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Research Papers

Occurrence of toxigenic fungi in maize and maize-gluten meal from Pakistan

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Summary. The present study was designed to isolate and identify toxigenic mycoflora of maize and maize-gluten meal. A total of 82 samples of maize and 8 samples of maize-gluten meal were collected from Faisalabad district of Pakistan over a period of two years. These samples were inoculated on different culture media. Fungal contamination of maize and maize-gluten was 56% and 75% of samples, respectively. Isolation frequencies of different genera isolated from maize were *Aspergillus* 33%; *Penicillium* 28%; *Fusarium* 10%; and *Alternaria* 1%. Isolation frequency among species was maximum for *P. verrucosum,* followed by *A. niger* aggregates, *A. ochraceous, A. flavus*, *P. chrysogenum*, *A. parasiticus*, *A. carbonarius, Fusarium* spp. and *Alternaria* spp. Relative density of *Aspergillus* isolates was maximum for *A. niger* aggregates and *A. ochraceous* (30% each) followed by *A. flavus* (26%), *A. parasiticus* (11%) and *A. carbonarius* (3%). Percentage of toxigenic fungi among *Aspergillus* isolates was 52%. Aflatoxigenic isolates of *A. flavus* and *A. parasiticus* were 43 and 67% and ochratoxigenic isolates of *A. carbonarius*, *A. ochraceous* and *A. niger* aggregates were 100, 63 and 38%, respectively. *Aspergillus parasiticus* produced higher concentrations of AFB1 (maximum 1374.23 ng g^{-1}) than *A. flavus* (maximum 635.50 ng g^{-1}). Ochratoxin A production potential of *A. ochraceous* ranged from 1.81 to 9523.1 ng g^{-1}, while in *A. niger* aggregates it was 1.30 to 1758.6 ng g^{-1}. Isolation frequencies of fungal genera from maize-gluten meal were *Aspergillus* (63%) and *Penicillium* (50%). *A. flavus* was the most frequently isolated species. Percentage of toxigenic fungi among *Aspergillus* isolates was 40%. Aflatoxigenic isolates of *A. flavus* were 33% and ochratoxigenic isolates of *A. ochraceous* were 100%.

Key words: *Aspergillus*, ochratoxin A, aflatoxins, wheat, wheat-bran, *Penicillium*.

Introduction

Pakistan has an agriculture-based economy; the agricultural share of national GDP is more than 23% (Anonymous, 2009). Maize is extensively used by humans in the form of different maize products and it is a major ingredient of the animal and poultry feed industry. Therefore, maize is grown on a vast area throughout the country, second only to wheat. This maize is purchased by feed mills for long storage to fulfill their future needs; if storage conditions are not proper it will lead to fungal contamination and consequently buildup of mycotoxins. Fungal growth and mycotoxin production are the consequence of an interaction among the fungus, the host and the environment. Contamination of agricultural commodities by fungi results not only in downgrading of quality, but toxigenic fungi also represent a health hazard for humans, livestock and poultry (Anderson and Thrane, 2006). Cereal crops are at high risk for fungal contamination at both pre-harvest and post-harvest stages. Mycotoxins are natural food and feed contaminants, mainly produced by moulds of genera *Aspergillus*, *Penicillium* and *Fusarium* (Zinedine *et al*., 2006). Maize is a cereal in which a range of mycotox-

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ins have been found, e.g. aflatoxins (Shotwell *et al*., 1973), ochratoxin A (Saleemi *et al*., 2010), zearalenone (Shotwell *et al*., 1970), deoxynivalenol (DON) (Gilbert *et al*., 1983), fumonisins (Gelderblom *et al*., 1988) and moniliformin (Shraman *et al*., 1991). Aflatoxins are produced by strains of *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* (Kurtzman *et al*., 1987). The incidence of aflatoxins in foods and feeds is relatively high in tropical and subtropical regions, where climatic conditions favor the growth of moulds (Rustom, 1997). Ochratoxin A is the second most important mycotoxin produced by *Aspergillus ochraceous, Aspergillus carbonarius* etc. It is receiving great attention worldwide because of the hazard it poses to animal and human health (Pitt *et al*., 2000). The contamination of cereal crops with ochratoxigenic fungi and ochratoxins is responsible for heavy losses in cereal crops. Devegowda reported that 25% of cereals approximately consumed in the world are contaminated with mycotoxins (Devegowda *et al*., 1998). Pakistan is situated in a subtropical to tropical region, with ideal environmental conditions and poor storage facilities that favor fungal growth. This fungal contamination of cereals, especially maize, leads to mycotoxin production. Mycotoxin contamination is a burning issue in Pakistan; the farming community and agrarian society in the country have serious concerns about this important matter. In Pakistan, so far, little published information is available about fungal mycobiota in agricultural products, particularly those used as ingredients in poultry and animal feeds. Only few reports covering short periods and smaller regions have described the presence of some toxigenic fungi or mycotoxins in agricultural products (Saleemullaha, 2006) and animal feed stuffs (Afzal *et al*., 1979; Hanif *et al*., 2006; Saleemi *et al*., 2010). There is inadequate research on fungi and mycotoxins in the country, but a few groups have recently started research work in this area. Keeping in view this problematic situation, this study was planned for isolation and identification of mycobiota of maize and its by-products and evaluation of their mycotoxigenic potential, because a problem can never be resolved until it is properly recognized.

