Research Article

Characterization of Aflatoxigenic and Nonaflatoxigenic Isolates of *Aspergillus flavus* by Cultural and Immunological Methods

Iftikhar Jan* and Sahib Alam

Department of Agricultural Chemistry and Biochemistry, the University of Agriculture, Peshawar, 25130, Pakistan.

Abstract | This study aimed to characterize different *Aspergillus* section *Flavi* isolates in search for nonaflatoxigenic strains for use as biocontrol agent. A total of 434 isolates of *A. flavus* were isolated from soil and stored maize grain samples collected from four agroecological zones (AEZ) i.e., AEZ-A, AEZ-B, AEZ-C and AEZ-D of Khyber Pakhtunkhwa (KP) province of Pakistan. The mycological analysis of the samples demonstrated that the highest population of *Aspergillus* section *Flavi* was existed in soil (n = 236) than in maize grain samples (n = 198). Across all the four zones, the highest population of *Aspergillus* section *Flavi* was observed in AEZ-C (n = 115) whereas the lowest population was found in AEZ-B (n= 99). The isolates of *Aspergillus* section *Flavi* were then characterized for their aflatoxigenicity and nonaflatoxigenicity by cultural (i.e., production of blue fluorescence, room temperature phosphorescence, ammonium hydroxide vapor induced color change and sclerotia production) and immunological methods i.e., enzyme linked immunosorbent assay (ELISA). All the four cultural methods successfully differentiated aflatoxigenic and nonaflatoxigenic isolates, however ammonium hydroxide vapor induced test was found to be the most efficient (80.29%) for segregation of the isolates. Among all isolates, thirty were screened for their total aflatoxin production in corn meal agar medium by using ELISA technique. The results showed that isolate AFS32 produced highest level of total aflatoxin (230.78 µg Kg^{-1}) while isolates AFS5, AFS17, AFS25 and AFS33 produced no aflatoxin. These nonaflatoxigenic isolates were considered as isolates of interest and could be used as biocontrol agent in agricultural fields for prevention of pre-harvest aflatoxins production in different crops.

Received | March 21, 2021; **Accepted** | June 17, 2021; **Published** | August 03, 2021
***Correspondence** | Iftikhar Jan, Department of Agricultural Chemistry and Biochemistry, the University of Agriculture, Peshawar, 25130, Pakistan; **Email:** ijan719@gmail.com
Citation | Jan, I. and S. Alam. 2021. Characterization of aflatoxigenic and nonaflatoxigenic isolates of *Aspergillus flavus* by cultural and immunological methods. *Sarhad Journal of Agriculture,* 37(3): 1062-1072.
DOI | https://dx.doi.org/10.17582/journal.sja/2021/37.3.1062.1072
Keywords | Aflatoxins, Maize, Soil, Aflatoxigenic, *Aspergillus flavus*, ELISA

Introduction

Aflatoxins represent a major class of mycotoxin that are highly toxic and carcinogenic in nature. They are mostly produced by closely related species of *Aspergillus* in many agricultural products worldwide (Pickova *et al*., 2021). The B-group aflatoxins i.e., AFB_1 and AFB_2 are typically produced by *Aspergillus flavus,* while most toxigenic strains of *Aspergillus parasiticus* have great potential in producing all the four kinds i.e., AFB_1, AFB_2, AFG_1 and AFG_2 of aflatoxins (Dorner, 2004). Aflatoxins are chemical derivatives of difuranocoumarin formed by fusion of two furans and one coumarin ring synthesized by polyketide pathway. Aflatoxins are classified into more than eighteen different types among which AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 are the most important and naturally occurring members found in different food commodities (Benkerroum, 2020). Many species in section *Flavi* also produce

a wide range of other mycotoxins besides aflatoxin like paspaline, aspergillic acid, cyclopiazonic acid (CPA) and kojic acid (Frisvad and Samson, 2000).

Aflatoxin causes aflatoxicosis was first reported in England during 1960s after the outbreak of 'Turkey X' disease which killed several thousand young Turkeys upon eating aflatoxin contaminated peanuts (Blount, 1961). Later on, same sort of incidence was investigated in ducklings, chickens, swine and calves (Asplin and Carnaghan, 1961; Loosmore and Harding, 1961; Harding *et al*., 1963). In 1961, mycotoxin producing mold was recognized as *A. flavus,* while the toxin was given the name "aflatoxin" by virtue of its origin (Ellis *et al*., 1991). A later study found that CPA was also present in peanut samples from the feed implicated in the outbreak. This latter observation proposed that CPA might have played an important role in the epidemic of Turkey X disease (Cole, 1986).

Aflatoxins are carcinogenic, mutagenic, immunosuppressive and growth inhibitory, affecting humans, livestock, poultry and wildlife (Tola and Kebede, 2016). The lethal effects of aflatoxins fluctuate and depend on ingested dose, toxin type, exposure duration, mode of action and defense mechanism of an organism. The disease known as aflatoxicosis caused by aflatoxins ingestion are predominantly linked with both acute and chronic toxicity in human and in animal populations (Bennett and Klich, 2003). The outbreak of acute aflatoxicosis in human was characterized by nausea, vomiting, abdominal pain, pulmonary edema, unconsciousness, convulsions and even death (Mwanda *et al*., 2005). However, chronic exposure to low doses may lead to liver cancer development (Claeys *et al*., 2020). Intake of aflatoxin can cause serious illness in animal particularly gastrointestinal disorders, reduced reproduction, alleviate milk and egg production, anemia and cancer of liver, colon and kidney (Peles *et al*., 2019). The International Agency of Research on Cancer (IARC) has categorized AFB1 and AFM1 as Group-1 and Group-2B human carcinogen because of high menace for hepatocellular carcinoma in individuals exposed to aflatoxins (Ostry *et al*., 2017).

Aflatoxin contamination is primarily observed in different agricultural commodities including corn, cereals, soybeans, sorghum, peanuts and other food and feed crops (Alshannaq and Yu, 2017). In many less developed countries aflatoxin is a common food contaminant particularly in their staple diets. It has been reported that the consumption of aflatoxin contaminated food in India and Kenya caused aflatoxicosis which accounted for the death of 100 and 125 people, respectively (Moss, 2002; Lewis *et al*., 2005). Therefore, the United States Food and Drug Administration (USFDA) has settled an allowable limit of total aflatoxin in corn destined for use as human food and animal feed at 20 ppb, while the guidelines in the European Union (EU) tolerate no more than 4 ppb of total aflatoxins (Van Egmond and Jonker, 2003). Any products found to have aflatoxins above the recommended limits are usually rejected for subsequent marketing. The US market losses from aflatoxin contamination alone are estimated to be in the hundreds of billions (Wu and Guclu, 2012). Due to molds and mycotoxins contamination, there is a huge pressure on Pakistan economy by rejection of shipment of agricultural produces in the international market. In this regard it appears very critical that research on detection and control of aflatoxins in food should be undertaken on priority basis in order to enable Pakistan to produce products comparable to World Trade Organization (WTO) standards as well as to safeguard its citizens from these toxic contaminants.

