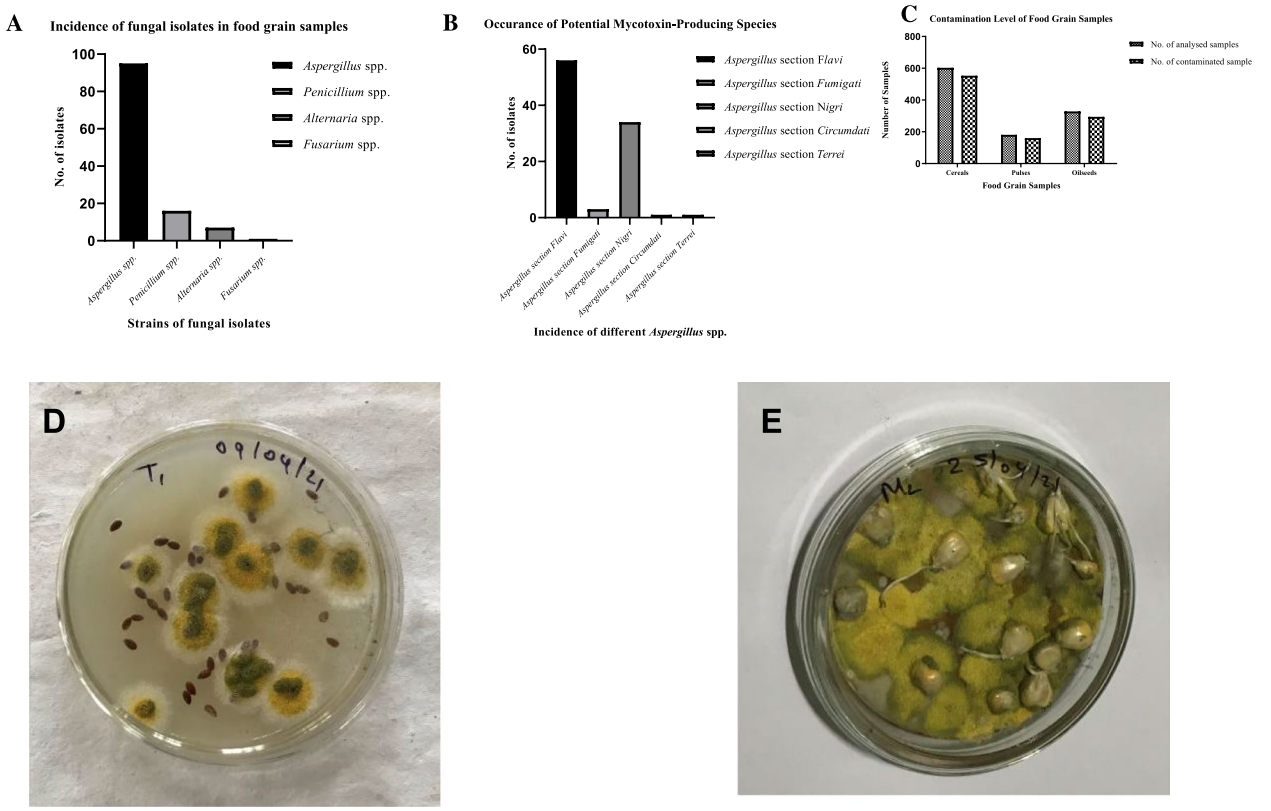


Fig. 2 Incidence of (**A**) fungal isolates in food grain samples (**B**) potential mycotoxin producing species (**C**) contamination level in different food grain samples (**D**) display of mould in tested flaxseed (T1) sample (**E**) display of mould in tested Maize (M2) sample

Table 1 Potential mycotoxicogenic fungal isolates and their incidence in tested food grain samples

Sample	Percentage incidence			
	***Aspergillus* spp.**	***Penicillium* spp.**	***Alternaria* spp.**	***Fusarium* spp.**
Rice (n = 15)	**68.75**	**21.875**	**9.375**	**-**
Wheat (*n* = 13)	**80**	**5**	**15**	**-**
Maize (*n* = 3)	**83.33**	**16.66**	**-**	**-**
Flax seed (*n* = 4)	**77.78**	**11.11**	**-**	**11.11**
Groundnut (*n* = 1)	**100**	**-**	**-**	**-**
Pigeonpeas (*n* = 7)	**91.66**	**8.33**	**-**	**-**
Chickpeas (*n* = 4)	**75**	**25**	**-**	**-**
Red lentils (*n* = 1)	**33.33**	**66.67**	**-**	**-**
Grasspeas (*n* = 2)	**100**	**-**	**-**	**-**
Yellow lentils (*n* = 2)	**75**	**25**	**-**	**-**
Black mustard (*n* = 3)	**100**	**-**	**-**	**-**
Black sesame (*n* = 1)	**100**	**-**	**-**	**-**
Yellow mustard (*n* = 5)	**92.85**	**-**	**7.14**	**-**
Total *n* = 61				

to be highly contaminated with 4 different species and in pulses samples (C3) chickpea, (A3) pigeon pea and (Mo1) yellow lentil were highly contaminated with three different species and in oilseed samples (S1) and (S2) of yellow mustard was found to be highly contaminated with five and three different species respectively.