Materials and methods

Study region and sample collection

Faisalabad is the third largest city of Pakistan, located in central Punjab province. It is located at latitude 31°- 26′ N, longitude 73°- 06′ E, altitude 184.4 m. It has a major grain market connected to neighboring agricultural towns. Also due to its road and railway connections to other cities, it receives grains from all agricultural areas of the country.

Eighty two samples of maize and eight samples of maize-gluten meal were collected over a period of two years from August 2005 to July 2007. These samples were collected from different grain markets, poultry feed ingredient suppliers, feed manufacturing units and home mix feed manufacturing poultry farms. These samples were mixed thoroughly and kept in polythene bags in quantities not less than 500 g and stored in refrigerator prior to inoculation onto culture media.

Isolation and identification of fungi

Intact and broken grains, granules and powders from different samples were inoculated in Petri plates containing different culture media. These were Czapek dox solution agar (Pitt and Hocking, 1997) for colony color and morphology for identification and yeast extract sucrose agar (Samson *et al*., 2004) for toxin production. The plates were incubated at 27°C in the dark for 7–10 days, then the representative colonies from different plates were sub-cultured on Czapek dox solution agar by culturing single conidia (Pitt and Hocking, 1997) for further studies of colony characters. Identification of fungi was made on the basis of their colony characters (diameter, color, fluorescence and texture of colonies) on different culturing media and microscopic characteristics on slide cultures using identification keys (Raper and Fennel, 1965; Klich and Pitt, 1988; Singh *et al*., 1991). The isolation frequency (Fr) and relative density (RD) of each species were calculated according to Gonazalez *et al*. (1995) as follow

$$\text{Fr}\ (\%) = \frac{\text{Number of samples with a species or genus} \times 100}{\text{Total number of samples}}$$

$$\text{RD}\ (\%) = \frac{\text{Number of isolates of species or genus} \times 100}{\text{Total number of samples}}$$

Determination of toxigenic potential of *Aspergillus* fungi

Toxigenic potential of pure cultures of *Aspergillus* fungi following inoculation on yeast extract sucrose

medium (YES) were determined by their fluorescence character in UV light at 364 nm wavelength (Yabe *et al.*, 1987). These plates were examined at 12, 24, 36, 48, 72h, and 4th, 5th and 7th day post inoculation. Both observed and reverse sides of the plates were examined under UV.

Aspergillus fungi isolates were further evaluated for their aflatoxin and ochratoxin A production potential by high pressure liquid chromatography (HPLC) with fluorescent detection. Fungal cultures were extracted by a modified micro-scale extraction method (Smedsgaard, 1997). Fungal isolates were inoculated on YES plates, at three equidistant points. These Petri-plates were incubated at 27°C for seven days in the dark. Culture extracts were made by cutting 18 plugs of 6 mm diameter, from the centre, at the rim of colonies and around colonies. These plugs were transferred to a 10-mL disposable autosampler screw-cap vial and 3 mL solvent mixture methanol-dichloromethane-ethyl acetate (1:2:3) containing 1% (v/v) formic acid was added. The plugs were extracted ultrasonically for 60 minutes. The extraction solvent mixture was similar to the extraction solvent used in the standardized HPLC method. These extracts were transferred into two vials using micropipettes, one containing 2.5 mL and another containing 0.5 mL. The 0.5 mL extract was used for analysis of mycotoxins. These extracts were evaporated to dryness under a gentle stream of nitrogen. The residues were re-dissolved ultrasonically for 10 minutes in 2.4 mL methanol containing 0.6% (v/v) formic acid, 0.02% (v/v) hydrochloric acid and 2.5% (v/v) water.

Analysis of aflatoxins and ochratoxin A was performed on the HPLC system (Prominence ™, Shimadzu®, Japan) equipped with fluorescent detector RF-10AXL® (Shimadzu, Japan) by using C-18 column, Mediterranea Sea18® 5µm 25cm × 0.46 Serial No. N45074 **(**Teknokroma, Spain).