It is pertinent to mention that not all strains of *A. flavus* produce aflatoxin and this prompts the adoption of multiple screening techniques to find out their real aflatoxigenic potential. The nonaflatoxigenic strains could be used to compete the aflatoxigenic strains in the field or stored grains for ecological niches. Thus, it is hypothesized that the *A. flavus* flora of different agroecological zones of Khyber Pakhtunkhwa province of Pakistan may have potential nonaflatoxigenic strains that could be used for the control of aflatoxigenic ones. Therefore, this study was carried out to (1) determine the occurrence frequency of *A. flavus* population in soil and stored maize grain samples collected from various parts of Khyber Pakhtunkhwa and to (2) characterize the isolated *A. flavus* strains into aflatoxigenic and nonaflatoxigenic by employing cultural and immunological techniques.

Materials and Methods

Samples collection

Samples of stored maize grain from farmer premises and soil of agricultural fields (0.5 kg each) were

collected from four agroecological zones (AEZs) i.e., AEZ-A (Swat (SWT), Dir (DIR) and Buner (BUN)), AEZ-B (Abbottabad (ABT), Mansehra (MAN) and Haripur (HAR)), AEZ-C (Charsadda (CHD), Mardan (MAR) and Peshawar (PES)) and AEZ-D (Tank (TAN), Dera Ismail Khan (DIK) and Lakki Marwat (LMT) districts) of Khyber Pakhtunkhwa (KP) province of Pakistan (Figure 1). The samples were collected from six locations in each district. A total of 144 samples (36 samples per zone) were collected in clean polyethylene Ziplock bags, shipped to laboratory and screened for *Aspergillus* Section *Flavi* strains.

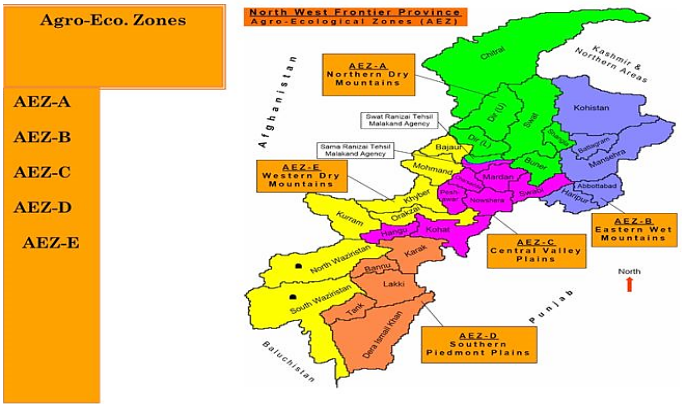

Figure 1: *Major agroecological zone of north western Pakistan. Source: ARP-II Diagnostic study 1992.*

Isolation and identification of Aspergillus section Flavi isolates

The soil and maize samples were screened on potato dextrose agar (PDA) medium to isolate *Aspergillus* section *Flavi* isolates using dilution plate technique (Rosa *et al*., 2006; Shah *et al*., 2010). In this method 1.0 g of each grounded sample was dissolved in 10 mL sterilized distilled water in glass Universal bottles containing 0.01% Tween 80. The content was mixed thoroughly and serial dilution (up to 4 times) were prepared by successively transferring 1 mL suspension to 9 mL sterilized distilled water. A 100 µL aliquot of suspension from 10^{-4} dilution was transferred to PDA media in Petri plates and seeded on the whole surface by using sterilized wire loop. All the Petri plates were incubated at 25 °C for 7-10 days. The pure cultures of *Aspergillus* section *Flavi* isolates were obtained by single spore culture technique. The isolates were morphologically identified on the basis of their macroscopic (mycelium color, colony reverse color and formation of sclerotia) and microscopic characteristics (conidiophores, vesicles, matulae, phialides and conidia) according to recommended synoptic key descriptions (Clayton, 1977; Klich, 2002; McClenny, 2005). The isolates were further cultured on Aspergillus Flavus Parasiticus Agar (AFPA) medium to confirm their identity as *A. flavus* and *A. parasiticus* strains (Pitt *et al*., 1983).

Selection of nonaflatoxigenic strains

All the *Aspergillus* section *Flavi* isolates were screened for aflatoxin production through cultural and immunological methods.

Cultural methods

The following cultural methods were employed for the identification of nonaflatoxigenic isolates.

Production of blue fluorescence on coconut cream agar (CCA)

In this technique a small, pointed inoculum of *Aspergillus* section *Flavi* was placed on 50% coconut cream agar medium (Dyer and McCammon, 1994). The Petri plates were incubated at 25 °C for 7 days after inoculation. All aflatoxigenic isolates emitted characteristic blue fluorescence on the reverse side of CCA medium plates under long-wave ultraviolet (UV) light (365nm) whereas the nonaflatoxigenic isolates did not produce any fluorescence under the same wavelength.

Room temperature phosphorescence

The aflatoxins production ability was further confirmed by growing each isolate on sterilized malt extract agar (MEA) medium modified with β-cyclodextrin (0.3%) and sodium deoxycholate (0.6%). A small portion of the actively growing part of colony of each isolate was inoculated in the center of modified MEA plate and incubated upright at 25 °C. The Petri plates were examined for room temperature phosphorescence on day 5th of incubation period. The plates were examined in such a way that they were placed upright over UV lamp. The appearance of bright green phosphorescence for a while represented positive indication of aflatoxigenicity after turning off the excitation source (Rojas-Duran *et al*., 2007).

Ammonium hydroxide vapor-induced color change

A single colony of *Aspergillus* isolates was inoculated in the center of PDA medium plates and incubated in the dark at 28 °C for seven days or until the fungal mats covered the Petri dish. The dishes were inverted, and concentrated ammonium hydroxide solution was sprayed on inside of the lids so that fungal mats were fully exposed to the vapors. The appearance of plum-red

color on underside of cultural plate indicated aflatoxin production ability of isolate (Abbas *et al*., 2004).