Table 2 Content of aflatoxins determined by TLC and HPLC-FLD in *Aspergillus* section *Flavi* isolates

Aspergillus section *Flavi* isolates	Positive isolates and their label	Content(µg/kg)of mycotoxins by HPLC-FLD					Content(µg/kg)of crude mycotoxins by TLC + UV-Spectrophotometer				
		AFB_1	AFB_2	AFG_1	AFG_2	Total AF	AFB_1	AFB_2	AFG_1	AFG_2	Total AF
Flax seed *n* = 3	0	-	-	-	-	-	-	-	-	-	-
Rice *n* = 14	R10G	416.13	-	-	-	416.13	1380	-	-	-	1380
Wheat *n* = 12	W7G	6907.47	68.88	-	-	6976.35	4390	1203	-	-	5593
Maize *n* = 3	0	-	-	-	-	-	-	-	-	-	-
Chickpeas *n* = 4	C3G	2219.25	45.79	-	-	2265.04	3680	860	-	-	4540
Pigeonpeas *n* = 5	0	-	-	-	-	-	-	-	-	-	-
Black mustard seeds *n* = 3	Ra2G	252.58	-	-	-	252.58	1317	-	-	-	1317
Grasspeas *n* = 2	0	-	-	-	-	-	-	-	-	-	-
Red lentils *n* = 1	0	-	-	-	-	-	-	-	-	-	-
Yellow lentils *n* = 1	0	-	-	-	-	-	-	-	-	-	-
Black sesame seeds *n* = 1	0	-	-	-	-	-	-	-	-	-	-
Yellow mustard seeds *n* = 6	S4G	335.07	11.22	-	-	346.3	1560	1099	-	-	2659
Groundnut *n* = 1	0	-	-	-	-	-	-	-	-	-	-

The *Aspergillus* and *Penicillium* isolates were found to be prevailing in 61 samples of 119 fungal isolates, which were inoculated in PDA medium. Some of the samples of rice, wheat, flax seed, and yellow mustard were found to be contaminated with other fungal isolates such as *Alternaria* and *Fusarium*. This indicates the different level of contamination of the grains, which was similar with previous findings (Choudhary et al. 2020; Wenndt et al. 2020). This study indicates the high percentage of mycotoxicogenic *Aspergillus* species such as, *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri*, *Aspergillus* section *Circumdati, Aspergillus* section *Fumigati* and *Aspergillus* section *Terrei.* Among all the isolates, the *Aspergillus* section *Flavi* was clearly dominating, which is consistent with previous studies (Choudhary et al. 2020). In summary, these results indicated that due to altered post-harvesting processes and storage, various fungal isolates infect the grain samples depending on their susceptibility towards different mycotoxigenic mycoflora.

Quantification of aflatoxins of fungal isolates

All the fifty-six fungal isolates of *Aspergillus* section *Flavi* of potential aflatoxigenic mycoflora were further analyzed with TLC, for presence of aflatoxin. It is confirmed by blue-fluorescence of aflatoxin-B_1 and aflatoxin-B_2 and green-fluorescence of aflatoxin-G_1 and aflatoxin-G_2 in the UV-trans illuminator with the aflatoxins standard represented in supplementry information S1. The Rf value of standards were matched with the positive samples. The AFB_1 were present in five isolates followed by AFB_2 in three isolates of *Aspergillus* section *Flavi* and this was confirmed by brilliant intensity of fluorescence. The concentration of this toxin was

