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NATURAL OCCURRENCE OF AFLATOXINS, ZEARALENONE AND TRICHOTHECENES IN MAIZE GROWN IN PAKISTAN

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Abstract

A total of 65 maize grain samples , from 7 maize producing areas of Pakistan, were assayed for 14 toxicologically significant mycotoxins viz., aflatoxin B_1 (AfB_1), aflatoxin B_2 (AfB_2), aflatoxin G_1 (AfG_1), aflatoxin G_2 (AfG_2), zearalenone (ZON), deoxynivalenol (DON), 3acetyl-deoxynivalenol (3A-DON), 15acetyl-deoxynivalenol (15A-DON), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2), diacetoxyscirpenol (DAS), neosolaniol (NEOS) and fusarenone-x (Fus-x). Quantification was made by using high performance thin layer chromatography (HPTLC). The frequently encountered mycotoxin was AfB_1 – 27.69 % (n=18, range: 5-850 µg kg^{-1}; mean: 192 µg kg^{-1}). Other mainly detected mycotoxins include AfB_2 (18.46 %), NIV (12.31 %), DON (9.23 %), and 3ac-DON (7.69 %) with average values of 40, 1326, 1549, and 356 µg kg^{-1} respectively. DAS, T-2 and HT-2 were detected in only 9.23 %, 6.15% and 6.15% of samples respectively with relatively low concentrations. A co-occurrence phenomenon was observed in 12 (18.46 %) samples with a combination of two or maximum three different mycotoxins. It is a first preliminary study report dealing with 14 important mycotoxins simultaneously in maize from main maize growing areas of Pakistan.

Introduction

Mycotoxins, toxic secondary fungal metabolites, are one of leading perils to food and feed safety all over the world in general and particularly in developing countries. A wide range of chemically diverse mycotoxins (> 400 known) is being produced by *Aspergillus*, *Fusarium* and *Penicillium* which implicate educe different biological effects on living beings including humans and animals. Worldwide, Aflatoxins (AFs), Zearalenone (ZON) and Trichothecenes have been found to be associated with number of human disorders like hepatocarcinoma, precocious puberty and Alimentary toxic Aleukia-ATA (Peracia *et al*., 1999; Galvano *et al*., 2005). They are also best known for their toxic effects in animals (Placinta *et al.,* 1999; Anon., 2003; Dawegoda & Murthi, 1998; Morgavi & Riley, 2007). In addition to clinical manifestations, sub-clinical problems such as immunosuppression are non specific disorders that can be linked with mycotoxin ingestion. Furthermore, co-contamination of mycotoxins is an emerging concern because of mycotoxin-mycotoxin interactions as in combination they can produce synergistic, additive or antagonistic effects (Smith & Seddon, 1998; Anon., 2003)

Maize (*Zea mays* L.) is an important multipurpose crop of Pakistan, ranked as third important cereal after wheat and rice (Rafiq *et al*., 2010). It is being used as human food (especially in mountainous regions), livestock feed and also in wet milling industry. Apart from that, maize based products (corn flakes, corn flour etc.) and its by-products (corn gluten, starch) have wider applications in food and feed enterprises. Owing to its vast utility, its production has been enhanced from 3313MT in year 2008 to 3487 MT in 2010 (Anon., 2010). The main growing areas are concentrated in two provinces i.e. Punjab and Khyber Pakhtunkhwa (KPK). Moreover, it is cultivated twice in a year i.e. autumn maize (July/September) and spring maize (February/March).

Maize, being the world's important staple food (Anon., 2002) has been extensively studied for mycotoxin contamination as it has been found (among cereals) a very good substrate for fungal growth and toxigenesis (Trung *et al*., 2008). Worldwide surveys indicate the contamination of maize with aflatoxins, ochratoxin A, trichothecenes and fumonisins (Janardhana *et al.,* 1999; Kpodo *et al.,* 2000; Domija *et al*., 2005; Sangare-Tigori *et al*., 2006; Schollenberger *et al*., 2006; Binder *et al.,* 2007). There are several reports in the literature on the levels of aflatoxins (Shah *et al.,* 1981; Khan *et al.,* 1984; Shah *et al*., 1985) and ochratoxin A (Karim, 1993) in local varieties of maize. Furthermore recent domestic surveys report the presence of trichothecenes in wheat (Khatoon & Hanif, 2006) and poultry feed (Hanif *et.al,* 2006). However literature surveys indicate the paucity of information on the incidence of commonly occurring important fusariotoxins in local maize grains.