Sclerotia formation

The isolates were further assessed for their aflatoxigenicity on the basis of sclerotia formation on $NaNO_3$ added Czapek yeast agar (CYA) medium. The isolates were cultured on the media for 21 days at 28 °C. The isolates which did not produce sclerotia were characterized as nonaflatoxigenic and those that produced sclerotia were marked as aflatoxigenic (Abbas *et al*., 2005).

Immunological method

The nonaflatoxigenicity of selected isolates (n= 30) were further confirmed through enzyme linked immunosorbent assay (ELISA) using commercially available test kit RIDASCREEN® Aflatoxin Total (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer instruction. The selected isolates were cultured on corn meal agar (CMA) medium for 10 days at 30°C. An agar plug (2 g) from the cultural medium was extracted with 10 mL of 70% methanol for 10 minutes at room temperature on high-speed reciprocal shaker. The extracts were centrifuged (10 min 3500g^{-1}), diluted with water and then analyzed by ELISA. The absorbance of spiked and test samples were measured by spectrophotometer at wavelength of 450 nm. Concentration of aflatoxin was calculated from calibration curve which was obtained using aflatoxin standards with the following concentrations: 0, 5, 15, 45, 135 and 405 ng L^{-1}. In this assay limit of detection for total aflatoxin was approximately 1.75 ng L^{-1}.

Statistical analysis

The data regarding cultural techniques and determination of aflatoxin were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test at 0.05 level of probability by using the statistical software package Statistix 8.1.

Results and Discussion

Isolation, identification and geographical distribution of Aspergillus section Flavi

A total of 434 isolates of *Aspergillus* section *Flavi* were isolated from soil and maize samples collected from four agroecological zones of KP province of Pakistan. The isolates were putatively identified as *A. flavus* on the basis of morphological characteristics and their orange red colored formation on reverse side of AFPA medium plates (Figure 2). Among all the isolates, 236 were obtained from soil samples and 198 from maize grains (Figure 3). In case of sample locations, the highest total member of section *Flavi* were isolated from samples collected from districts CHD (*n* = 44) and DIK (*n* = 42) while the lowest number were found in district BUN (*n* = 32). Similarly, maximum (*n* = 25) number of section *Flavi* isolates among all soil samples was recorded in DIK while their minimum (*n* = 16) population occurred in district HAR, ABT and TAN. In case of maize samples, BUN showed the highest (*n* = 22) while HAR possessed the lowest (*n* = 12) number of section *Flavi* isolates. Across all the four AEZ, the highest population of *Aspergillus* section *Flavi* was observed in AEZ-C (*n* = 115) followed by AEZ-D (*n* = 113), AEZ-A (*n* = 107) and AEZ-B (*n* = 99). Apart from *Aspergillus* section *Flavi*, colonies of other molds were also observed in soil and maize samples (Figure 4). In soil samples, *Pencillium* spp. (31.28%) and *Rhizopus* (17.25%) ranked the highest while *Alternaria* spp (4.63%) was the lowest among all the isolated fungi. Likewise, maize samples had high relative occurrence of *Fusarium* spp (26.0%) and *Paecilomyces* spp (22.67%) but less incidence of *Cladosporium* spp (2.33%).

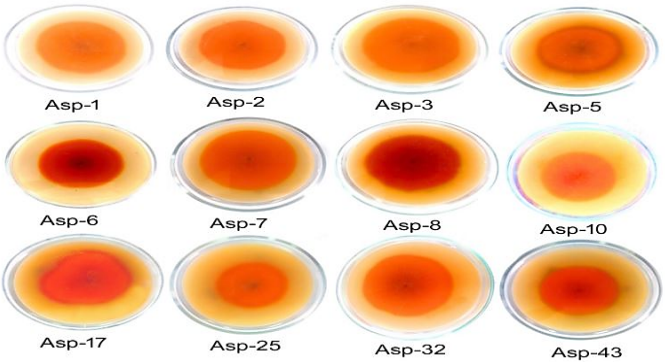

Figure 2: *Formation of orange red color of selected Aspergillus section Flavi isolates on reverse side of AFPA medium plate.*

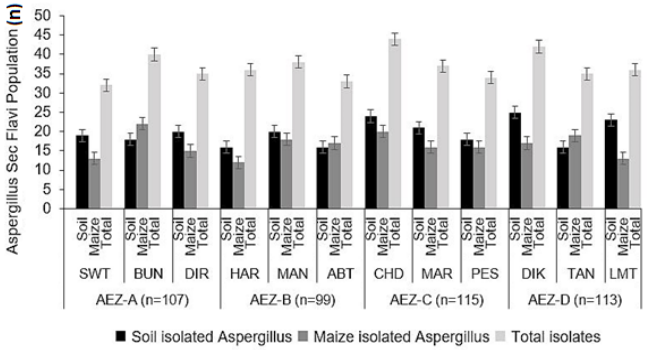

Figure 3: *Total number of Aspergillus section Flavi population isolated from soil and stored maize grains sample taken from different agroecological zones of Khyber Pakhtunkhwa.*

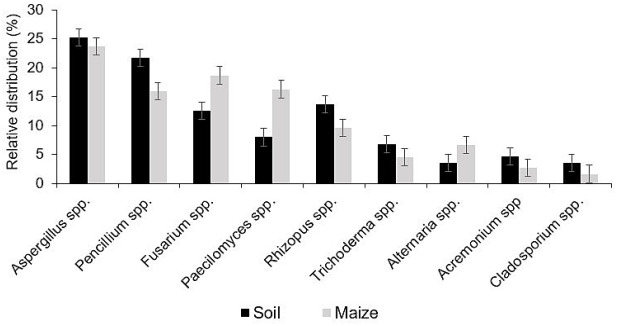

Figure 4: *Relative distribution of common dominant fungal genera isolated from field soil and stored maize grains samples taken from different agro-ecological zones of Khyber Pakhtunkwa.*

Detection of nonaflatoxigenic aspergillus isolates by cultural techniques

The blue fluorescence technique revealed that 71.76% of all the *A. flavus* isolates isolated from maize grain samples and 66.04% of the soil dwelling isolates were aflatoxigenic in nature (Figure 5). Among all soil samples, PES district of AEZ-C had abundant (87.07%) while DIR district of AEZ-A had the least (53.33%) BF emitting isolates. In case of maize samples, most BF producers population occurred in district MAR (85.42%) of AEZ-C, however their lowest percentage was examined in district BUN (54.55%) of AEZ-A. Across all the zones, AEZ-C (78.92%) showed more BF producer's isolates than AEZ-B (70.73%), AEZ-D (64.53.09%) and AEZ-A (61.41%).