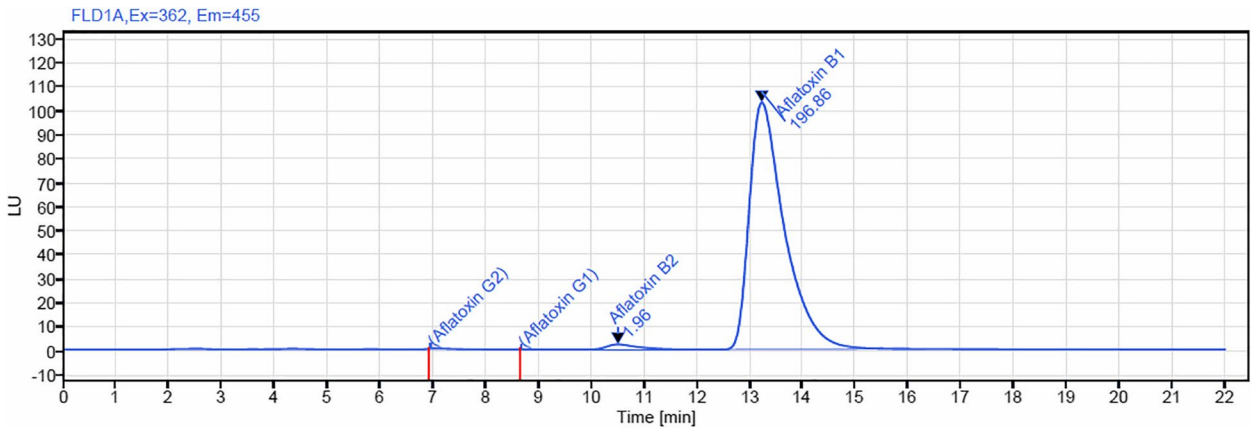
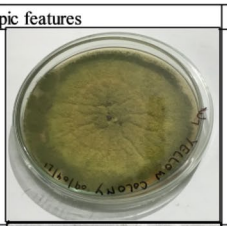
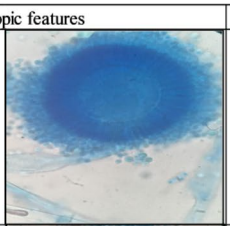
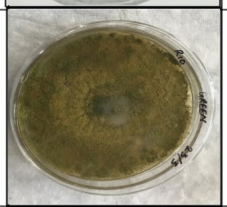
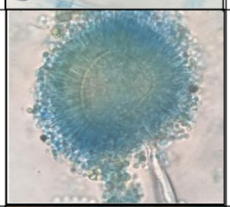
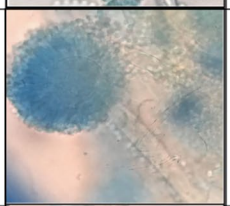
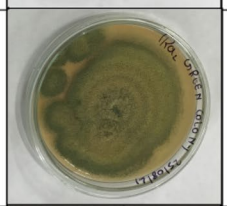
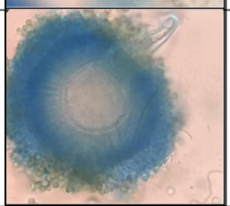
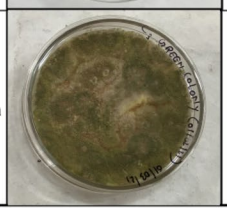
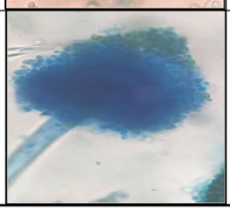


Fig. 3 HPLC chromatogram of W7G isolate of *Aspergillus* section *Flavi* representing aflatoxins (AFS) concentration

determined by UV spectrophotometer at 360 nm for AFB_1 and 362 nm for AFB_2 and it is shown in Table 2. Among the five positive isolates, the intensity of fluorescence was very high in W7G isolate. The chromatogram and elution times of AFs standards in HPLC-FLD is in the sequence of AFG_2(8.83 min), AFG_1(11.02 min), AFB_2(14.01 min) and AFB_1(17.90 min) represented in supplementry information S2. The real concentration of analytes (such as AFs) in samples is corrected using spiked studies (Trucksess et al. 2011). For recovery study, three different concentration of mixed standard solution 5, 7.5 and 10 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2 were directly added to the crude extract of fungal isolates which did not produce aflatoxins. The spiked samples were removed, cleaned up in accordance with the aforementioned technique, and the average recovery was examined by HPLC-FLD coupled with Kobra cell® were 100.05% for 5 µg/kg, 99.13% for 7.5 µg/kg and 103.6% for 10 µg/kg having RSD less than 2.5%, which complied with European Union (EU) requirements and AOAC (AOAC 2006) (Zhu et al. 2013). This method has been validated in agreement with EU Commission regulation EC No. 401/2006 (European Commission 2006). The limit of detection (LOD) is found to be 0.3 µg/kg and limit of quantification (LOQ) is found to be 0.5 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2.

