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ASSESSMENT OF FUSARIOTOXINS AND TOXIGENIC POTENTIAL OF *FUSARIUM SPP.* IN MAIZE GRAINS COLLECTED FROM PAKISTAN

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ABSTRACT

A total of 65 maize grain samples (*Zea Mays L.*) collected from cold and hot climatic zones of Pakistan lead to isolation of 10 *Fusarium* supposing deep freezing method. Occurrence of *fusarium spp.* was high (75%) in cold climatic zones as compared to hot zones (25%). Fumonisin were recorded in both climatic zones, though their incidence was higher in hot climatic zone (range 4,379-9,990 µg/kg). Incidence of A and B trichothecenes was higher in cool climatic zone. Zearalenone (ZON) with mean level 500 µg/kg was positive in only 10% samples of hot climatic zone. Furthermore, co-contamination of fusariotoxins was also observed for fumonisins with ZON, A and B trichothecenes. Among 33 recovered isolates, *F. proliferatum*, *F. tricinctum*, *F. subglutinans*, *F. poae*, *F. nivale* and *F. acuminatum* were found toxigenic.

Keywords: fumonisins, synergism, climatic distribution, health hazards.

INTRODUCTION

Maize (*Zea mays L.*) is the third largest cereal crop of Pakistan, after wheat and rice. It is grown in irrigated as well as rain-fed areas. Its production has increased from 3,593 to 4,631 MT between 2008 and 2013 (Economic Survey, 2014). Punjab and Khyber Pakhtunkhwa (KPK) provinces are the major maize growing areas. Maize is a very versatile cereal, providing food, feed, adhesives, oil, syrups, flakes, alcoholic and non-alcoholic drinks, starch, ethanol and pop-corn, etc. (El-Imam *et al.*, 2012). Many feed mills utilize maize as the principal raw material. This important food source can be infested by different parasites, pests/ insects and fungi in field as well as during storage (Fandhon *et al.*, 2003; Matny, 2014). Among these, fungi rank second in deteriorating the maize quality (Saleemi *et al.*, 2012). Maize is considered as an ideal

substrate for fungal growth because of its high carbohydrate contents (Trung *et al.*, 2008). Spores of fungi are ubiquitously present in air and soil. Whenever, they find optimum conditions, they germinate and produce 'Mycotoxins' (Niaz and Dawar, 2009) chemically diverse group of toxic secondary metabolites of certain pathogenic fungi (Sultana *et al.*, 2013). The major toxigenic genera commonly recorded on maize in tropical regions, including Pakistan, are *Aspergillus*, *Penicillium* and *Fusarium* (Orisi *et al.*, 2000; Saleemi *et al.*, 2012). *Aspergillus* and *Penicillium* are mainly responsible for aflatoxins and ochratoxin A production. *Fusarium spp.* produces a wide range of mycotoxins, *viz.*, fusariotoxins (Tahira *et al.*, 2015).

Fusarium is a major genus of the filamentous fungi widely distributed in soil in association with plants, particularly cereal

grains. They may colonize in maize at all stages of their development, from the early hours of kernel germination to harvest time and even postharvest decay of grains (Fandhon *et al.*, 2003). Infection and contamination of maize by *Fusarium spp.* and fusariotoxins are generally influenced by many factors, including environmental conditions (climate, temperature and humidity), insect infestation, pre- and post-harvest handling (Bakan *et al.*, 2002). Several phytopathogenic species of *Fusarium* have been associated with maize, including, *F. verticillioides*, *F. proliferatum*, *F. graminearum* and *F. anthophilum* (Schollenberger *et al.*, 2006; Saleemi *et al.*, 2012).

Fusarium species have been extensively reviewed due to its pathogenicity and toxic nature (Fandhon *et al.*, 2003). The main toxins produced by *Fusarium* species are fumonisins (B₁, B₂ and B₃) and trichothecenes, i.e., Type A {T-2, HT-2, Diacetoscripenol (DAS), Neosolaniol (NEOS)}, B {(Fusarenon-x (Fus-x), Deoxynivalenol (DON), 3acetyl-DON (3ac-DON), 15acetyl-DON (15ac-DON), Nivalenol (NIV)} and Zearalenone (ZON). These have well documented deleterious effects, i.e. emesis, protein synthesis inhibition, hyperestrogenism and esophageal cancer in human and animals (CAST, 2003; Galvano *et al.*, 2005; Khatoon *et al.*, 2012; Sultana *et al.*, 2013).