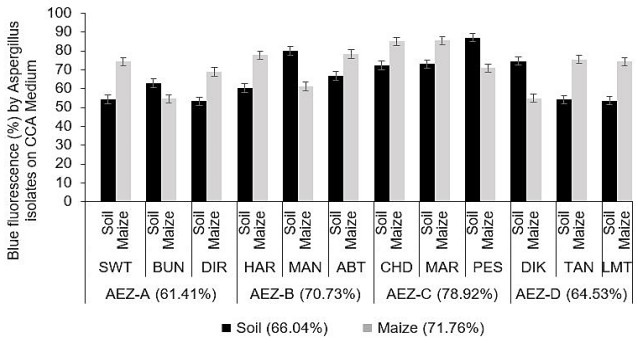

Figure 5: *Blue flourescence (%) on coconut cream agar (CCA) medium of Aspergillus section Flavi isolated from different agro-ecological zones of Khyber Pakhtunkwa.*

Likewise, significantly higher percentage of RTP emitting isolates were examined in maize (64.71%) as compared to soil (66.04%) samples (Figure 6). Among all soil samples, MAN and MAR districts of AEZ-B and AEZ-C had abundant (80.0% and 77.78%, respectively) while BUN district of AEZ-A had the lowest (42.59%) RTP producing isolates. Regarding maize samples, abundant RTP emitting isolates were noted in ABT (70.79%) and CHD (70.0%) districts of AEZ-B and AEZ-C, whereas SWT region of AEZ-A had less RTP producing isolates (43.59%). Among all zones, the highest population of phosphorescence producing isolates were found in AEZ-B (65.80%) followed AEZ-C (63.36%) AEZ-D (57.09%) and AEZ-A (55.10%).

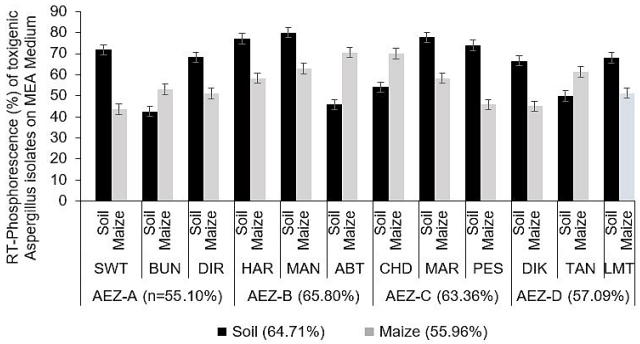

Figure 6: *Room temperature phosphorescence (%) on malt extract agar (MEA) medium modified with β-cyclodextrin (0.3%) and sodium deoxycholate (0.6%) of Aspergillus section Flavi isolated from different agro-ecological zones of Khyber Pakhtunkwa.*

The results of ammonium hydroxide vapor-induced (AV) induced color change (Figure 7) showed that AEZ-C had the highest aflatoxigenic isolates (87.71%) followed by AEZ-B (81.42%) while the lowest population was recorded for AEZ-D (75.80%). Regarding sample sources, promising toxigenic *Aspergillus* isolates were found in maize (82.40%) as compared to soil (78.18%) samples. Among all the soil samples, the highest aflatoxigenic isolates were found in the samples from PES (87.04%) district of AEZ-C followed by SWT (85.96%) of AEZ-A, whereas fewer isolates were noted from TAN (64.58%) district of AEZ-D. In case of maize samples, abundant toxigenic isolates were observed in PES (93.75%) and CHD (90.0%) districts of AEZ-C, while the lowest number were examined in DIK (64.71%) district of AEZ-D.

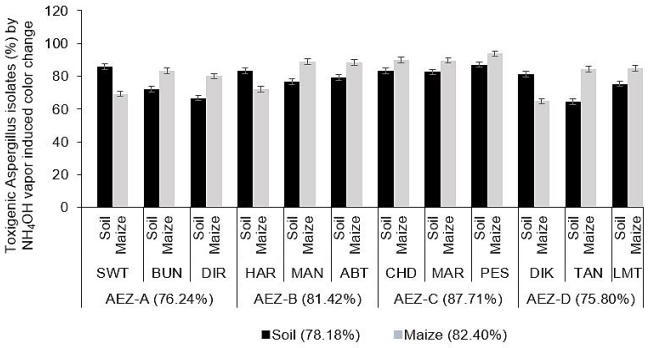

Figure 7: *Ammonium hydroxide (NH4OH) vapor induced color change on coconut cream agar (CCA) medium of toxigenic strains (%) of Aspergillus section Flavi isolated from different agro-ecological zones of Khyber Pakhtunkwa.*

The aflatoxigenicity of *Aspergillus* isolates were also confirmed on the basis of their sclerotia formation on CYA medium (Figure 8). Among all the zones, AEZ-D contained the highest sclerotia forming isolates (35.52%) as compared to AEZ-B (31.31%), AEZ-C (28.84%) and AEZ-A (26.33%). It was observed that more sclerotia forming isolates were present in soil (40.15%) than in maize (20.85%) samples. Among all the soil samples, TAN district of AEZ-D possessed comparatively higher (58.33%) population of sclerotia forming isolates followed by LMT (52.17%) of AEZ-D, whereas BUN district of AEZ-A had the least (18.52%) isolates. In all maize samples, the highest population of *Aspergillus* that developed sclerotia were recorded for DIK (43.14%) of AEZ-D and BUN (33.33%) of AEZ-A, while the lowest percentage was observed for LMT (10.26%) district of AEZ-D.