It has been previously known that under favorable condition *Aspergillus* section *Flavi* produces aflatoxins (Ayofemi 2020). Utilizing the tested HPLC-FLD-Kobra cell® technique, the positive fungal strain from TLC was tested for presence of AFB_1, AFB_2, AFG_1 and AFG_2. The AFB_1 was identified in *Aspergillus* section *Flavi* isolates of rice (R10), wheat (W7), black mustard (Ra2), yellow mustard (S4) and chickpea (C3) was represented in Table 2. The AFB_2 were detected in *Aspergillus* section *Flavi* isolates of wheat (W7), yellow mustard (S4) and chickpea (C3). Among all the isolates wheat (W7) isolate chromatogram represented

Table 3 Main macro - and microscopic features of *Aspergillus* section *Flavi* isolates after 7 days of culture on CYA at room temperature and Nucleotide accession numbers for ITS sequences

Isolates	Maroscopic features		Microscopic features		r-RNA gene
W7G	Yellowish-green colony , orange in reverse		Long conidiophore Globose smooth conidia, biseriated heads		OQ100087
R10G	Olive green colony, with reddish-brown reverse		Long conidiophore, rough mostly on distal part		OQ091357
S4G	Green colony,orange in reverse		Smooth conidiophores Small subglobose smooth conidia		OQ099838
Ra2G	Yellowish-green colony , pale-yellow in reverse		Long conidiophore Globose smooth conidia,		OQ099803
C3G	Olive green colony, with reddish-brown reverse, with white rough margine		Elongated vesicles with smooth conidia		OP810687

in Fig. 3, produces high amount of aflatoxin-B_1 as 6907.47 µg/kg. The black mustard (Ra2) isolate was found to be low producer of aflatoxin-B_1 as 252.58 µg/kg. Aflatoxin-B_2 were found to be in high amount in wheat (W7) isolate as 68.88 µg/kg and in low amount in yellow mustard (S4) isolate as 11.22 µg/kg. AFG_1 and AFG_2 were not present in any isolates. All the toxicogenic isolates which were found in this study produced very high amount of aflatoxins which was found to be higher from previous reporting's done by Rajarajan et al. 2021. The reported AFB_1 and AFB_2 are considered to be the most important dietary risk factor due to being a class-I carcinogen compound and found to be hepatotoxic in nature. This investigation is in confirmation with other investigators (Fakruddin et al. 2015; Kong et al. 2014). Present study indicates excessive level of aflatoxins in mycoflora isolated from seed samples of non-industrialized area is of major concern