Conditions for analysis of aflatoxins and ochratoxin A

Extracts of samples were derivatized prior to analysis (Anonymous, 2000). For aflatoxins**,** a mixture of acetonitrile **:** methanol: water (22.5 : 22.5 : 55) was used as the mobile phase with a flow rate of 1.0 mL min^{-1}, and the temperature of column oven was 30°C. The Mediterranea sea 18 (Teknokroma) 25 cm × 0.465µm column was used for both. The emission and excitation wavelengths were 360 and 440 nm, respectively. For OTA, a mixture of acetonitrile: water: acetic acid (57 : 41 : 2) was used as the mobile phase with a flow rate of 1.0 mL min^{-1}, and the temperature of column oven was 40°C. The emission and excitation wavelengths were 333 nm and 477 nm, respectively. Confirmation of OTA was done by determining methyl ester formation (Zimmerli and Dick, 1995). All the samples were analyzed three times and the mean of these values was taken as the representative value.

The limit of detection for aflatoxin was 0.02 ng g^{-1} and for ochratoxin A was 0.05 ng g^{-1}. The mycotoxin standards of Supelco USA for AFs and OTA were purchased from local suppliers and their specifications and concentrations were (Ochratoxin A, 50 µg mL^{-1}, Cat: 4-6912, Lot: LB46579 and Aflatoxin Mix Kit-M, 0.3–1 µg mL^{-1} Cat: 46304-U, Lot: LB44458, Supelco, USA).

Results

Out of a total of 82 samples of maize, 46 (56%) yielded fungi and 36 (44%) samples yielded no fungus. Isolated fungi belonged to genera *Aspergillus, Penicillium, Fusarium and Alternaria* and their isolation frequencies were 33, 28, 11 and 1 percent, respectively. Relative densities of different fungal genera on the basis of total fungal isolates (n=60) were *Aspergillus,* 45%; *Penicillium,* 38%: *Fusarium,* 15%; and *Alternaria,* 2% (Table 1). Isolation frequency and relative density among species was maximum for *P. verrucosum* followed by *A. niger* aggregates, *A. ochraceous, A. flavus, P. chrysogenum, A. parasiticus, A. carbonarius, Fusarium* spp. and *Alternaria* spp. Among *Aspergillus* species, *A. niger* aggregates and *A. ochraceous* were most frequently isolated, followed by *A. flavus, A. parasiticus* and *A. carbonarius.*

Out of 27 isolates of *Aspergillus*, 14 (52%) were toxigenic. Aflatoxigenic isolates included *A. flavus* (3/7, 43%), and *A. parasiticus* (2/3, 67%), as presented in Table 3. Ochratoxigenic isolates included *A. carbonarius* (1/1, 100%), *A. niger* aggregates (3/8, 38%), and *A. ochraceous* (5/8, 62%), as presented in Table 4.

Maize-gluten meal

Out of 8 samples of maize-gluten meal, 6 (75%) yielded fungi, while 2 (25%) samples yielded no fungus. Isolated fungal genera included *Aspergillus* and *Penicillium*. Isolation frequencies of these genera were 62 and 50 %, and relative densities were 56 and 44 %, respectively.

Table 1. Isolation frequency and relative density of different fungal genera and species isolated from maize samples (n=82).

Fungi isolated	No. of isolates	Isolation frequency (%)	Relative density (%) based upon total isolates (n=60)	Relative density (%) of *Aspergillus* spp. based upon total *Aspergillus* isolates (n=27)
Aspergillus	27	32.94	45	
A. carbonarius	1	1.22	1.67	3.70
A. flavus	7	8.54	11.67	25.93
A. niger aggregates	8	9.76	13.33	29.63
A. ochraceous	8	9.76	13.33	29.63
A. parasiticus	3	3.66	5	11.11
Alternaria	1	1.22	1.67	
Fusarium	9	10.97	15	
Penicillium	23	28.05	38.33	
P. chrysogenum	4	4.88	6.67	
P. verrucosum	19	23.17	31.67	

Table 2. Isolation frequency and relative density of different fungal genera and species isolated from maizegluten meal samples (n=8).

Fungi isolated	No. of isolates	Frequency (%)	Relative density (%) based upon total isolates (n=9)	Relative density (%) of *Aspergillus* spp. based upon total *Aspergillus* isolates (n=5)
Aspergillus	5	62.5	55.56	
A. ochraceous	1	12.5	11.11	20
A. flavus	3	37.5	33.33	60
A. niger aggregates	1	12.5	11.11	20
Penicillium	4	50	44.44	
P. chrysogenum	2	25	22.22	
P. verrucosum	2	25	22.22	

Table 3. Toxigenic potential of aflatoxigenic fungi isolated from maize .

Fungal species	No. of Isolates	Aflatoxigenic (%)	Aflatoxin levels AFB1(ng g^{-1}) Range
A. flavus	7	3 (43)	12.7 – 635.50
A. parasiticus	3	2 (67)	26.68 – 1374.23

Table 4. Toxigenic potential of ochratoxigenic fungi isolated from maize.