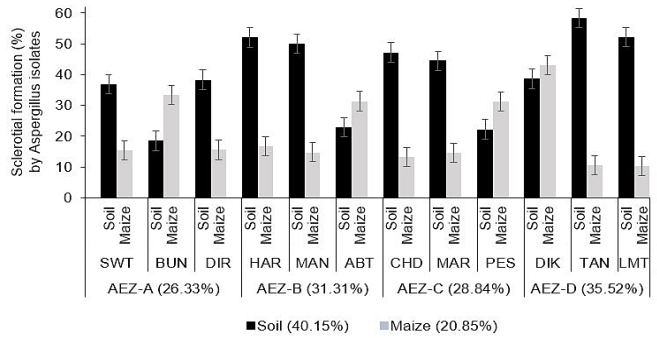

Figure 8: *Sclerotial production (%) on czapek yeast agar (CYA) medium of aflatoxigenic isolates of Aspergillus section Flavi isolated from different agro-ecological zones of Khyber Pakhtunkwa.*

The comparative analysis of all four cultural techniques divulged that soil had overall higher mean toxigenic isolates (62.27%) than maize grain samples (57.74%). Further observation showed that among all soil samples, MAN district of AEZ-B had the highest (71.67%) and BUN district of AEZ-A had the lowest (49.07%) average number of aflatoxigenic isolates. In case of maize samples, ABT district of AEZ-B was found to possess a mean higher number (67.16%) of toxigenic *Aspergillus* section *Flavi* isolates, while SWT district of AEZ-A had relatively less number of toxigenic isolates (50.64%). Regarding sample locations, maximum average aflatoxigenic *Aspergillus* section *Flavi* isolates were found in MAR (65.71%) and CHD (64.41%) districts followed by MAN (64.31%), while less aflatoxigenic isolates (52.57%) were examined in maize grain samples from BUN district (Table 1). The findings (Table 2) further revealed that AEZ-C had higher (64.84%) mean aflatoxigenic isolates than AEZ-B (62.32%), AEZ-D (58.37%) and AEZ-A (54.77%). With respect to cultural techniques, all the four methods successfully differentiated aflatoxigenic and nonaflatoxigenic isolates, however NH_4OH vapor induced color change of the reverse side of colonies was found more efficient (80.29%) than RTP (68.90%), blue fluorescence (60.40%) and sclerotia formation (30.50%).

Table 1: *Occurrence (%) of aflatoxigenic Aspergillus section Flavi isolates in stored maize grain and soil samples collected from different Agroecological zones (AEZs) of Khyber Pakhtunkhwa.*

Agroecological zone	Location/ District	Soil	Maize	Mean
AEZ-A	SWT	62.28	50.64	56.46def
	BUN	49.07	56.06	52.57f
	DIR	56.67	53.89	55.28ef
AEZ-B	HAR	68.23	56.25	62.24abc
	MAN	71.67	56.94	64.31ab
	ABT	53.65	67.16	60.40bcd
AEZ-C	CHD	64.24	64.58	64.41ab
	MAR	69.44	61.98	65.71a
	PES	67.56	60.42	64.0ab
AEZ-D	DIK	65.33	51.96	58.65cde
	TAN	56.77	57.89	57.33de
	LMT	62.32	55.13	58.72cde
Mean	-	62.27a	57.74b	-

Mean values in row and column followed by different alphabets are statistically significant at p<0.05.

Table 2: *Detection of aflatoxigenic isolates (%) of Aspergillus section Flavi using cultural techniques of blue fluorescence (BF), room temperature phosphorescence (RTP), ammonium hydroxide vapor induced color change (AV) and sclerotia formation (SF) on CCA, modified MEA, PDA and CYA medium.*

Origin of *A. flavus* isolate	Cultural technique				Mean
	BF	RTP	AV	SF	
AEZ-A	61.41	55.10	76.24	26.33	54.77c
AEZ-B	70.73	65.80	81.42	31.31	62.32a
AEZ-C	78.92	63.36	87.71	28.84	64.71a
AEZ-D	64.53	57.09	75.80	35.52	58.37b
Mean	68.90b	60.34c	80.29a	30.50d	

Mean values in row and column assigned with identical alphabets are statistically non-significant at p<0.05.

Detection of nonaflatoxigenic Aspergillus isolates by immunological technique

The ELISA results showed that the highest total aflatoxins content (230.78 µg Kg^{-1}) was recorded

for isolate labeled as AFS32 whereas no aflatoxins were produced by isolates AFS5, AFS17, AFS25 and AFS33. In this technique, the recovery rate of total aflatoxins was between 95.50-105.42% with relative standard deviation (RSD) value ranged from 3.65-4.68% (Table 3).

Table 3: *Aflatoxin level (µg Kg^{-1}) of selected A. flavus isolates determined by ELISA.*

S. No.	*A. flavus* isolate	Total aflatoxin (µg Kg^{-1})	S. No.	*A. flavus* isolate	Total aflatoxin (µg Kg^{-1})
1.	AFS1	146.37±1.76	16.	AFS25	0.00
2.	AFS2	170.58±2.08	17.	AFS26	192.48±2.65
3.	AFS3	55.55±0.90	18.	AFS27	142.18±2.34
4.	AFS5	0.00	19.	AFS29	160.86±1.22
5.	AFS6	158.38±1.49	20.	AFS31	95.65±1.25
6.	AFS7	184.51±2.10	21.	AFS32	230.78±1.54
7.	AFS9	130.22±2.48	22.	AFS33	0.00
8.	AFS10	84.55±1.16	23.	AFS35	130.44±2.91
9.	AFS12	65.34±2.24	24.	AFS36	198.54±2.14
10.	AFS13	210.62±2.25	25.	AFS39	110.46±1.49
11.	AFS17	0.00	26.	AFS43	6.43±0.57
12.	AFS18	50.86±1.10	27.	AFS44	226.22±2.58
13.	AFS19	31.37±0.72	28.	AFS45	175.77±1.18
14.	AFS21	165.72±2.33	29.	AFS47	220.42±2.24
15.	AFS23	112.37±1.62	30.	AFS49	168.36±1.89

Each value is mean of three replications ± standard deviation.

In this study, the population diversity and geographic distribution pattern of *Aspergillus* section *Flavi* were determined to identify indigenous nonaflatoxigenic strains as potent biocontrol candidate for prevention of aflatoxin contamination in agricultural crops. A total of 434 isolates of *Aspergillus* section *Flavi* were isolated from field soil and maize grains collected from farmers' storage structures across four agroecological zones in Khyber Pakhtunkhwa province of Pakistan. All soil and maize samples contained *Aspergillus* section *Flavi* isolates, though abundant population was observed in soil samples. Additionally, the incidence of section *Flavi* varied widely among districts and AEZ, however, the prevalence of *Aspergillus* was more consistent in the southern region i.e., AEZ-C and AEZ-D than in the northern region i.e., AEZ-A and AEZ-B. The climatic conditions of the southern part of Khyber Pakhtunkhwa ranges from warm and sub-humid in Zone C to hot and arid in Zone D. Similarly, drought stress, less rainfall and high temperature was more common in the southern region as compared to the northern part (EPA, 2016). All these factors and many other micro-climatic factors usually contributed in higher population of *Aspergillus* section *Flavi* in both AEZ-C and AEZ-D. A comparative study conducted in major almond producing areas of California, USA, showed higher frequencies of *A. flavus* L strains in the southern region as compared with the northern region (Donner *et al.*, 2015). Similar results associated with high predominance of *A. flavus* in the soil ecosystem were reported from West Africa, Nigeria and Thailand (Cardwell and Cotty, 2002; Ehrlich *et al.*, 2007; Donner *et al.*, 2009). Likewise, fluctuation in prevailing environmental conditions of temperature, rainfall and relative humidity had significant influence on the distribution, density and structure of aflatoxin producing fungi as well as vulnerability of crop plants to such communities (Van der Fels-Klerx *et al.*, 2016; Mmongoyo *et al.*, 2017).