In view of foregoing, the primary objective set forth for the present study was to assess the natural occurrence, co-relation with origin/geography and co-occurrence of different mycotoxins in indigenous stored and freshly harvested maize samples collected from different maize growing areas.

Materials and Methods

Sampling: A total of 65 samples (0.5–1kg) of maize grains were collected in two batches for present study. Samples were randomly collected from open markets and farmers during the period of February to September, 2007 from major maize growing areas i.e., from five cities/ localities of Punjab (Murree, Rawalpindi, Faisalabad, Sahiwal, Multan) and two of KPK (Peshawar, Swat). Out of 65, thirty five samples were comprised of stored maize grains, corresponding to 2006 harvest and thirty freshly harvested samples were collected at the time of harvest belonging to spring crop of 2007. In both batches five samples were obtained from each region except Multan owing to non cultivation of spring maize in this area. All maize samples were apparently in good condition.

Chemicals and mycotoxin standards: All mycotoxin standards were procured from Biopure, Austria. All other organic solvents of GR grade were purchased from Merck AG, Germany. Clean up cartridges were procured from Romer Labs, Inc. (1301 Stylemaster Drive Union MO 63084, USA)

Mycotoxin quantification: Upon the arrival of samples in laboratory, the moisture content of the whole grains was determined by using a digital moisture meter (Draminski Moisture meter Model No. GMM 10330/04) as can be seen in Table 5. Afterwards the grains were milled by using RAS mill (Romer Labs, Inc., 1301 Stylemaster Drive, Union, MO 63084, USA). The ground samples' 75% could pass through a sieve of 20-mesh size. The ground samples, packed in air tight polythene bags covered by paper bags, were stored at 4 °C for future mycotoxins analyses.

Mycotoxin analysis was performed for AfB_1, AfB_2, AfG_1, AfG_2, ZON and trichothecenes including Type-A (NEOS, DAS, HT-2 and T-2 toxin) and Type-B (Fus-x, NIV, DON and its derivatives i.e. 3ac-DON, 15ac-DON). Samples were extracted and chromatographed according to previously published procedure (Hanif *et al*., 2006). In brief, extraction was performed with a mixture of acetonitrile and water (84:16; v/v). Clean up was carried out by using clean up cartridges i.e. MycoSep® 226 for aflatoxins and ZON, MycoSep® 227 and MultiSep® 216 for trichothecenes (Romer Labs. Inc., MO, USA). Quantification was accomplished by HPTLC at 365 nm. Other conditions are given in Table 1.

Meteorological data: Climatic information regarding mean temperature, rainfall and relative humidity of the relevant study areas were collected from Pakistan Meteorological Department, Islamabad (Table 2).

Table 1. Procedure outline for different mycotoxins in maize samples (Hanif *et al*., 2006).

Mycotoxin	Extraction solution (mL)	Clean up mode	TLC Plate	Re-dissolving solutions (ml)	Developing solution (ml)	LOQ (µg/kg)
Aflatoxins (B_1, B_2, G_1, G_2)	Acetonitrile: Water (84:16)	MycoSep® 226	Silica Gel 60_{F254}	Toluene: Acetonitrile (97/3)	Chloroform: Acetone (9:1)	B_1/ G_1= 1 B_2/ G_2 = 0.5
Zearalenone				Toluene: Acetonitrile (95/5)	Toluene: Acetone (1: 1)	ZON= 125
Type A Trichothecenes		MycoSep® 227 & MultiSep® 216 (Dual Clean up)	KC18-Silica gel	Toluene: Acetonitrile (97/3)	Methanol: Water: Acetic Acid (25: 15: 1)	T-2/HT-2= 100 DAS = 250 NEOS= 500
Type B Trichothecenes			Silica Gel 60_{F254}	Methanol: Acetone (1: 2)	Toluene: Acetone (1: 2)	DON/3 &15ADON = 100 NIV/FUSX = 500

Table 2. Meteorological data of sampling areas under study for 2006-2007[a].

Mean climatic conditions	Sampling areas						
	Punjab					KPK	
	Murree	Rawalpindi	Faisalabad	Sahiwal	Multan	Peshawar	Swat
Humidity (%)	65.22	54.44	61.75	63.92	49.63	59.75	69.72
Rainfall (mm)	95.34	106.91	47.39	39.12	17.03	22.53	117.22
Temperature (°C)	15.04	23.01	26.18	31.07	27.68	25.15	18

[a]Source: Pakistan Meteorological Department, Islamabad

Statistical analysis: SPSS 10.0 statistical software was used for statistical analysis. Multivariate analysis was performed for analyzing interactions of climate, area/ zone and type of maize on parameters (mycotoxin levels).