In Pakistan, comprehensive research work has been carried out on seed borne mycoflora of different maize varieties (Niaz and Dawar, 2009; Saleemi *et al.*, 2012). Data concerning mycotoxins in maize grains of Pakistan were well reported (Khatoon *et al.*, 2012). However, toxicogenic potential of *Fusarium spp.* and scenario of fusariotoxins, particularly fumonisins, are missing. In view of foregoing, present study was

planned to investigate the *Fusarium* mycoflora, fusariotoxigenic species and fusariotoxins in maize collected from different climatic zones.

MATERIALS AND METHODS

Sampling

Randomly 65 maize grains samples (1.0kg) were collected from major maize growing areas. On the basis of meteorological data, sampling areas were divided into cold (Swat, Murree) and hot (Peshawar, Faisalabad, Sahiwal and Multan) climatic zones. This study was carried out at the premises of Romer Labs, Pakistan and plant pathology department (Arid Agriculture University Rawalpindi). The maize samples were preserved at -20°C till analysis to prevent further fungal contamination and mycotoxin production.

Mycological Analysis

For the *Fusarium spp.* isolation, deep freezing method was used as described by Mostafa *et al.* (2011). Surface of maize grains was disinfected by 1% Sodium-hypochlorite for one minute and rinsed twice in sterile distilled water for 30 seconds. Samples were plated @ 12 seeds/sample /plate on three layered sterilized moistened filter paper in 9 cm glass Petri dishes and incubated at 22±0.5 °C for 24 hours. Plates were then placed at -20°C for next 24 hours followed by incubation at 22 ± 0.5°C for five days. After incubation, slides were prepared using Lactophenol cotton blue stain (LCBS) as mounting reagent and examined under low (20x) and high (100x) magnification for the presence of mycelia and conidial structures and identified following Ninreberg *et al.* (1982).

Fusariotoxins Analysis

Total fumonisins (FB₁, FB₂ and FB₃) were analyzed using direct competitive ELISA. Samples were prepared following manufacturer's protocol provided along with AgraQuant[®] ELISA kit (Romer Labs Inc, USA). Weighed quantity (20g) of homogenized maize was mixed with 100 ml of 70% methanol, vortexed vigorously for two minutes and filtered through syringe filter (0.45 µm; MiniSart[®], Germany). Absorbance was read at 450 nm and differential filter at 630nm (ELISA reader BioTek Elx808, USA). Optical density (OD) was recorded and data was computed by using BIOTEK[®] Gen5 software (BioTek[®], USA). Limit of detection was 0.25-5 mg/kg for total fumonisins. Other fusariotoxins including Zearalenone (ZON), A and B trichothecenes were analyzed following Sultana *et al.* (2013).

Mycotoxigenic Potential

To assess the mycotoxigenic ability, 20 gm of rice was taken in 500 ml Erlenmeyer flask and 100 ml distilled water and autoclaved at 121°C for 30 minutes. Each flask was inoculated and incubated at 15 °C with daily shaking, for the first 3-4 days for even distribution of inoculums (Khatoon *et al.*, 2008). The detection and quantification of fusariotoxins in fungal cultures was carried out by using ELISA for fumonisins and HPTLC for ZON, A and B-trichothecenes respectively.

RESULTS

Maize grain samples collected from cold zone exhibited a higher (75%) infestation rate of *Fusarium spp.* as compared to hot climate zone (25%). Among *Fusarium spp.*, *F. proliferatum* was the most prevalent specie in samples

collected from both climatic zones (Table 1). Fusariotoxins analysis showed higher incidence of fumonisins, followed by B-trichothecenes, A-trichothecenes and ZON. Fumonisin concentration ranged from 4379 to 9990 µg/kg in samples of both zones. Type A-trichothecenes, i.e. DAS (1250 µg/kg; 10%), T-2 (500 µg/kg; 10%) and HT-2 (236 µg/kg; 30%) was more frequent in cold climate samples (Swat and Murree). In hot climate DAS (354 and 369 µg/kg; 10% and 15%), and T-2 toxin (450 µg/kg; 10%) were recorded only in Sahiwal and Peshawar samples. B-trichothecenes i.e. NIV and DON, 3ac-DON and 15ac-DON were isolated from cold climatic zone. ZON was detected in only 10% samples from Sahiwal with mean concentration of 500µg/kg (Table 2). Co-contamination of fusariotoxins was also observed in both climatic zones with different percentage incidences (Table 3). Recovered isolates (33) of *Fusarium*, belonged to 10 species proceed further for the mycotoxigenic potential assessment and twenty-two (66.66%) isolates exhibited their toxigenicity. Moreover, 30.30% isolates produced only single fusariotoxin, while 36.36% produced multi-mycotoxins (Table 4).

