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The Occurrence of Aflatoxins in Maize and Distribution of Mycotoxin-Producing Fungi in Eastern Kenya

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Abstract: Aflatoxin poisoning resulting from consumption of contaminated maize has continued to recur in a yearly pattern in Eastern Kenya. The largest mycotoxin-poisoning epidemic in the last decade was reported in Kenya in 2004. Therefore, this study was carried out to determine the occurrence and levels of mycotoxin-producing fungi and aflatoxin B_1 in maize and soils from Eastern Kenya. Maize, soils and mill dust samples were collected from farmers and traders in Machakos to determine the incidence of mycotoxin-producing fungi and aflatoxins during the 2007 harvest season. Fungal isolation was done by plating on agar medium, while aflatoxin B1 was determined by ELISA. The most frequently isolated fungi were *Fusarium* and *Aspergillus* species and the *Aspergillus* species identified were *A. flavus*, *A. niger*, *A. terreus* and *A. versicolor*. *Aspergillus flavus* was frequently isolated from mill dust and soils from under the stores. Aflatoxin levels of up to 160 μg kg^{-1} were detected in samples from areas with high *A. flavus* isolation and in whole maize than in semi-processed grain. Most mill dust samples were contaminated with aflatoxin up to 80 μg kg^{-1}. The results indicate that *A. flavus* is the main producer of aflatoxins in maize Machakos and high inoculum levels of the fungus are present in soils, near stores and maize mills. Therefore, management of aflatoxin poisoning should include reduction of *A. flavus* inoculum in farms and storage environment.

Key words: Aflatoxicosis, *Aspergillus flavus*, contamination, inoculum

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by fungi and contaminate various agricultural commodities either before harvest or under post-harvest conditions (FAO, 1991). The most important mycotoxins are aflatoxins, ochratoxins, deoxynivalenol (DON), zearalenone, fumonisin, T-2 toxin and T-2 like toxins. However, food borne mycotoxins likely to be of greatest significance in tropical developing countries are the fumonisins and aflatoxins (Kumar *et al*., 2008; WHO, 2006). About 4.5 billion people in developing countries are chronically exposed to aflatoxin (Williams *et al*., 2004) and the CODEX recommended sanitary and phytosanitary standards set for aflatoxins adversely affect grain trade in developing countries (Gebrehiwet *et al*., 2007).

Aflatoxins are produced by *Aspergillus flavus* and the fungus forms sclerotia which allow it to survive in soil for extended periods of time (Scheidegger and Payne, 2003). The sclerotia are the principal sources of primary inoculum. Conditions such as high temperatures and moisture, unseasonal rains during harvest and flash floods lead to fungal proliferation and production of mycotoxins (Bhat and Vasanthi, 2003; Thomson and Henke, 2000). Poor harvesting practices, improper storage and less than optimal conditions during transportation, marketing and processing can also contribute to fungal growth and increase the risk of mycotoxin production. While, aflatoxins occur mostly in maize and groundnuts, the prevalence of fumonisins in maize is 100% (Bankole *et al*., 2006; Wagacha and Muthomi, 2008). Mycotoxins have negative impact on human health, animal productivity and trade (Wagacha and Muthomi, 2008; WHO, 2006; Wu, 2006). Aflatoxin B_1 is the most toxic and is associated with liver cancer and immune suppression (Shephard, 2008). Exposure to large doses (>6000 mg) of aflatoxin may cause acute toxicity with lethal effect, whereas exposure to small doses for prolonged periods is carcinogenic (Groopmann and Kensler, 1999). There may be an interaction between chronic mycotoxin exposure and malnutrition, immuno-suppression, impaired growth and diseases such as malaria and HIV/AIDS (Gong *et al*., 2003, 2004; Williams *et al*., 2004). Mycotoxin poisoning may be

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compounded by the co-occurrence of aflatoxins with other mycotoxins such as fumonisins, zearalenone and deoxynivalenol (Kimanya *et al*., 2008; Pietri *et al*., 2009).

However, the presence of mycotoxins in food is often overlooked due to public ignorance about their existence, lack of regulatory mechanisms, dumping of food products and the introduction of contaminated commodities into the human food chain during chronic food shortage due to drought, wars, political and economic instability. The largest mycotoxin-poisoning epidemic in the last decade occurred in Kenya in 2004 (Lewis *et al*., 2005; CDC, 2004). Aflatoxin poisoning was associated with eating home grown maize stored under damp conditions (Lewis *et al*., 2005). Acute aflatoxin poisoning has continued to occur severally in Eastern and Central provinces of Kenya (Lewis *et al*., 2005; CDC, 2004; Ngindu *et al*., 1982). In the 2004 aflatoxin-poisoning outbreak, the concentrations of aflatoxin B1 in maize was as high as 4,400 ppb, which is 220 times greater than the 20 ppb regulatory limit. The outbreak covered more than seven districts and resulted in 317 case-patients and 125 deaths (Lewis *et al*., 2005). Other smaller outbreaks were reported in 2005 and 2006, with about 30 and 9 deaths, respectively (Ministry of Agriculture, 2006, 2007). The maize implicated in the 2004 aflatoxicosis outbreak was harvested in February during off-season, early rains.

