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Fungal Infections and Aflatoxin Contamination in Maize Grains Collected from West Showa and East Wallega Zones, Ethiopia

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ABSTRACT

Mycotoxin affects the world's food crops and creates a large economical loss in the developed and developing countries. Aflatoxins are a group of mycotoxins that mainly produced by *Aspergillus* species viz., *A. flavus*, and *A. parasiticus*. An aflatoxins contamination of maize grains has exhibiting a serious threat to human and animal health over the past two decades. Toprotect the safety of food commodities, regular monitoring and diagnosis of the presence and amount of non-permissible levels of aflatoxins in food is necessary to take appropriate management measures. Maize grain samples were collected from Ilu Galan and Bako districts of West Shoa and Gobu Sayo district of East Wollega zones of Oromiya; from different grain storage types. About 500 gr of maize grains were sampled from each sampling spot. PDA media was used for isolation of associated maize grains sample associated fungi. Sun-culturing and purification of the associated fungi were done and preserved using agar slant technique. The associated fungal mycoflora were characterized based on morphological and growth sporulation properties. Enzyme Linked Immuno Sorbent Assay (ELISA) diagnostic kit were used for identification and quantification of aflatoxins. *Aspergillus, Fusarium Penicillium* and *Trichoderma* species were identified and characterized. Aflatoxin B1 was identified and quantified from zero to 381.6µg/kg. About 34.4% of the samples were positive to aflatoxin B1 compared to Food and Drug Administration (20µg/kg) and European Union (4µg/kg), respectively. The management of mycotoxigenic fungi, improvement of storage methods, development of resistant maize varieties and awareness creations could be possible solutions

Key Words: Maize grain, Storage methods, Fungi, Identification, Detection, Quantification and AflatoxinB1

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by certain fungi viz., *Aspergillus*, *Fusarium, Alternaria* and *Penicillium* spp. in agricultural products that are susceptible to mold infestations (Morenoa*et al.*, 2009). Now a day mycotoxin effectis attracting the worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade (Wagacha and Muthomi, 2008). These toxins are highly toxic and carcinogenic to animals and humans which lead to hepatotoxicity, teratogenicity, immunotoxicity and even death (Wen *et al*., 2004).Children below five years remain most vulnerable, with exposure damaging their immunity and causing stunted growth (www.aatf-africa.org). Mycotoxins contaminate and reduce crops quality through discolorations and reduction of nutritional values (Waliyar*et al*., 2008). Regulations on mycotoxins have been set and strictly enforced by most agricultural commodities importing countries, thus affecting international trade(FAO, 1988). In some developing countries where agricultural commodities account for about 50 percent of the total national exports, the economic importance of mycotoxins is considerable. FAO (2014) has estimated that about 25% of agricultural crops worldwide contaminated by mycotoxins. Similarly, the Center for Disease Control (CDC) has estimated that more than 4.5 billion people in the developing world are exposed to aflatoxins (CDC, 2004). The total allowable level of aflatoxin (µg/kg) in human food in different countries were reported i.e., Australia, china, European union, India, Kenya, Taiwan and USA is 15, 20, 4-15, 30, 20, 50, and20, respectively (FAO, 2004). The estimated

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crop lost due to aflatoxin is $225 million per year, out of the $932 million crop lost in each year in the USA (Betran and Isakeit, 2003).

Many studies across the world showed that the maize grains highly contaminated with aflatoxins that mostly caused by *Aspergillus flavus* and *Aspergillus parasiticus*(Patten, 1981; Munkvold, 2003). Aflatoxins can produced by fungal action during production, harvest, transportation, storage and food processing (CAST, 2003; Murphy *et al*., 2006). According to Befekadu and Berhanu (2000) the maize crop is the third most important crop in Ethiopia after wheat and teff which accounts for the largest share in total crop production. The maximum quantities of maize produced are stored under poor storage conditions. The traditional storage of maize in Ethiopia made up of mud, bamboo strips, and pits (Chauhan *et al*., 2016). Storage of maize grains under poor storage methods enhances the growth of fungi and promotes the production of mycotoxins (Chauhan*et al*.,2008).Despite the fact that maize is a crucial food to Ethiopia and it's vulnerable to aflatoxin risk due to different geographical and climatic conditions and poor handling (*Alemu et al*,2008). There are limited reports on the relationship between fungal infections and aflatoxins contamination in maize crop in Ethiopia.