DISCUSSION

Fusarium Incidence

Maize is considered as the best substrate for germination of fungal spores because of its high carbohydrates contents (Fandhon *et al.*, 2005). Spores may spread in several ways, viz., birds, insects and wind, etc. Fungal spores may enter maize via silk hairs of comb and may create wounds in grains and grow (Matny, 2013 and 2014). In present study a higher incidence of *Fusarium spp.* infestation in cold zone (75%) was observed. It might be due to low temperatures (15-25°C) and

Table 1: Incidence (%) and distribution of different *Fusarium spp.* in maize samples collected from different climatic zones.

<i>Fusarium Spp.</i>	Incidence (%)	Cold Climatic Zone			Hot Climatic Zone		
		Swat	Murree	Peshawar	Faisalabad	Sahiwal	Multan
<i>F. proliferatum</i>	57.14	+	+	+	+	+	+
<i>F. subglutinans</i>	14.29	+	+	+	+	-	+
<i>F. graminearum</i>	7.14	+	+	+	+	+	-
<i>F. Poae</i>	7.14	-	+	-	-	-	+
<i>F. Nivale</i>	7.14	+	+	+	+	+	+
<i>F. chlamydosporum</i>	7.14	+	+	+	-	-	-
<i>F. acuminatum</i>	7.14	+	+	+	-	-	-
<i>F. tricinctum</i>	7.14	+	+	+	+	-	-
<i>F. semitectum</i>	7.14	+	+	+	+	+	-
<i>F. anthophilum</i>	7.14	+	+	+	-	+	+

Table 2: Incidence (%) and contamination levels (µg/kg) of fusariotoxins in maize samples collected from different cities (Pakistan).

Climatic Zones	Cities	Fumonisin (B ₁ +B ₂ +B ₃) (µg/kg)	A-Trichothecenes (µg/kg)				B-Trichothecenes (µg/kg)				ZON (µg/kg)	
			NEOS	DAS	HT-2	T-2	Fus-x	NIV	DON	3-ac DON		15-ac DON
Cold	Swat	51147 (60)	<125	<100	236 (30)	500 (10)	182 (10)	285 (10)	475 (10)	100 (10)	111 (10)	<30
	Murree	5019 (73)	<125	1250 (15)	<100	<100	<100	2650 (10)	876 (10)	312 (10)	<100	<30
	Peshawar	4379 (80)	<125	369 (15)	<100	<100	<100	1950 (10)	<100	<100	<100	<30
Hot	Faisalabad	9610 (100)	<125	<100	<100	<100	<100	<100	<100	2250 (10)	<100	<30
	Sahiwal	9990 (90)	<125	354 (10)	<100	450 (10)	<100	<100	<100	<100	850 (10)	500 (10)
	Multan	4700 (90)	<125	<100	<100	<100	<100	<100	<100	<100	<100	<30
EC Legislation, 2007		<1000	*NA	NA	NA	NA	NA	NA	1250	NA	NA	<75

*NA – Not Available; '<' shows limits of method

Table 3: Co-contamination of various fusariotoxins in maize grains.

Climatic Zones	Co-Contamination of Fusariotoxins					
	Two		Three		Four	
	Toxins	%	Toxins	%	Toxins types	%
Cold	DAS + Fum	6.60	DON+T-2+ Fum	3.33	3-Ace-DON +DON+DAS+ Fum	3.3
	DAS+ Fum	10	NIV+DAS+Fum	3.33	NIV+DAS+DON+Fum	3.3
	HT-2 +Fum	3.33	NIV+DAS+Fum	6.60		
Hot	ZON +Fum	1.17	15ac +T-2 +Fum	2.80	DAS+ T-2+15Ace+ Fum	2.80
	NIV +Fum	5.71	DAS +T-2+15 ac-DON	2.80	ZON +DAS + T-2+Fum	2.80
	DAS+Fum	3.30	ZON +DAS + T-2	2.80		

Table 4: Toxicogenic potential of isolated *Fusarium* species.