Results

A widespread occurrence of different mycotoxins was observed in domestic maize samples. Mean mycotoxin contamination levels and incidence of positive samples are shown in Table 3. Overall 37 (56.92%) samples were tainted with different mycotoxins. Most prevalent mycotoxins were aflatoxins followed by type B trichothecenes. Out of the 18 (27.69%) AFB_1 positive samples 13 (20%) were in the range of 1-200 μgkg^{-1} while five samples showed the highest levels greater than 200 μgkg^{-1}. In addition, 12 (18.46%) samples were also found positive for AFB_2 with a range of 3-187 μgkg^{-1}. Nivalenol was predominant trichothecenes detected in 8 (12.31%) samples at mean concentration of 1326 μgkg^{-1}. Of them five samples were heavily contaminated having NIV more than 1000 μgkg^{-1}. Six (9.23%) samples were harbored with DON at a mean level of 1549 μgkg^{-1}. With the exception of two samples, others had DON less than 500 μgkg^{-1}. In type A trichothecenes, DAS (9.23%), T-2 and HT-2 (each 6.15%) were detected in relatively low contamination level i.e., ≤ 500 µg/kg (Tables 3 & 4).

Table 3. Occurrence of mycotoxins in maize samples collected from different areas of Pakistan.

Toxin	Incidence (%)/ (No. of positive samples[n])	Min.–Max. (µg/kg)	Mean[a] (µg/kg)
AfB_1	27.69 (18)	5-850	192
AfB_2	18.46 (12)	3 -187	40
AfG_1	1.3 (2)	8-11	9
AfG_2	<0.5	-	-
ZON	1.3 (1)	1250	1250
DON	9.23 (6)	136-2625	1549
3ac-DON	7.69 (5)	100-850	356
15ac-DON	1.3 (1)	100	100
NIV	12.31(8)	500-2650	1326
DAS	9.23 (6)	364-750	516
HT-2	6.15 (4)	100-500	236
T-2	6.15 (4)	143-1125	506
Fus-X	<500	-	-
NEOS	<500	-	-

n = total samples (65); [a]mean of all positive samples

Data was also computed to analyze the distribution of mycotoxin contamination in two batches (Table 5). Aflatoxins, NIV and DAS were more prevalent, in terms of pervasiveness and mean levels, in freshly harvested maize samples of 2007 as compared to stored maize samples 2006. HT-2 and T-2 were detected in only stored maize samples.

The distribution of mycotoxins in maize grains of different geographic origin is shown in Table 6. *Aspergillus* toxins (AfB_1 and AfB_2) were pervasive in all areas except Murree where all samples were found negative. Highest incidence was observed in samples collected from Peshawar (10.77%). Whereas elevated trichothecenes incidence were observed in samples collected from Murree (DON- 7.69%, NIV- 3.08%, 3ac-DON and DAS-1.54%), Swat (HT-2 - 6.15%, T-2 - 3.08%, DON, 3ac-DON, 5ac-DON and NIV- 1.54%), Rawalpindi (NIV- 6.15%) and Peshawar (DAS-6.15%). Twelve samples (18.46 %) were found to be co-contaminated with multiple mycotoxins at different areas (Fig. 1).

Non significant ($p>0.05$) interactive effects of factors such as season and sampling zone/ area, during the survey, were observed on mycotoxin levels (except NIV) in maize.

Table 4. Frequency distribution of mycotoxin contamination (µg/kg) in maize grains collected from different areas of Pakistan.

Aflatoxins					
	<1[a]	**>1-50**	**>50-200**	**>200-400**	**>400-850**
AfB_1	47 (72.31 %)	7 (10.77 %)	6 (9.23 %)	3 (4.61)	2 (3.08)
AfB_2	53 (81.54)	10 (15.38)	1 (1.54)	1 (1.54)	-
Trichothecenes					
	<100*	**>100-500**	**>500-1000**	**>1000-1500**	**>1500-4500**
DON	59 (90.77)	4 (6.15)	-	-	2(3.08)
3ac-DON	60(92.31)	4(6.15)	1(1.54)	-	-
T-2	61(93.85)	3(4.62)	-	1(1.54)	-
HT-2	61(93.85)	4(6.15)	-	-	-
	<250*	**>250-500**	**>500-750**	**>750-1000**	**>1000-1250**
DAS	59(90.77)	4(6.15)	2(3.08)	-	-
	<500*	**>500-1000**	**>1000-1500**	**>1500-2000**	**>2000-3000**
NIV	57(87.69)	3(4.62)	1(1.54)	1(1.54)	3(4.62)

[a] Denotes the detection limits of the analytical procedure.