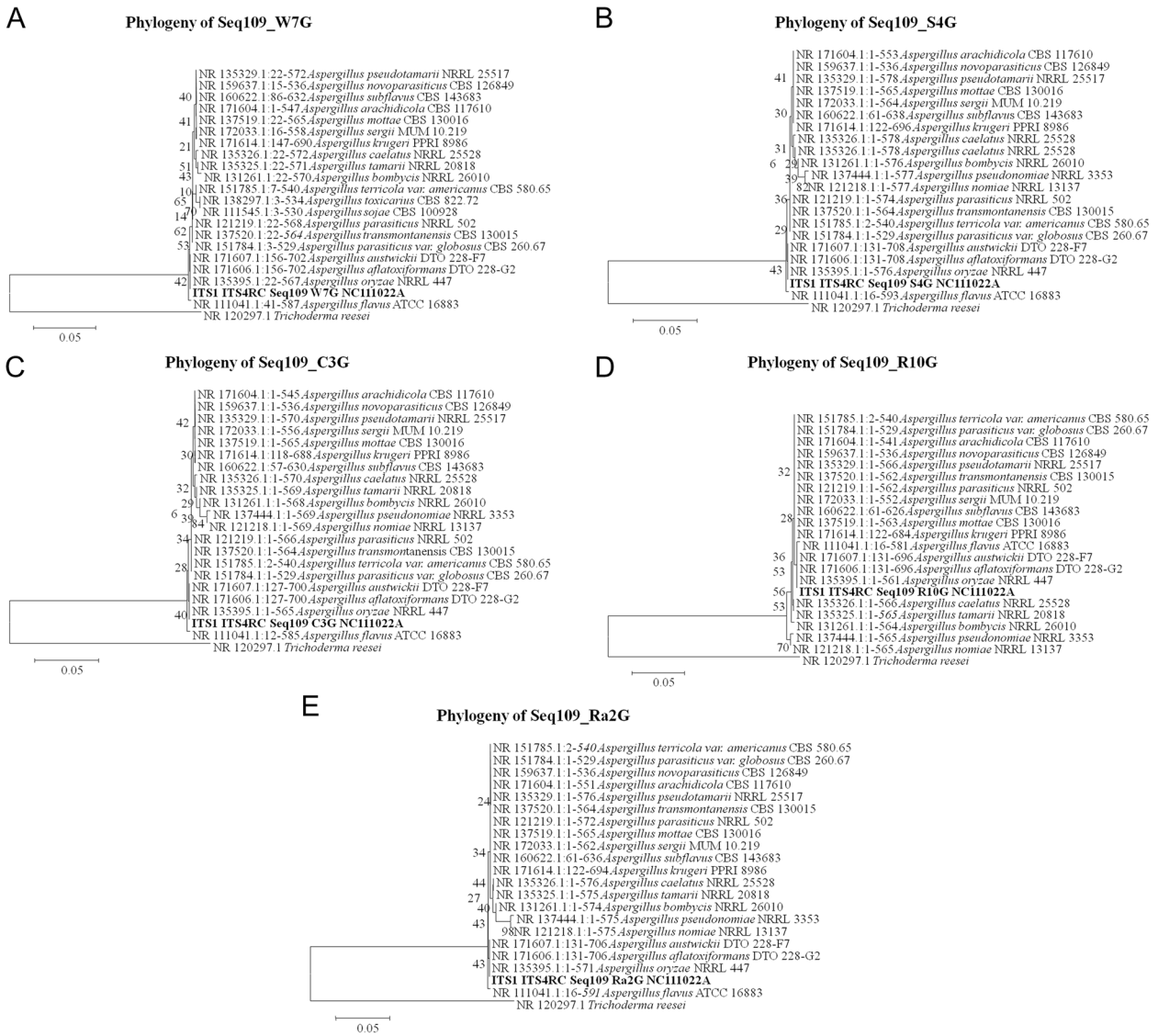


Fig. 4 **A** The optimal tree with the sum of branch length = 0.31896953 is shown. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 353 positions in the final dataset. **B** The optimal tree with the sum of branch length = 0.31893937is shown. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 363 positions in the final dataset. **C** The optimal tree with the sum of branch length = 0.32050524 is shown. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset. **D** The optimal tree with the sum of branch length = = 0.37832727is shown. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 349 positions in the final dataset. **E** The optimal tree with the sum of branch length = 0.38177994 is shown. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 361 positions in the final dataset

because, post-harvest treatments were not adequate for aflatoxins removal such as alkalization, ammonization and heat or gamma radiation.

Molecular characterization of *Aspergillus* section *Flavi*

Isolates of yellow mustard (S4), chickpea (C3), rice (R10), black mustard (Ra2) and wheat (W7) were found to be positive for aflatoxin production and hence its internal transcribed spacer (ITS) region sequencing were done for its identification. All fungal morphotypes were determined based on their 18S rDNA-ITS region sequence (Arifah et al. 2023; Sukmawati et al. 2018). The sequence result of all the five positive isolates compared with the nucleotide base sequence stored in the NCBI genotype by using the BLAST program to identify closely related species of S4, C3, R10, Ra2 and W7 isolates. The sequence alignment showed that S4 isolate has the highest sequence homology with *A. aflatoxiformans* DTO 228-G2 and *A. austwickii* DTO 228-F7 with the sequence similarity of 99.83%, for C3 isolate 100%, for R10 isolate 99.82%, for Ra2 isolate 99.65% and for W7 isolate 99.63%. The retrieved sequences of PCR product were deposited with accession numbers in the gene bank shown in Table 3. Based on phylogenetic tree analysis (Fig. 4), isolates of S4, C3, R10, Ra2 and W7 are shown that they belong to the *A.* section *Flavi*.

Recommendations

The government-sponsored *Anganwadi* (Rural) program, which is present in every village in India, could be the problem solving by involving the ASHA (Accredited Social Health Activist) workers in creating awareness of the mycoflora contamination and the health risk associated with it along with community screening for mycotoxin exposure risk assessment. This was also suggested previously by Wenndt et al. 2020 and we strongly recommend this.