<i>Fusarium Spp.</i>	Number of isolates (n)	ZON	Toxicogenic Potential (mg/kg)									
			A-Trichothecenes				B-Trichothecenes					Fumonisin (B ₁ +B ₂ +B ₃)**
			NEOS	DAS	HT-2	T-2	DON	3ac-DON	15ac-DON	NIV	Fus-x	
Single mycotoxin producing <i>Fusarium Spp.</i>												
<i>F. proliferatum</i>	17	<0.03	<0.1	<0.1	<0.1	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	*12 (116.64)
<i>F. subglutinans</i>	03	<0.03	<0.1	<0.1	<0.1	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	(11.74)
<i>F. nivale</i>	02	<0.03	*1 (44.44)	<0.1	<0.1	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25
Multi mycotoxin producing <i>Fusarium Spp.</i>												
<i>F. acuminatum</i>	01	*1 (137)	<0.1	<0.1	<0.1	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	*1 (28.0)
<i>F. tricinctum</i>	03	*1 (71.43)	ND	*1 (145)	*1 (110)	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25
<i>F. Poae</i>	02	*1 (55.6)	*2 (38.38)	<0.1	<0.1	<0.10	<0.1	<0.1	<0.1	<0.1	1* (104)	<0.25
<i>F. anthropilum</i>	01	<0.030	(57.14)	<100	*1 (210)	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25
Non-toxicogenic producing <i>Fusarium Spp.</i>												
<i>F. semitectum</i>	01	<0.03	<0.1	<0.10	<0.10	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25
<i>F. graminearum</i>	01	<0.03	<0.1	<0.10	<0.10	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25
<i>F. chlamydosporum</i>	02	<0.03	<0.1	<0.10	<0.10	<0.10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.25

*Toxicogenic isolates; in parenthesis values are of mean level of toxin produced in mg/kg. '<' symbol indicates the detection limits of method (Sultana et al., 2013). ** Analysis by ELISA.

higher relative humidity levels which provides favorable environment for *Fusarium spp.* growth (Mangan *et al.*, 2010). Furthermore, water activity of maize samples also play key role for the colonization of fungi. Generally, water activity ($a_w > 0.7$) leads to fungal growth and < 0.7 considered as a measure to prevent fungal growth (Mostafa *et al.*, 2011). Time of sampling may also be an important contributing factor for this variation (Backhouse and Burgess, 2002). Present samples from hot zone (Faisalabad Sahiwal and Multan) were collected in July when temperature and relative humidity were $> 40^\circ\text{C}$ and $\leq 55\%$, respectively. However, in cold zone, harvesting of spring maize starts at the end of September (having temperature $\leq 30^\circ\text{C}$ and $\sim 65\%$ relative humidity). The higher temperature and lower relative humidity could be the reason for low (25%) incidence of *Fusarium spp.* infestation in hot zone.

This is the first report from Pakistan suggesting fumonisins as the most frequent fusariotoxin. A total of 28 fumonisins analogues have been identified and characterized so far, but FB_1 , FB_2 and FB_3 are abundantly found in naturally contaminated food (Pietri *et al.*, 2004). Fumonisins are more abundant in maize as compare to sorghum, rice and spices (Fandhon *et al.*, 2005; Saleemi *et al.*, 2012). The occurrence of fumonisins is associated with warmer and dry climate (Marsasa *et al.*, 2001; Pietri *et al.*, 2004; Galvano *et al.*, 2005; Fandhon *et al.* 2005; Binder *et al.*, 2007), which explains their higher incidence in Sahiwal and Faisalabad having hot climate. Detected levels of fumonisins in present samples were found far beyond (4.3 to 9.99 times) the allowable limit of EC, i.e., $1000 \mu\text{g}/\text{kg}$ (EC, 2007). These are strongly associated with esophageal cancer in human, leukoencephalomalacia (ELEM) in horses,

pulmonary edema syndrome (PES) in pigs and liver cancer in livestock and poultry (Shephard *et al.*, 2000; Gelderblom *et al.*, 2001; Matny, 2014). The international agency for research on cancer (IARC), has classified fumonisins as potential human carcinogen (type 2B carcinogen; IARC, 2002).