Table 5. Moisture and mycotoxins levels in stored and freshly harvested maize.

	Moisture (%)	Mycotoxins (µg/kg)							
		AfB_1	AfB_2	DON	3ADON	NIV	DAS	HT-2	T-2
Stored maize									
No. of samples/ Incidence (%)	-	6/ 17.14	3/ 8.57	3/ 8.57	4/ 11.43	3/ 8.57	1/ 2.86	4/ 11.43	4/11.43
Mean	12.74	96	51	765	441	500	750	236	506
Range	11.78-14.63	9-250	11-92	312-1584	250-850	500	-	100-500	143-1125
Fresh maize									
No. of samples/ Incidence (%)	-	12/ 40	9/30	3/10	1/ 3.33	5/16.67	5/16.67	ND	ND
Mean	14.61	226	36	1079	100	1710	458	-	-
Range	12.47-18.83	5-850	3-187	136-2625	-	800-2650	364-625	-	-

Table 6. Distribution of different mycotoxins in maize grain samples collected from different areas of Pakistan.

Areas	Mycotoxins							
	AFB1	AFB2	DON	3ac-DON	NIV	DAS	HT-2	T-2
Murree	-	-	+	+	+	+	-	-
Peshawar	+	+	-	-	-	+	-	-
Swat	+	-	+	+	+	-	+	+
Rawalpindi	+	+	-	-	+	-	-	-
Multan	+	+	-	+	-	-	-	-
Sahiwal	+	+	-	+	+	+	-	-
Faisalabad	+	+	-	+	-	-	-	+

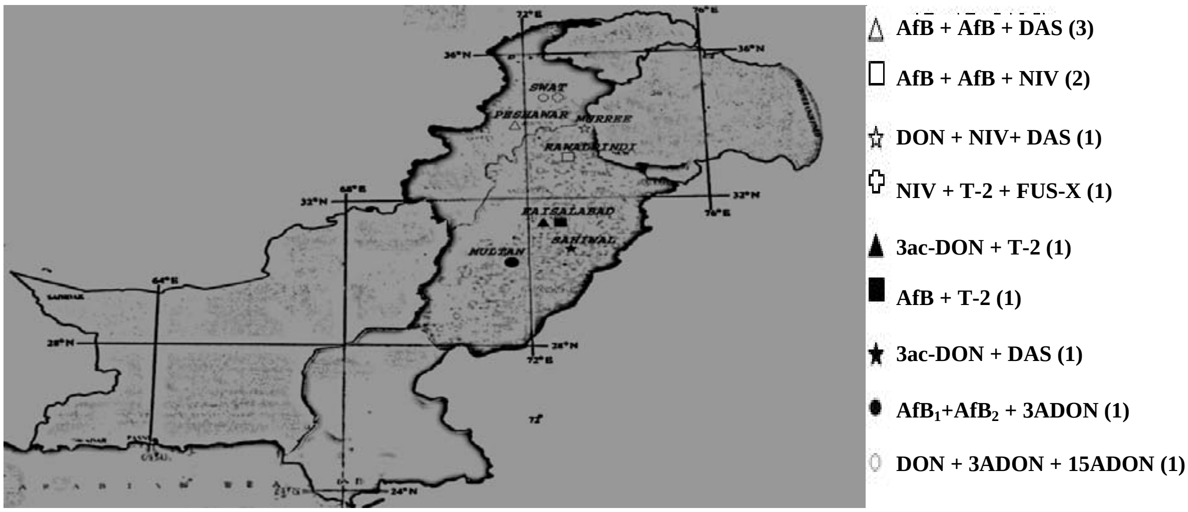

Fig. 1. Map showing co-occurrence of mycotoxins in seven study areas of Pakistan. Digits in parenthesis denote the number of samples contaminated with respective combinations.

Discussion

In Pakistan, maize is being grown under different topographical and ecological conditions. Rainy season and moderate temperature during pre-harvest period, intermittent showers during harvesting, traditional harvesting practices and inadequate storage facilities induce fungal contamination and accumulation of mycotoxins (Memon *et al*., 2011). In the present study an attempt has been made to determine the natural occurrence of various commonly occurring mycotoxins in sixty five (65) maize samples collected from different agro-climatic regions of Pakistan.