In Pakistan, huge amount of maize grains are generally stored for extended time period under improper and insufficient storage conditions which consequently result in degradation of the grains quality because of pest attack and mold infestation. Therefore, in this research work we underlined the incidence and prevalence of aflatoxigenic fungal isolates in stored maize grains samples collected from traditional farmer's storage structures across the four AEZ of KP. The data obtained in this study divulged that all stored maize grain samples were at risk of fungal infestation and mycotoxins contamination. The data obtained showed that all grains were primarily contaminated with *Aspergillus* section *Flavi* isolates, though co-contamination with many field and storage fungi occurred in most of the samples. The analysis further indicated high predominance of some toxigenic fungal genera including *Aspergillus, Fusarium*, *Paecilomyces* and *Penicillium* as compared with other fungal isolates. This widespread distribution and prevalence of *Aspergillus* section *Flavi* in stored maize were observed previously in China (Xing *et al.*, 2017), Nigeria (Muhammad *et al.*, 2019) and Spain (Alborch *et al.*, 2012). The development of these fungi could be affected by moisture content of the product (Gtorni *et al.*, 2009). However, some other factors like temperature, water activity (Alam *et al.*, 2009), physical condition of the grains (Hocking, 1991) and post-harvest management practices (Hell and Mutegi, 2011; Akowuah *et al.*, 2015) also support fungal growth in maize grain during storage.

The cultural methods used for the detection

of aflatoxins were applied to 434 members of section *Flavi* included blue fluorescence, RTP phosphorescence, NH_4OH vapor induced color change and sclerotia formation. The data obtained from combined cultural techniques showed high (60.0%) frequency of aflatoxigenic *A. flavus* than nonaflatoxigenic (40.0%) isolates with reference to substrates types and geographical origin. A significant ($P<0.05$) higher aflatoxin producers isolates of *A. flavus* were observed in soil than in maize samples. The relative proportion of toxigenic and atoxigenic strains of *A. flavus* varied significantly ($p<0.05$) among the districts sampled during the survey. Across districts, the highest prevalence of aflatoxin producers was observed in Mardan, Charsadda and Mansehra, while the lowest occurrence of aflatoxigenic strains was noted in district Buner. Similarly, the frequencies of aflatoxigenic isolates were significantly ($P<0.05$) higher than that of nonaflatoxigenic isolates in all the four AEZs. The incidence of aflatoxigenic isolates in AEZ-C and AEZ-B were comparatively greater than that of aflatoxigenic strains in AEZ-A and AEZ-D. The present data finds supportive evidence from previous investigation conducted in USA, and Italy which showed high proportion of aflatoxigenic isolates in soil and maize samples than nonaflatoxigenic isolates (Giorni *et al.*, 2007; Horn and Dorner, 1999). The relative proportion of aflatoxigenic and nonaflatoxigenic isolates of *A. flavus* observed in this study was influenced by many factors such as geographical regions, soil condition, soil type, crop sequence history, crop management practices, day length, insect presence, fungicide application history and irrigation source and frequency (Orum *et al.*, 1997).

In the course of this study we also compared the efficacy of four cultural techniques used for differentiation of aflatoxigenic and nonaflatoxigenic isolates of *A. flavus*. The current findings confirmed that NH_4OH vapor induced color change had greater (80.29%) potential in the distinction of aflatoxigenic isolates followed by blue fluorescence (68.90%) and RTP (68.90%). Moreover, 30.50% of the total isolates produced sclerotia was considered as positive isolates with respect to their aflatoxin production. It is evident from the present results that the occurrence of blue fluorescence, emission of RTP, orange-red pigment formation of isolates after exposure to NH_4OH vapor as well as formation of sclerotia were strongly an indication of aflatoxin producing ability of the isolates. These findings were in concordance to the previously published reports (Abbas *et al.*, 2004, 2005; Rojas-Duran *et al.*, 2007).

This study also presents a distinctive method for direct extraction of aflatoxin from solid nutrient medium and their simultaneous quantification by ELISA. The highly specialized and sensitive method of ELISA was applied on selected 30 out of 434 isolates for their aflatoxins production in CMA medium. The ability of *A. flavus* to produce total aflatoxins varied significantly ($p<0.05$) among different strains. The results showed that AFS32, AFS44, AFS47 and AFS13 produced greater amount of total aflatoxin (>200µg Kg^{-1}) as compared with other isolates. The isolates AFS5, AFS17, AFS25 and AFS33 produced no aflatoxins when analyzed with ELISA. The variation among aflatoxigenic *Aspergillus* isolates in production of aflatoxins determined in the present study had also been reported previously (Fraga *et al.*, 2007).

Conclusions and Recommendations

The isolates AFS5, AFS17, AFS25 and AFS33 confirmed for their nonaflatoxigenicity by using cultural and ELISA methods were considered as isolates of interest and could be used as candidate biocontrol agents for prevention of pre-harvest aflatoxin production in different crops. It was also suggested that cultural and immunological techniques should be used in combination with analytical and molecular techniques for authentic segregation of nonaflatoxigenic isolates of *A. flavus* from aflatoxigenic isolates.

Acknowledgements

We are thankful to the Higher Education Commission (HEC) of Pakistan for providing financial grant under the Project No. 8688/KPK/NRPU/R&D/HEC/2017. Thanks, are also extended to the University of Liverpool, UK for research facilitation to successfully complete this study.

Novelty Statement

This is the first study of its type in Khyber Pakhtunkhwa to explore indigenous *Aspergillus flavus* flora for nonaflatoxigenic strains to be used as biocontrol agents for prevention of aflatoxin contamination in different crops.

Author's Contribution

Iftikhar Jan: Conducted the experimental work and wrote the manuscript.
Sahib Alam: Designed and supervised the study.