MATERIALS AND METHODS

Description of the study areas and sample collections

The maize grain samples were collected from West Showa and East Wallega Zones of Oromia regional state which is located at about 250km to the West of Addis Ababa along the main road to Asossa. Ilu Gelan and Bako Tibe districts from West Shoa Zone; Gobu Sayo district from East Wallega Zone were selected for this studies based on their maize production potential and available storage systems. These areas annual rainfall and temperature range from 800 – 1000 mm and 15°C – 29°C, respectively. A total of 90 maize grain samples were collected; about 30 samples were collected from each district i.e., Ilu Gelan, Bako Tibe and Gobu Sayo. Maize grains were sampled from six traditional grain storage types. The samples were collected from different storage positions across different storage types. About 500gm maize grains were sampled from each sampling spot from the top, middle and bottom of each type of storage. The samples were temporary stored in the paper bag and transported to Ambo plant protection research center laboratory within 72 hour for fungal Microflora and Aflatoxins analysis.

Mycotoxigenic fungi isolation, characterization and identification

Agar plate method was used to determine the number and kind of fungi present. About 48 undamaged kernels of each sample was taken by a spatula directly into sterilized flasks and surface sterilized with 2% hypochlorite solution for 3 minutes and then rinsed in sterile distilled water. Potato dextrose agar (PDA) medium containing 100µg chloramphenicol per ml was used. About 8seeds per plate were cultured to isolate and detect the associated fungi. The plates were incubated at 25°C and the presence of *A. flavus* and other common fungi were observed after one week. The suspected mycotoxigenic fungi colonies were further purified individually by sub culturing on PDA plate and then on PDA slant. Isolated fungi were then identified according to Raper and Fennel (1965), Nelson *et al.* (1983), Rechard (1996), and Klich (2002) based on colony characteristics and morphology under light microscope.

Detection, identification and quantification of Aflatoxin B1

Specific ELISA kit was used for the detection, identification and quantifications aflatoxin B1. The samples preparation, extraction and purification were done according to the instruction given by the company (RIDASCREEN®Aflatoxin B1, Germany). ELISA reader was employed for the quantification of aflatoxin B1. Finally, detected and quantified aflatoxin B1 was used for analysis across each samples, grain and storage types.

Statistical Analysis

Statistical analysis on aflatoxin B1 concentration and standardized curve of determination was performed using mini tab version 20 and all the graph and percentage was done by excel.

Results

Assessment and identification of fungal microflora

The assessment of associated and identified fungi *spps*. were provided below in the Fig. 1. A total of nine species of fungi were isolated and identified as *Aspergillus flavus, Aspergillus parasiticus, Fussarium verticilliodes, Penecillium notatum, Penecillium verrucosum, Fussarium proliferatum,Fussarium gramminearum, Aspergilus niger, and Trichoderma Spp*. The most common fungi isolated from the maize grains were *Aspergillus flavus (25.7%)* and *Aspergillus parasiticus (18.9%)* from the total of ninety maize grain samples.

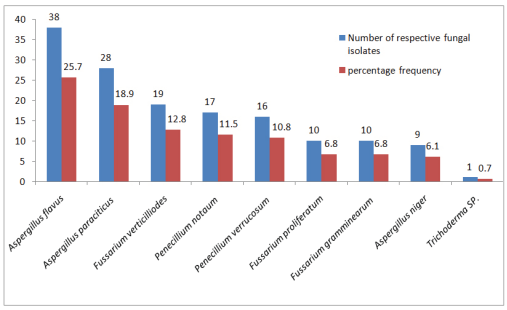

Figure 1: Identified and percentage frequency of associated fungi mycoflora.

Occurrences of mycotoxigenic fungi in different maize storage types

Mycotoxigenic fungal occurrence were assessed in all maize samples collected from the six of storage types; namely open above ground(OAG), sack in open air(SOA), sack in house(SH), open sorghum stalk (OSS), improved gottera(IG), and in house ground (IHG) storages (Table1). Accordingly, the highest occurrence of mycotoxigenic fungi were seen in OAG storage typewhich has been accounted about 44(29.7%) followed by SOA 27(18.2%) and few mycotoxigenic fungal 5(3.4%) was isolated from IG. *Aspergillus* species; *A. flavus, A. parsiticus* and *Fussarium* species; *F. gramminearum*, *F. verticilliodes* were the most prevalent mycotoxigenic fungi in OAG and SOA storage types.