Conclusion

In the present investigation, the occurrence of mycotoxigenic fungi obtained from the grain samples from farmers' storage sites in the Madhubani and Sitamarhi districts of Bihar, India were studied. A total of $n=119$ fungal isolates from 5 distinct genera were isolated in PDA and CYA. The *Aspergillus* spp. were the dominant with 95 isolates out of 119 isolates with occurrence of *Aspergillus* section *Flavi* (58.95%), *Aspergillus* section *Nigri* (35.79%), *Aspergillus* section *Fumigati* (3.16%), *Aspergillus* section *Terrei* (1.05%) and *Aspergillus* section *Circumdati* (1.05%). Among all the isolates, only 8.92% were aflatoxin producers by HPLC-FLD and TLC. The isolates of *Aspergillus* section *Flavi* were found to be aflatoxins producers specially AFB_1 and AFB_2. AFG_1 and AFG_2 were not present in any of these isolates. In summary, to our knowledge, this is the initial report concerning the detection of aflatoxin producing *A.* section *Flavi* from grain samples of flood prone districts of Bihar, India. It suggests time-to-time monitoring is required for having track record about the presence of toxigenic mycoflora in food grain samples in these flood prone areas. As conventional agricultural practices prevented the essential precautions from being taken during the processing of food grain samples, guidelines for toxigenic fungi must be established. Moreover, the state Government should ensure the distribution of mycotoxin free grains in the market so that the health related issued involved can be prevented.

Abbreviations

HPLC-FLD	High Performance Liquid Chromatography with Fluorescent Detector
ITS	Internal transcribed spacer
AFB_1	Aflatoxin-B_1
AFB_2	Aflatoxin-B_2
AFG_1	Aflatoxin-G_1
AFG_2	Aflatoxin-G_2
AFS	Aflatoxins
TLC	Thin Layer Chromatography
HPTLC	High Performance Thin Layer Chromatography

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40550-025-00109-x.

Additional file 1: S1:- TLC plate representing standards and positive samples. S2:- HPLC chromatogram of aflatoxins (AFS) standard solution. S3:- Proforma for sample collection. S4:- Aflatoxin standard calibration.

Acknowledgements

The authors are thankful to Department of Botany, Patna University, Patna for infrastructural facilities and TUV India food lab, Pune, for HPLC-FLD analysis.

Authors' contributions

K.G.C. did all the experimental work and did all the analysis and manuscript writing. C.S. did Proof reading of the manuscript. A.K. did all the final proof reading and data interpretation. All authors reviewed the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

As the objective of the survey was only to document the mycoflora and mycotoxin associated with post-harvest storage and processing of grain samples, this study does not require any ethical clearance since it does not directly involve human participants.

Consent for publication

Not applicable.

Competing of interests
The authors declare no competing interests.

Received: 30 July 2024 Accepted: 23 February 2025
Published online: 16 April 2025