Conflict of interest
The authors declare no conflict of interest.

References

Abbas, H.K., M.A. Weaver, R.M. Zablotowicz, B.W. Horn and W.T. Shier. 2005. Relationships between aflatoxin production and sclerotia formation among isolates of *Aspergillus* section *Flavi* from the Mississippi Delta. Europ. J. Plant Pathol., 112: 283-287. https://doi.org/10.1007/s10658-004-4888-8

Abbas, H.K., R.M. Zablotowicz, M.A. Weaver, B.W. Horn, W. Xie and W.T. Shier. 2004. Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. Can. J. Microbiol., 50: 193-199. https://doi.org/10.1139/w04-006

Akowuah, J.O., D.M. Lena, C. Chian and R. Anthony. 2015. Effects of practices of maize farmers and traders in Ghana on contamination of maize by aflatoxins: case study of Ejura Sekyeredumase municipality. Afr. J. Microbiol. Res., 9: 1658-1666. https://doi.org/10.5897/AJMR2014.7293

Alam, S., H.U. Shah and N. Magan. 2009. Water availability affects extracellular hydrolytic enzymes production by *Aspergillus flavus* and *Aspergillus parasiticus*. World. Mycotox. J., 2: 313-322. https://doi.org/10.3920/WMJ2008.1108

Alborch, L., M.R. Bragulat, G. Castella, M.L. Abarca and F.J. Cabanes. 2012. Mycobiota and mycotoxin contamination of maize flours and popcorn kernels for human consumption commercialized in Spain. Food Microbiol., 32: 97-103. https://doi.org/10.1016/j.fm.2012.04.014

Alshannaq, A., and J. Yu. 2017. Occurrence, toxicity, and analysis of major mycotoxins in food. Int. J. Environ. Res. Public. Health, 14: 632. https://doi.org/10.3390/ijerph14060632

Asplin, F.D., and R.B.A. Carnaghan. 1961. The toxicity of certain groundnut meals for poultry with special references to their effect on ducklings and chickens. Vet. Rec., 73: 1215-1219.

Benkerroum, N., 2020. Aflatoxins: Producing-molds, structure, health issues and incidence in Southeast Asian and Sub-Saharan African countries. Int. J. Environ. Res. Publ. Health, 17: 1215. https://doi.org/10.3390/ijerph17041215

Bennett, J.W., and M. Klich. 2003. Mycotoxins. Clin. Microbiol. Rev. 16: 497-516. https://doi.org/10.1128/CMR.16.3.497-516.2003

Blount, W.P., 1961. Turkey "X" disease. Turkeys. 9: 62-61.

Cardwell, K.F., and P.J. Cotty. 2002. Distribution of *Aspergillus* section *Flavi* among soils from the four agroecological zones of the Republic of Benin, West Africa. Plant. Dis., 86: 434-439 https://doi.org/10.1094/PDIS.2002.86.4.434.

Claeys, L., C. Romano, K.D. Ruyck., H. Wilson, B. Fervers, M. Korenjak, J. Zavadil, M.J. Gunter, S.D. Saeger, M.D. Boevre and I. Huybrechts. 2020. Mycotoxin exposure and human cancer risk: A systematic review of epidemiological studies. Compr. Rev. Food Sci. Food Saf., 19: 1449-1464. https://doi.org/10.1111/1541-4337.12567

Clayton, Y.M., 1977. Medically important fungi: a guide to identification. Proc. R. Soc. Med., 70: 359. https://doi.org/10.1177/003591577707000519

Cole, R.J., 1986. Etiology of turkey "X" disease in retrospect: A case for the involvement of cyclopiazonic acid. Mycotoxin Res., 2: 3-7. https://doi.org/10.1007/BF03191956

Donner, M., J. Atehnkeng, R.A. Sikora, R. Bandyopadhyay and P.J. Cotty. 2009. Distribution of *Aspergillus* section *Flavi* in soils of maize fields in three agroecological zones of Nigeria. Soil. Biol. Biochem., 41: 37-44. https://doi.org/10.1016/j.soilbio.2008.09.013

Donner, M., P.S.F. Lichtemberg, M. Doster, A. Picot, P.J. Cotty, R.D. Puckett and T.J. Michailides. 2015. Community structure of *Aspergillus flavus* and *A. parasiticus* in major almond-producing areas of California, United States. Plant Dis., 99: 1161-1169. https://doi.org/10.1094/PDIS-05-14-0450-RE

Dorner, J.W., 2004. Biological control of aflatoxin contamination of crops. J. Toxicol. Toxin Rev., 23: 425-450. https://doi.org/10.1081/TXR-200027877

Dyer, S.K., and S. McCammon. 1994. Detection of toxigenic isolates of *Aspergillus flavus*

and related species on coconut cream agar. J. Appl. Bactiol., 76: 75-78. https://doi.org/10.1111/j.1365-2672.1994.tb04418.x

Ehrlich, K.C., B.G. Montalbano and P.J. Cotty. 2007. Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. FEMS Microbiol. Lett., 268: 231-236. https://doi.org/10.1111/j.1574-6968.2006.00588.x

Ellis, W.O., J.P. Smith, B.K. Simpson and J.H. Oldham. 1991. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection and methods of control. Crit. Rev. Food Sci. Nutr., 30: 403-439. https://doi.org/10.1080/10408399109527551

EPA (Environmental Protection Agency). 2016. Government of Khyber Pakhtunkhwa forestry, environment and wildlife department, Khyber Pakhtunkhwa, Pakistan.

Fraga, M.E., F. Curvello, M.J. Gatti, L.R. Cavaglieri and A.M. Dalcero, 2007. Potential Aflatoxin and Ochratoxin A production by *Aspergillus* species in poultry feed processing. Vet. Res. Comm., 31: 343-357. https://doi.org/10.1007/s11259-006-3434-x

Frisvad, J.C., and R.A. Samson. 2000. *Neopetromyces* gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus Circumdati. Studies in Mycol., 45: 201-207.