Table 1: Mycotoxigenic fungi occurrence in relation to storage types in number and percentage

Fungal type	Occurrence across all storage	Open above ground	Sack in open air	Sack in house	Open sorghum stalk	Improved 'Gottera'	In house ground
Aspergillus flavus	38	10(26.3)	7(18.4)	8(21.1)	6(15.8)	2(5.3)	5(13.2)
Aspergillus parasiticus	28	7(25)	3(10.7)	4(14.3)	7(25)	1(3.6)	6(21.4)
Fussarium verticilliodes	19	5(26.3)	7(36.8)	1(5.3)	4(21.1)	0	2(10.5)
Penecillium notatum	17	6(35.3)	2(11.8)	3(17.6)	2(11.8)	1(5.9)	3(17.6)
Penecillium verrucosum	16	5(31.3)	3(18.8)	2(12.5)	3(18.8)	0	3(18.8)
Fussarium proliferatum	10	4(40)	2(20)	2(20)	1(10)	0	1(10)
Fussarium gramminearum	10	5(50)	3(30)	0	2(20)	0	0
Aspergillus niger	9	2(22.2)	0	3(33.3)	1(11.1)	1(11.1)	2(22.2)
Trichoderma Spp	1	0	0	0	0	0	1(100)
Total	148	44(29.7)	27(18.2)	23(15.5)	26(17.6)	5(3.4)	23(15.5)

Aflatoxins detection and identification in maize grain samples

Aflatoxins B1 has been identified from the maize samples detected in laboratory using Enzyme Linked Immuno Assay (ELISA). The results of ELISA has reveal that the mean31 (34.4%) of 90 maize grain sample were above and shown toxicity higher than those recommended by Food and Drug Administration (2004) and European Union (2018)standards which states that maximum permissible level of afaltoxin B1 in maize should be 20µg/kg and 4µg/kg, respectively. Higher aflatoxin B1 concentration (73.3%) was observed in maize grains sampled from Gobu Sayo district followed by-Ilu Gelan(20%)(Table 2). The determination of aflatoxinB1 concentration was done by developing a curve from the supplied aflatoxin B1standard which has ranged from 0-4.5ppb (Fig. 2).

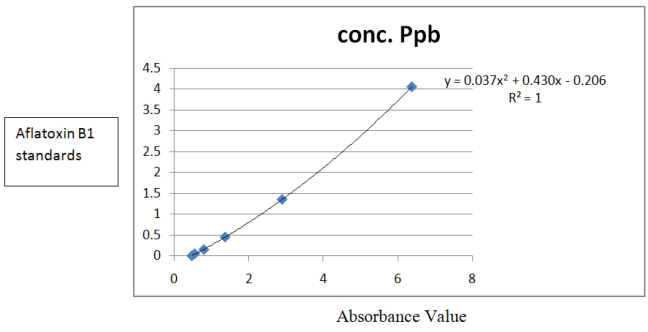

Figure 2: Aflatoxin B1 determination curve.

Table 2: Prevalence of AflatoxinB1in the three districts across all maize grain samples

District	No. of maize grain samples	No. of aflatoxin B1positive samples
Ilu Gelan	30	6 (20%)
BakoTibe	30	3 (10%)
GobuSayo	30	22 (73.3%)
Total	90	31 (34.4%)

Aflatoxin B1 concentration across storage and grain types

The level of aflatoxin B1 concentration is presented in Table 3. It was observed that the level of aflatoxin B1 in sample 2,14, 57,71,74,75,78,82 and 83 was detected and measured between 3.9-381.6 ppb and its corresponding storage type were open above ground, open sorghum stalk and sack in house. It is also observed that the aflatoxin B1 level was varied with the maize grain storage types. Open above ground, open sorghum stalk and sack in house storage types were exposed to rain and high temperature which has been created conducive environment for mycotoxigenic fungi growth and development.

Table 3: Aflatoxin B1 concentration across each maize grain sample and storage type