Present study revealed Aflatoxins (AfB_1 & AfB_2) to be the primary contaminants. Eighteen (18) of total samples (27.69%) were found positive for AfB_1. These results are in congruent with the previously reported studies in Pakistan (Shah *et al*., 1981, 1985; Khan *et al*., 1984; Hanif *et al*., 2006) and other Asian countries (Janardhana *et al*., 1999; Binder *et al*., 2007; Tangendjaja *et al.,* 2008). International Agency for Research on Cancer has classified it as group 1 carcinogen and one of the main etiological factors in hepatocelluler carcinoma (HCC) of humans (Anon., 1993). About three quarters of the cases of liver cancer are found in Southeast Asia. Various studies, from different countries of the region, associated high frequency of HCC with aflatoxin contamination (Lihua *et al*., 2002; Murugavel *et al.,* 2007).

In the present study, presence of fusariotoxins in maize samples was also assessed. The results of present investigation indicated the presence of trichothecenes in maize samples. Nivalenol (12.31%) and DON (9.23%) were found as the principal mycotoxins. Aflatoxins are considered to be the major contaminants of maize. It is therefore, local literature is sparse about occurrence of trichothecenes. Albeit, these results are in line with the findings of Schollenberger *et al.,* (2006). Who reported the same mycotoxins of group A (T-2, HT-2, DAS) and B (DON, NIV) as major contaminant of maize. Similarly Adejumo *et al.,* (2007) has reported the occurrence of DON, 3ac-DON and DAS in Nigerian maize. These trichothecenes mycotoxins are endowed with both acute and chronic aspects of toxicity. Main biochemical and cellular level effects include protein synthesis inhibition, induction of apoptosis particularly in lymphatic and hematopoietic tissue (Anon., 1999, 2000c, 2001a). Physiologically trichothecenes mycotoxins pose health risk to humans and animals by causing growth retardation, leucopenia/ reduced antibody production, reproductive defects and increased susceptibility to infections. Zearalenone, other important eutropic Fusarium mycotoxins, has been frequently documented in maize cereal. Presently its incidence was rare (1.53%) converse to previously reported studies (Yamashita *et al*., 1995; Lauren *et al.,* 1996; Pietri *et al.,* 2004; Schollenberger *et al*., 2006). The reason for this variation might be the climatic conditions and existence of different chemotypes of toxigenic species in different part of the world. *Fusarium asiaticum*, capable of producing NIV, DON and its derivatives, was found to be predominant species in China and other Asian countries whereas *Fusarium graminearum*- ZON and DON producer, is reported in Europe and North America (Gale *et al.,* 2002; Zhang *et al*., 2007).

The production of mycotoxins in agricultural commodities depends on such factors as geography, season and environmental conditions. In certain geographical areas of the world some mycotoxins are produced more readily than others (Devegowda *et.al.,,* 1998; Ratcliff, 2002). Presently two batches of maize seed samples were analyzed from seven different areas. Statistically no differences were observed in stored crop of 2006) and fresh crop of 2007 harvested samples of different origins. Surprisingly high frequency and levels of aflatoxins and trichothecenes were observed in freshly harvested samples as compared to stored samples. Yearly and seasonal variation in mycotoxins incidence has been observed (Broggi *et al*., 2007; Tangendjaja *et al*., 2008). Unusual rains and other ever changing stress factors may attribute to this variation.

Mycotoxin co-contamination is another concern as mycotoxins in combination appear to exert greater negative impact on the health in comparison with their individual effects. For humans, the toxicological implications of the concurrent mycotoxins are unknown but experimental studies on animals show that simultaneous exposure to these toxic agents (with different mechanism of action) raises the problem of addition or synergy (Anon., 2003). Presently, 12 samples (18.46%) were observed to be simultaneously contaminated with 2 or 3 mycotoxins. Most (n = 5) of the frequent combination was aflatoxin and trichothecenes (either type A or type B). Though, co-contamination phenomenon (with respect to aflatoxin and trichothecenes) has been sporadically documented. However, Wang *et al*., (1995) has reported the co-contamination of Vietnam maize samples with AfB_1, DON and NIV.

This study demonstrated that trichothecenes (particularly DON & NIV) in addition to aflatoxins are a concern in maize samples of Pakistan. Inevitable exposure of human population and livestock to these toxins with probable adverse outcomes require scientific based mangemental practices on this subject to minimize the opportunity for toxigenic fungi to produce mycotoxins. Furthermore, mycotoxin monitoring system, development and implementation of legislation is required to come across the mycotoxins menace at farmer/producer level. It is a first preliminary survey report dealing with fourteen commonly occurring mycotoxins simultaneously in maize samples collected from maize growing areas of Pakistan. This study may be used as a base for the further studies/assessment for other cereals as well.

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