References

Adejumo TO, Adejoro DO (2014) Incidence of aflatoxins, fumonisins, trichothecenes and ochratoxins in Nigerian foods and possible intervention strategies. Food Science and Quality Management 31:127–146
Alameri MM, Kong AS-Y, Aljaafari MN, Ali HA, Eid K, Sallagi MA, Cheng W-H, Abushelaibi A, Lim S-HE, Loh J-Y, Lai K-S (2023) Aflatoxin contamination: an overview on health issues. Detection and Management Strategies Toxins 15(4):246. https://doi.org/10.3390/toxins15040246
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
Arifah F, Aini LQ, Muhibuddin A (2023) Molecular and morphological characterization of fungi isolated from nutmeg (Myristica fragrans) in North Sulawesi, Indonesia. Biodiversitas J Biol Diversity. 24(1):d240151. https://doi.org/10.13057/biodiv/d240151
Ayofemi Olalekan Adeyeye S (2020) Aflatoxigenic fungi and mycotoxins in food: a review. Crit Rev Food Sci Nutr. 60(5):709–721. https://doi.org/10.1080/10408398.2018.1548429
Bennett JW, Klich M (2003) Mycotoxins. Clin Microbiol Rev 16(3):497–516. https://doi.org/10.1128/CMR.16.3.497-516.2003
Chen X, Abdallah MF, Landschoot S, Audenaert K, De Saeger S, Chen X, Rajkovic A (2023) Aspergillus flavus and Fusarium verticillioides and Their Main Mycotoxins: Global Distribution and Scenarios of Interactions in Maize. Toxins 15(9):577. https://doi.org/10.3390/toxins15090577
Choudhary AK (2020) Present status, prevalence and seasonal variations of aflaxtoxin in cattle feed, Bihar, India. Ind J Sci Technol 13(17):1738–1745. https://doi.org/10.17485/ijst/v13i17.288
Darby AC, Chandler SM, Welburn SC, Douglas AE (2005) Aphid-symbiotic bacteria cultured in insect cell lines. Appl Environ Microbiol 71(8):4833–4839. https://doi.org/10.1128/AEM.71.8.4833-4839.2005
Das A, Jayaraman A, Bhattacharya S, Muthusamy P (2012) Evaluation of process parameters influencing aflatoxin B1 synthesis from aspergillus flavus MTCC 2798 using rice straw under submerged fermentation. Int J Pharm Biol Sci. 2:94–105
Davis ND, Diener UL, Eldridge DW (1966) Production of aflatoxins B1 and G1 by Aspergillus flavus in a semisynthetic medium. Appl Microbiol 14(3):378–380. https://doi.org/10.1128/am.14.3.378-380.1966
Đuričin S, Savić S, Bodroža D, Cvijanović G, Đorđević S (2016) Climate change impacts on agricultural water management: challenge for increasing crop productivity in serbia. Economics of Agriculture 63(4):1333–1346. https://doi.org/10.5937/ekoPolj1604333D
European Commission (2006) Regulation (EC) No. 401/2006 of 23 February 2006: Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of European Union L70:12–34
Ezekiel CN, Ayeni KI, Akinyemi MO, Sulyok M, Oyedele OA, Babalola DA, Ogara IM, Krska R (2021) Dietary risk assessment and consumer awareness of mycotoxins among household consumers of cereals, nuts and legumes in North-Central Nigeria. Toxins 13(9):635. https://doi.org/10.3390/toxins13090635
Fakruddin M, Chowdhury A, Hossain MN, Ahmed MM (2015) Characterization of aflatoxin producing Aspergillus flavus from food and feed samples. SpringerPlus. 4(1):159. https://doi.org/10.1186/s40064-015-0947-1
FAO (Food and Agriculture Organization of the United Nations), The FAO Strategy on Climate Change. FAO, Rome 2017).
Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution; Int J Organic Evol. 39(4):783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x
Fried B (1996) Practical thin-layer chromatography: a multidisciplinary approach (1st ed.). CRC Press. https://doi.org/10.1201/9780203742365
Gertz E (2005) BLAST scoring parameters
Girish GK, Goyal RK (1986) Bull Gain Tech 24(2):157–177
Girma G, Abebaw M, Zemene M, Mamuye Y (2016) A review on aspergillosis in poultry. J Vet Sci Technol. 07(6):382. https://doi.org/10.4172/2157-7579.1000382
"GSI – Eastern Region". Geographical Survey of India, Government of India. 11 June 2014.
Horwitz W (2006) Official methods of analysis of AOAC International. AOAC International
Hulin M, Bemrah N, Nougadère A, Volatier JL, Sirot V, Leblanc JC (2014) Assessment of infant exposure to food chemicals: the French Total Diet Study design. Food Additives & Contaminants: Part A 31(7):1226–1239. https://doi.org/10.1080/19440049.2014.921937
I.C.A.R. , 2017 Database http://www.iiwm.res.in/pdf/Bulletin_ICAR_IWMI.pdf. Accessed 7 February 2021
I.C.A.R., 2018 Database https://icar.org.in/content/agricultural-resilience-flood-prone-areas-through-post-flood-crop-management-and-ibfi. Accessed 20 May 2021
I.A.R.C 1993 Working Group on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon (FR): International Agency for Research on Cancer (I.A.R.C. Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 56.) Available from: https://www.ncbi.nlm.nih.gov/books/NBK513574/
I.A.R.C. 2016. New I.A.R.C. report urges action against widespread mycotoxin contamination in developing countries. Lyon: IARC, Press release N: 242.
Kansal M, Kishore K, Kumar P (2017) Impacts of flood and its management – a case study of bihar. Int J Adv Res 5(3):1695–1706. https://doi.org/10.21474/IJAR01/3681
Karlin S, Altschul SF (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc Natl Acad Sci 87(6):2264–2268
Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
Klich MA (2007) Aspergillus flavus: the major producer of aflatoxin. In Mol Plant Pathol 8(6):713–722. https://doi.org/10.1111/j.1364-3703.2007.00436.x
Kong W, Wei R, Logrieco AF, Wei J, Wen J, Xiao X, Yang M (2014) Occurrence of toxigenic fungi and determination of mycotoxins by HPLC-FLD in functional foods and spices in China markets. Food Chem 146:320–326. https://doi.org/10.1016/j.foodchem.2013.09.005
Kumar P, Mahato DK, Kamle M, Mohanta TK, Kang SG (2017) Aflatoxins: A global concern for food safety, human health and their management. Front Microbiol. 7:2170. https://doi.org/10.3389/fmicb.2016.02170
Milićević DR, Milešević J, Gurinović M, Janković S, Đinović-Stojanović J, Zeković M, Glibetić M (2021) Dietary Exposure and Risk Assessment of Aflatoxin M1 for Children Aged 1 to 9 Years Old in Serbia. Nutrients 13(12):4450. https://doi.org/10.3390/nu13124450
Myers EW, Miller W (1988) Optimal Alignments in Linear Space Computap-plbiosci 4:11–17
National Disaster Management Authority (2008) Government of India, National Disaster Management Guidelines, Management of Floods, New Delhi, India. https://ndma.gov.in/sites/default/files/PDF/Guidelines/flood.pdf
Okoth S, De Boevre M, Vidal A, Diana Di Mavungu J, Landschoot S, Kyallo M, Njuguna J, Harvey J, De Saeger S (2018) Genetic and toxigenic variability within Aspergillus flavus population isolated from maize in two diverse environments in Kenya. Front Microbiol 9:57
Pandey MK, Kumar R, Pandey AK, Soni P, Gangurde SS, Sudini HK, Fountain JC, Liao B, Desmae H, Okori P, Chen X, Jiang H, Mendu V, Falalou H, Njoroge S, Mwololo J, Guo B, Zhuang W, Wang X, Liang X, Varshney RK (2019) Mitigating aflatoxin contamination in groundnut through a combination of genetic resistance and post-harvest management practices. Toxins 11(6):315. https://doi.org/10.3390/toxins11060315
Pitt J, Hocking A (2009) Fungi and Food Spoilage. Springer. 519:388. https://doi.org/10.1007/978-0-387-92207-2
Pitt J, Hocking A (2022) Fungi and Food Spoilage. https://doi.org/10.1007/978-3-030-85640-3
Rajarajan P, Sylvia K, Periasamy MP, Subramanian M (2021) Detection of aflatoxin producing Aspergillus flavus from animal feed in Karnataka, India. Environ Anal Health Toxicol 36(3):20. https://doi.org/10.5620/eaht.2021017