A and B trichothecenes were detected in maize grain samples from both cold and hot zones. Among these, European commission has defined regulatory limits for DON i.e. $1250 \mu\text{g}/\text{kg}$ (EC, 2007). In present study, DON with mean level of $913 \mu\text{g}/\text{kg}$ was observed and found below the EC regulatory limits. Trichothecenes are associated with various acute and chronic symptoms in human and animals. In human, trichothecenes are mainly responsible for impaired fertility, protein metabolism disturbance, lung diseases and cancer (Khatoun *et al.*, 2012; Sultana *et al.*, 2013; Kos *et al.*, 2014). Maize unfit for human consumption is usually consumed in animal feed as an energy source. Contaminated maize can cause vomiting, diarrhea, feed refusal, skin inflammation, hemorrhagic syndrome in internal organs, cellular damage to bone marrow, thymus and spleen (immunosuppressant), and disturbance of nervous system in animal (CAST, 2003; Galvano *et al.*, 2005).

Zearalenone, an important fusariotoxin, has been well recognized as maize contaminant. ZON induces hyperestrogenism in animal and human (CAST, 2003; Khatoun *et al.*, 2012). It is also declared as hepatotoxic in human (Zindine *et al.*, 2007). Due to its toxicity, European Commission (EC) has defined its permissible level in maize ($75 \mu\text{g}/\text{kg}$; EC, 2007). In present study only 10% samples collected from Sahiwal (hot zone) were positive for ZON. Although, the incidence

of ZON in present study was 10% but positive samples were contaminated with 6.66 folds higher level than the EC legislation (EC, 2007).

Co-contamination of mycotoxins is another aspect that has been highlighted because of mycotoxin-mycotoxin interactions. Various research studies revealed that combination of mycotoxins exerts synergistic, additive or antagonistic effects in consumers (Streit *et al.*, 2012; Sultana *et al.*, 2013). Fusariotoxins data of present study suggested that fumonisins were co-contaminated with trichothecenes. This co-contamination may prove more lethal even at lower levels (CAST, 2003; Striet *et al.*, 2013). Similar findings were reported from Vietnam (Wang *et al.*, 2000) and South East Asia (Yamashita, *et al.*, 1995). The multiple mycotoxin contamination in kernels may be due multi-species infestation of *Fusarium*. Furthermore, *Fusarium* does not grow alone, rather it forms complex either with other *Fusarium* or non-*Fusarium* species (Diaz and Borenman, 1994; Matny, 2014).

Mycotoxigenic Potential

In the past, researchers revealed *Fusarium spp.* as the main phytopathogenic fungi. However, since last two decades, interest for the investigation was turned over to its toxicity (Saleemi *et al.*, 2012). Various studies conducted in different regions of the world revealed that maize is highly susceptible to the invasion of pathogenic fungi, particularly *Fusarium* and *Aspergillus spp.* (Marsasa *et al.*, 2001; Verga *et al.*, 2005; Hussain *et al.*, 2013). During course of present study, 17 isolates of *F. proliferatum* recovered from maize grain sample collected from both climatic zones. It was due to its wider range of optimum conditions. Twelve (12) isolates

of *F. proliferatum* produced fumonisins with mean of 116.64 mg/kg. Only one isolate of *F. subglutinans* (out of 3) produced fumonisins with mean level of 11.74 mg/kg, though a previous study suggested it as a non-fumonisins producing species (Acuna *et al.*, 2005). This discrepancy may be due to differences in strains; some strains hold ability to produce toxins while others lack such an ability (Niaz And Dawar., 2009). Presently, *F. nivale*, *F. tricinctum* and *F. poae* were trichothecenes producers. *F. acuminatum* produced ZON and fumonisins, whereas *F. Anthophilum* produced NEOS and HT-2 toxins. These findings are in agreement with previously reports (Langseth *et al.*, 2000; CAST, 2003). *F. gramineum* is considered as fusariotoxin producers, but in present study, it was a non-toxigenic, which may an inter-strain variation or culture media differences (Matny, 2014).

Conclusion

Fusarium spp. were toxigenic and can produce different mycotoxins. Co-occurrence of mycotoxins may exaggerate the situation. This is a matter of great concern because maize and its products/ by-products have a significant place in human food and animal feed. Mycotoxins, particularly fumonisins, may pose a serious health concern to human and animals via their exposure to contaminated maize. A continuous monitoring is needed in order to avoid negative impact on human and animal health. Moreover, a detailed investigation at biochemical and molecular levels is suggested to give a broader understanding of the biodiversity of toxigenic *Fusarium species* in Pakistan and mechanism of mycotoxins production needs to be elucidated. Good agricultural practices, i.e. irrigation/ water supply, timely harvesting activity, proper drying and storage, are important tools for minimizing mycotoxin

contamination. An indigenous data based on the toxigenic nature of all commonly occurring toxigenic and non-toxigenic fungal species should also be generated.

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