Sr. no.	Sample ID.	Variety	Storage type	Con.(ppb)	Sr.no.	Sample ID.	Variety	Storage	Con.(ppb)
1	IG-1	BH660	OAG	0.00	27	IG-27	LIMU	SOA	0.0033
2	IG-2	BH661	OAG	3.9	28	IG-28	LIMU	SH	0.068
3	IG-3	LIMU	SOA	0.002	29	IG-29	LIMU	SOA	0.0017
4	IG-4	LIMU	OAG	0.0034	30	IG-30	BH661	SH	0.0012
5	IG-5	LIMU	OAG	0.0068	31	BT-1	BH661	SH	1.3
6	IG-6	LIMU	SOA	0.0046	32	BT-2	LIMU	OSS	0.0026
7	IG-7	BH540	SOA	0.0008	33	BT-3	LIMU	OSS	0.0068
8	IG-8	BH660	OSS	0.0005	34	BT-4	LIMU	OSS	0.00033
9	IG-9	LIMU	SH	0.0003	35	BT-5	ND	OSS	0.0017
10	IG-10	LIMU	SOA	0.0027	36	BT-6	LIMU	SH	0.00035
11	IG-11	BH540	IG	0.0062	37	BT-7	LIMU	SH	0.0023
12	IG-12	BH661	OAG	0.00003	38	BT-8	LIMU	IHG	0.37
13	IG-13	shone	IG	0.0021	39	BT-9	BH540	IHG	0.0018
14	IG-14	BH540	SOA	17.43	40	BT-10	LIMU	IHG	0.0071
15	IG-15	LIMU	SOA	0.0009	41	BT-11	LIMU	SH	0.0002
16	IG-16	BH540	IG	0.075	42	BT-12	LIMU	SH	0.00004
17	IG-17	BH660	IG	0.093	43	BT-13	LIMU	SH	0.00027
18	IG-18	LIMU	IG	0.8	44	BT-14	LIMU	OAG	0.00027
19	IG-19	LIMU	OAG	0.0033	45	BT-15	LIMU	SH	0.00003
20	IG-20	BH540	OAG	2.2	46	BT-16	LIMU	OAG	0.0018
21	IG-21	BH661	IHG	0.016	47	BT-17	BH540	OAG	0.00025
22	IG-22	LIMU	SOA	0.015	48	BT-18	BH541	OAG	0.00003
23	IG-23	BH660	SOA	0.0037	49	BT-19	LIMU	OAG	0.19
24	IG-24	BH661	SOA	0.0039	50	BT-20	LIMU	OAG	0.0017
25	IG-25	BH661	OAG	1.2	51	BT-21	LIMU	OSS	0.0016
26	IG-26	LOCAL	OAG	1.4	52	BT-22	LIMU	OSS	0.0018

Sr. no.	Sample ID.	Variety	Storage	Con.(ppb)	Sr.no	Sample ID.	Variety	Storage	Con.(ppb)
53	BT-23	LIMU	OSS	0.0002	72	GS-12	LIMU	SH	1.7
54	BT-24	BH540	OSS	0.0003	73	GS-13	LIMU	OAG	2.97
55	BT-25	LIMU	OSS	0.0082	74	GS-14	BH540	OSS	19.05
56	BT-26	LIMU	OSS	0.00038	75	GS-15	LIMU	SH	4.07
57	BT-27	LIMU	SH	5.09	76	GS-16	QPM	OSS	1.18
58	BT-28	ND	OAG	0.00045	77	GS-17	LIMU	OSS	2.6
59	BT-29	LIMU	IHG	2.25	78	GS-18	LIMU	SH	6.1
60	BT-30	SHONE	IHG	0.00019	79	GS-19	LIMU	OAG	0.002
61	GS-1	BH660	OSS	1.3	80	GS-20	BH540	SOA	0.0003
62	GS-2	BH660	SH	0.001	81	GS-21	BH660	SH	0.00006
63	GS-3	BH661	SH	0.00003	82	GS-22	BH660	SH	6.3
64	GS-4	LIMU	IHG	0.008	83	GS-23	BH660	OSS	30.04
65	GS-5	LIMU	OSS	0.002	84	GS-24	BH660	SOA	0.007
66	GS-6	BH661	IG	0.002	85	GS-25	BH660	SOA	1.5
67	GS-7	BH661	SH	0.000007	86	GS-26	BH540	OSS	0.99

Table 3: *(Continued)*

68	GS-8	LOCAL	OAG	1.7	87	GS-27	BH661	OSS	0.9
69	GS-9	BH540	OAG	0.002	88	GS-28	BH660	OSS	0.9
70	GS-10	LIMU	OAG	1.6	89	GS-29	BH661	OSS	0.2
71	GS-11	LIMU	OAG	381.6	90	GS-30	LIMU	SOA	0.5

Notice: OAG; open above ground, OSS; open sorghum stock, SH; sack in house, SOA; sack in open air, IG; improved gottera, IGH, in house ground storage

The highest concentration of aflatoxinB1was detected and quantified in LIMU variety (8.9 ppb) followed by BH660 (4.0ppb) and BH540 (1.6ppb). Out of the total 90 sample about 34.4 % (31 samples) were possessed more than 0.05ppb concentration of aflatoxins B1 while 65.6 % (59 samples) have had the aflatoxin B1 level less than 0.05 ppb. Whereas, results of ELISA has demonstrated that 0.2 ppb as a mean aflatoxin B1 concentration for all 90 maize grain samples tested(Table 4).