Ranjan KS, Sinha AK (1991) Occurrence of mycotoxigenic fungi and mycotoxins in animal feed from bihar, india. J Sci Food Agric 56:39–47
Reddy KR, Reddy CS, Muralidharan K (2009) Detection of Aspergillus spp. and aflatoxin B1 in rice in India. Food Microbiol 26(1):27–31. https://doi.org/10.1016/j.fm.2008.07.013
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
Sashidhar RB, Ramakrishna YB, Bhat RV (1992) Moulds and mycotoxins in sorghum stored in traditional containers in India. J Stored Prod Res 28:257–260. https://doi.org/10.1016/0022-474X(92)90006-C
Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG (1966) Production of aflatoxin on rice. Appl Microbiol 14(3):425–428. https://doi.org/10.1128/am.14.3.425-428
Singh T, Tyagi RPS, Srivastava SK, Girish GK (1983) Bull Grain Tech 21(1):63–73
States DJ, Gish W, Altschul SF (1991) Improved Sensitivity of Nucleic Acid Database Searches Using Application-Specific Scoring Matrices. Methods 3:66–70. https://doi.org/10.1016/S1046-2023(05)80165-3
Sukmawati D, Setyaningsih A, Handayani K, Rahayu T, Rustam S, Moersilah YH, Wahyudi P, Husna SN (2018) Isolation and characterization of aflatoxigenic Aspergillus spp from maize of livestock feed from Bogor. IOP Conference Series: Materials Science and Engineering 434:012105
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30(12):2725–2729. https://doi.org/10.1093/molbev/mst197
Thomas F, Eppley RM, Trucksess MW (1975) Rapid screening method for aflatoxins and zearalenone in corn. J Assoc off Anal Chem 58(1):114–116
Trucksess MW, Abbas HK, Weaver CM, Shier WT (2011) Distribution of aflatoxins in shelling and milling fractions of naturally contaminated rice. Food Additives and Contaminants - Part A 28(8):1076–1082. https://doi.org/10.1080/19440049.2011.576441
Wenndt A, Sudini HK, Pingali P, Nelson R (2020) Exploring aflatoxin contamination and household-level exposure risk in diverse Indian food systems. PLoS ONE 15(10):e0240565. https://doi.org/10.1371/journal.pone.0240565
White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315–322 In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.
WHO Mycotoxins (2018) Available online: https://www.who.int/news-room/fact-sheets/detail/mycotoxins. Accessed on 10 Dec 2024
Zhu Z, Liu G, Chen Y, Cheng J (2013) Assessment of aflatoxins in pigmented rice using a validated immunoaffinity column method with fluorescence HPLC. J Food Compos Anal 31(2):252–258. https://doi.org/10.1016/j.jfca.2013.05.011

Publisher's Note