Giorni, P., N. Magan, A. Pietri, T. Bertuzzi and P. Battilani. 2007. Studies on *Aspergillus* section *Falvi* isolated from maize in northern Italy. Int. J. Food Microbiol., 113: 330-338. https://doi.org/10.1016/j.ijfoodmicro.2006.09.007

Gtorni, P., P. Battilani and N. Magan. 2009. Effect of solute and matric potential on *in-vitro* growth and sporulation of strains from a new population of *Aspergillus flavus* in Italy. Fungi Ecol., 1: 101-106. https://doi.org/10.1016/j.funeco.2008.07.001

Harding, J.D.J., J.T. Dones, G. Lewis and R. Allcroft. 1963. Experimental groundnut poisoning in pigs. Res. Vet. Sci., 4: 217-229. https://doi.org/10.1016/S0034-5288(18)34864-1

Hell, K., and C. Mutegi. 2011. Aflatoxin control and prevention strategies in key crops of Sub-Saharan Africa. Afr. J. Microbiol. Res., 5: 459-466.

Hocking, A.D. 1991. Xerophillic fungi in intermediate and low moisture foods. In: D.K. Arora, K.G. Mukerji and E.H. Marth. (Eds.). Handbook of Applied Mycology: Foods and Feeds. Vol. III. Marcel and Dekker, New York. pp. 69-92.

Horn, B.W., and J.W. Dorner. 1999. Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl. Environ. Microb., 65: 1444–1449. https://doi.org/10.1128/AEM.65.4.1444-1449.1999

Klich, M.A., 2002. Identification of common *Aspergillus* Species. Centraalbureau voor Schimmelcultures: Utrecht, The Netherlands. pp. 426-432.

Lewis, L., M. Onsongo, H. Njapau, H. Schurz-Rogers, G. Luber, S. Kieszak, J. Nyamongo, L. Backer, A.M. Dahive, A. Misore, K.C. DeCock. Rubin and the Kenya Aflatoxicosis Investigation Group. 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. Environ. Health Perspect., 113: 1763-1767. https://doi.org/10.1289/ehp.7998

Loosmore, R.M., and J.D.J. Harding. 1961. A toxic factor in Brazilian groundnut causing liver damage in pigs. Vet. Rec., 73: 1362-1364.

McClenny, N., 2005. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: The traditional approach. J. Med. Mycol., 43: S125-S128. https://doi.org/10.1080/13693780500052222

Mmongoyo, J.A., F. Wu, J.E. Linz, M.G. Nair, K. Mugula, R.J. Tempelman and G.M Strasburg. 2017. Aflatoxin levels in sunflower seeds and cakes collected from micro- and small-scale sunflower oil processors in Tanzania. PLoS One, 12: e0175801. https://doi.org/10.1371/journal.pone.0175801

Moss, M.O., 2002. Mycotoxin review-1. *Aspergillus* and *Penicillium*. Mycology, 16: 116-119. https://doi.org/10.1017/S0269915X02003014

Muhammad, H.K., D.O. Apeh, H.L. Muhammad, Y.B. Olorunmowaju, E. Ifeji and H.A. Makun. 2019. Mycoflora of maize in Niger state, Nigeria. Adv. Res. Life Sci., 3: 40-45. https://doi.org/10.2478/arls-2019-0009

Mwanda, O.W., C.F. Otieno and E. Omonge. 2005. Acute aflatoxicosis: Case report. East. Afr. Med. J., 82: 320-324. https://doi.org/10.4314/eamj.v82i6.9305

Orum, T.V., D.M. Bigelow, M.R. Nelson, D.R.

Howell and P.J. Cotty. 1997. Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma County, Arizona, Soils. Plant Dis., 81: 911-916 https://doi.org/10.1094/PDIS.1997.81.8.911.

Ostry, V., F. Malir, J. Toman and Y. Grosse. 2017. Mycotoxins as human carcinogens-the IARC Monographs classification. Mycotoxin Res., 33: 65-73. https://doi.org/10.1007/s12550-016-0265-7

Peles, F., P. Sipos, Z. Gyori, W.P. Pfliegler, F. Giacometti, A. Serraino, G. Pagliuca, T. Gazzotti, and I. Pocsi. 2019. Adverse effects, transformation and channeling of aflatoxins into food raw materials in livestock. Front. Microbiol., 10: 2861. https://doi.org/10.3389/fmicb.2019.02861

Pickova, D., V. Ostry and F. Malir. 2021. A recent overview of producers and important dietary sources of aflatoxins. Toxins, 13: 186. https://doi.org/10.3390/toxins13030186

Pitt, J.I., A.D. Hocking and D.R. Glenn. 1983. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J. Appl. Bacteriol., 54: 14-109. https://doi.org/10.1111/j.1365-2672.1983.tb01307.x

Rojas-Duran, T.R., C.A. Fente, B.I. Vazquez, C.M. Franco, A. Sanz-Medel and A. Cepeda. 2007. Study of a room temperature phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media. Int. J. Food Microbiol., 115: 149-158. https://doi.org/10.1016/j.ijfoodmicro.2006.10.018

Rosa, C.A.R., J.M.M. Ribeiro, M.J. Fraga, M. Gatti, L.R. Cavaglieri, C.E. Magnoli, A.M. Dalcero and C.W.G. Lopes. 2006. Mycoflora of poultry feeds and ochratoxin producing ability of isolated *Aspergillus* and *Penicillium* species. Veter. Microbiol., 113: 89-96. https://doi.org/10.1016/j.vetmic.2005.10.031

Shah, H.U., T.J. Simpson, S. Alam, K.F. Khattak and S. Parveen, 2010. Mould incidence and mycotoxin contamination in maize kernels from Swat Valley, North West Frontier Province of Pakistan. Food Chem. Toxicol., 48: 1111–1116. https://doi.org/10.1016/j.fct.2010.02.004

Tola, M. and B. Kebede. 2016. Occurrence, importance and control of mycotoxins: A review. Cog. Food. Agri. 2: 1191103.

Van der Fels-Klerx, H.J.C., C. Liu and P. Battilani. 2016. Modelling climate change impacts on mycotoxin contamination. World Mycotox. J., 9: 717-726. https://doi.org/10.3920/WMJ2016.2066

Van Egmond, H.P., and M.A. Jonker. 2004. Worldwide regulations for food and feed in 2003. FAO Food and Nutrition Paper (FAO). 81: 1728-3264.

Wu, F., and H. Guclu. 2012. Aflatoxin regulations in a network of global maize trade. PLoS One, 7: e45151. https://doi.org/10.1371/journal.pone.0045151

Xing, F, X. Liu, L. Wang, J.N. Selvaraj, N. Jin, Y. Wang and Y. Liu. 2017. Distribution and variation of fungi and major mycotoxins in pre- and post-nature drying maize in North China Plain. Food. Contr., 80: 244-251. https://doi.org/10.1016/j.foodcont.2017.03.055