Table 4: Aflatoxin B1concentration across maize grain types

Variety type	Number of sample	AFTB1 ppb or (µg/kg)
LIMU	46	8.9
BH540	13	1.6
BH660	12	4.00
BH661	11	0.68
Local	2	1.55
Shone	2	0.0012
Unknown	2	0.0011
QPM	1	1.18
BH541	1	0.00
Total	90	17.9
Mean	90	0.2

The effect of maize grain storage types on the growth of mycotoxigenic fungi which in turn favor the production of aflatoxin B1were analyzed from the data of mycological results. There were six types of maize grain storage were observed during sample collection (Fig. 3). The occurrences of aflatoxin B1in different storage types were recognized different in concentrations. Accordingly, the highest aflatoxin B1 concentration were recorded in open ground (18.03 ppb) and the lowest aflatoxin B1 concentration were observed in maize grain stored in improved gottera (0.16ppb) as stated in Table 5.

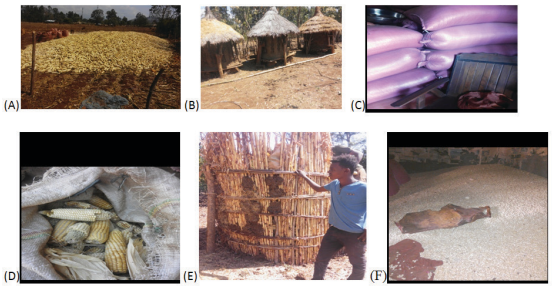

Figure 3: Open above ground(A), improved gottera(B), sack in house(C), sack in open air (D), Open sorghum stalk (E), in house ground (F).

Table 5: Aflatoxin B1 concentration across storage types

Storage type	Number of sample	AFTB ppb or (µg/kg
Open above ground	22	18.03
Sack in open air	15	1.3
Sack in house	19	1.3
Open sorghum stalk	21	1.5
Improved gottera	6	0.16
In house ground	7	0.38
Total	90	22.67
Mean	90	3.77

DISCUSSIONS

Several fungal species have been isolated from the maize grain sampled from three districts. Mycotoxigenic fungi were isolated and identified from the maize grain samples as well as the associated aflatoxin B1 was detected and quantified. *Aspergillus* spp. were the most predominant mycotoxigenic fungi with 50.7% frequency of occurrence fol-

lowed by *Fussarium* spp. with 26.4%, and *Penicillium* spp with 22.3%.*Trichoderma* species was also isolated in trace amount(1.07%). Similar studies were done in Gedeo zone by Chauhan *et al*. (2016)and in Kewot Provence by Geremew Tassew *et al*. (2016). The higher frequency of fungal infection specifically *Aspergillus* spp. was due to poor storage types and longtime storage greater than two yearsas similarly reported by Habtamu *et al*.(2001).The maize grain aflatoxigenic fungi contamination started from the fields before harvest and continued across storage, consumptions and marketing which is similarly reported by Bhat *et al*. (1997) and Gao *et al*. (2007)in different maize growing countries like Ethiopia Kenya, Somalia, Uganda and Sudan. The prevalence of maize grain aflatoxin B1 contamination has reached 34.4% and higher aflatoxin B1 concentration 22 (73.3%) were observed in Gobu Sayo province followed by Ilu Gelan6 (20%).About 3.3 %and 7.7% maize samples had aflatoxinB1 higher than those recommended by Food and Drug Administration (FDA; 20µg/kg) and European Union (EU; 4µg/kg) regulatory levels respectively. The observed aflatoxin B1 concentration was very low compared to the reports of Chauhan *et al*. (2016) that has stated mean aflatoxins concentration for a two year stored maize grain samples53 ppb with 100% contamination in aflatoxin. The highest aflatoxin B1 concentration were recorded in open ground (18.03 ppb) this could be due to the exposure of the grain to favorable temperature and rain which in turn facilitate the growth of aflatoxigenic fungi, and the lowest aflatoxin B1 concentration were observed in grain stared in improved gottera(0.16ppb).Generally, there should be the management of mycotoxigenic fungi starting from the field, harvesting, transport, and storage through the development of mycotoxins resistant maize varieties, improvement of grains storage types and awareness creations.

CONCLUSIONS

The fungi isolated in the present study were from the different genera that are common in maize grain. *A. flavus* was the predominant one while other toxin-producing species such as *Aspergillus parasiticus, Fussarium verticiloide* and *Penecillium notatum* occurred at relatively at the higher levels. The aflatoxin B1 concentration in the majority of the sample are below the recommended level however, in few of the sample its level is much higher than the EU standard. The level of aflatoxinB1 concentration is higher in maize grain stored in open ground field.

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