Fungal species	No. of Isolates	Ochratoxigenic (%)	Ochratoxin A (ng g^{-1}) Range
A. carbonarius	1	1 (100)	3.3
A. niger aggregates	8	3 (38)	1.30 – 1758.6
A. ochraceous	8	5 (62)	1.81 – 9523.1

Isolation frequency and relative density among species was maximum for *A. flavus* followed by *P. chrysogenum, P. verrucosum, A. niger* aggregates and *A. ochraceous*. Among *Aspergillus* species, *A. flavus* was the most frequently isolated, followed by *A. niger* aggregates and *A. ochraceous;* their isolation frequencies were 60, 20 and 20 percent, respectively (Table 2).

Out of 5 *Aspergillus* isolates, 2 (40%) were toxigenic. Aflatoxigenic isolates included *A. flavus* (1/3, 33 %). Ochratoxigenic isolates included *A. ochraceous* (1/1, 100%).

Discussion

In maize, isolation frequency among fungal genera was highest for *Aspergillus*, followed by *Penicillium*, *Fusarium,* and least for *Alternaria*. *P. verrucosum* was the most frequently isolated species, followed by *A. niger* aggregates, *A. ochraceous, A. flavus, P. chrysogenum, A. parasiticus, A. carbonarius, Fusarium* and *Alternaria* spp. Kpodo *et al.* (2000) in Ghana isolated *Aspergilli* as the predominant species followed by *Penicillium*, *Fusarium* and other fungi. Similar results have been reported from China (Gao *et al*., 2007) and Nigeria (Adebajo *et al*., 1994). Predominance of *Aspergillus* species followed by yeasts and *Fusarium* species has also been reported from maize silage in Argentina (Gonzalez *et al*., 2007). In their study, predominant species were *A. flavus* followed by *A. fumigatus* and *A. niger* aggregates. Atehnkeng *et al.* (2008) reported that the predominant fungal genus in maize was *Aspergillus,* followed by *Fusarium*. Pacin *et al*. (2002) from Ecuador reported *Fusarium* as the predominant genus isolated from maize followed by *Penicillium* and *Aspergillus*. In contrast to our study, Lamboni and Hell (2009) from Togo and Benin reported *Fusarium* spp. as predominant fungi and *Aspergillus* spp. were minimal from maize. Sampling strategy might be responsible for these differences because these workers collected samples from freshly harvested maize or silage while in our studies samples were collected from stored maize. Under storage conditions, *Aspergillus* and *Penicillium,* being storage fungi, are most active (Amadi and Adeniyi, 2009). Similarly, Pitt *et al*. (2000) reported that *Aspergillus* species are more commonly found as contaminants of commodities and foods during drying and subsequent storage.

Out of 27 isolates of *Aspergillus*, 51.8% were toxigenic. Aflatoxigenic isolates included *A. flavus* (42.9%) and *A. parasiticus* (66.7%). Ochratoxigenic isolates included *A. carbonarius* (100%), *A. niger* aggregates (37.5%), and *A. ochraceous* (62.5%). *Aspergillus parasiticus* produced higher concentrations of AFB1 (maximum 1374.2 ng g^{-1}) than *A. flavus* (maximum 635.5 ng g^{-1}). Giorni *et al*. (2007) reported that 70% strains of *A. flavus* and *A. parasiticus* were aflatoxigenic and their AF production potential varied from less than 10 ng g^{-1} (22.8%) to more than 1000 ng g^{-1} (24.3%). Similarly, Wicklow *et al*. (1998) reported that 53% of *Aspergillus* species produced aflatoxins. Variation among aflatoxigenic *Aspergillus* isolates in production of one or more of the four AFs (B1, B2, G1 and G2), as observed in the present study, has also been reported previously (Fraga *et al*., 2007).

OTA production potential of *A. ochraceous* ranged from 1.81 to 9523.1 ng g^{-1} while for *A. niger* aggregates it was 1.30 to 1758.6 ng g^{-1}. Magnoli *et al.* (2006) from Argentina reported that 30 (25%) out of 112 strains of black *Aspergilli* produced OTA, whereas only one strain out of four *A. ochraceous* produced OTA; however, maximum OTA produced by their isolates was 31.5 ng mL^{-1}, which was lower than that in the present study.

In, maize-gluten meal 75% samples were contaminated with fungi. *Aspergilli* were the most frequently isolated fungi followed by *Penicillium.* The most frequently isolated species was *A. flavus,* followed by *P. verrucosum*, *P. chrysogenum, A. ochraceous* and *A. niger* aggregates. *A. ochraceous* was 100% ochratoxigenic, while *A. flavus* was 33.3% aflatoxigenic. Information about maize-gluten meal fungal contamination is scant in available literature. This was first extensive study about mycobiota of maize and maize-gluten meal and their toxigenic potential.

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