The repeated outbreaks of aflatoxicosis in Eastern Kenya have worsened the food security situation, since maize is the main staple in almost every household. Information on the levels of *Aspergillus flavus* inoculum in the environment and contamination of maize with aflatoxins would be valuable in the development of mycotoxin management strategies. Therefore, this study was carried out to determine the occurrence and levels of fungi and aflatoxin B_1 in maize and soils from greater Machakos Region.

MATERIALS AND METHODS

Sample collection: The study was conducted in 2007 after the harvest of the 2006-2007 cropping season in the greater Machakos districts of Eastern Kenya where recurrent aflatoxin poisoning is common. Samples were collected from nine agro-ecological zones (lower highland zones 2 and 3, upper midland zones 2, 3, 4 and 6 and lower midland zones 3, 4 and 5). The nine agro-ecological zones fall under the administrative divisions of Kathiani (LH 2 and LH 3), Central (UM 2 and UM 6), Matungulu (UM 3 and UM 4), Ndithini (LM 3), Katangi and Yathui (LM 4 and LM 5). About 1 kg samples of maize grain, semi-processed grain, mill dust and soils were collected from at least five farmers per ecological zone and two processors per division from Central, Katangi, Kathiani, Matungulu, Ndithini and Yathui divisions. In addition, soils from under the stores were collected from each farmer. The samples were stored at 4°C until fungal isolation and aflatoxin analysis.

Isolation, culturing and identification of fungi: Sub-samples of maize kernels or semi processed grains were surface sterilized in 2.5% sodium hypochlorite with 2-3 drops of Tween 20 for 2-3 min and rinsed off three times in sterile distilled water. A total of 100 kernels per sample were plated on Czapek Dox agar amended with 25 ppm of antibiotics chloramphenicol, streptomycin, penicillin and fungicide pentachloronitrobenzene (PCNB). Five kernels were aseptically plated in each plate and incubated at 25°C for 7-10 days. One gram of soil samples and mill dust were serialy diluted in sterile distilled water and 1 mL of 10^{-3} and 10^{-4} dilutions plated in Czapek Dox agar. The number of infected kernels and number colonies for each fungus type was recorded. The number of colony forming units (cfu g^{-1}) was calculated for the soil samples and mill dust. The fungi were identified based on cultural characteristics like growth rate, colour, aerial mycelium and reverse surface. *Aspergillus* and *Penicillium* colonies were sub-cultured on Czapek dox agar and identified to species level based on morphological and cultural characteristics as described by Gilman (1957) and Gao *et al*. (2007). *Fusarium* colonies were sub-cultured on Potato Dextrose Agar (PDA) and identified to genus level on basis of morphological characteristics as described by Nelson *et al*. (1983).

Aflatoxin analysis in maize and mill dust samples: Aflatoxin content in the samples was analyzed by direct competitive Enzyme-Linked Immunosorbent Assay (ELISA), (AOAC, 1995; Gathumbi, 2001; Gathumbi *et al*., 2001). Maize grain, semi-processed grains and mill dust were ground to fine powder and five grams of the powder extracted with 25 mL methanol: water (50:50 v/v). The extract was defatted with 10 mL hexane and the mixture centrifuged at 1500 g for 10 min. The methanolic extract was diluted 1:5 in Phosphate Buffered Saline (PBS) and again 1:4 in methanol-PBS (9:1) before ELISA analysis. Micro-titre polystyrene plates were coated by adding 100 μL of anti-aflatoxin antibody in bicarbonate buffer to each well and incubated overnight in moist chamber. The plates were emptied and free protein binding sites blocked by addition of 200 μL of 3% bovine serum albumin in PBS for 20 min. The plates were then washed thrice with Tween 20 solution and semi dried. Four aflatoxin standard concentration levels (1, 0.333, 0.111 and 0 ng mL^{-1}) were

used. Then 50 μL of the sample extract and 50 μL those of the calibrated aflatoxin standards were incubated simultaneously with aflatoxin-enzyme conjugate solution in wells of the coated microtitre plate. The plates were covered with aluminium foil and incubated for 2 h at room temperature. The wells were washed thrice with NaCl-Tween and semi dried. The amount of the bound aflatoxin-enzyme conjugate was determined by incubation with 100 μL of enzyme substrate solution and color was allowed to develop for 20 min. Enzyme reaction was stopped by adding 100 μL of 1M H_2SO_4 and the resultant colour intensity determined with a spectrophotometer ELISA reader (model Uniskan 11 type 364 Labystems, Finland) at A450 nm. The percentage inhibition for each standard and sample was calculated as B/B_0%, where B is mean absorbance reading of the sample and B_0 is the mean absorbance reading of blank standard. Absorbance values of aflatoxin standards dilutions were used to construct a standard curve and the aflatoxin content of sample was determined by interpolating on the curve.

Data analysis: All data were subjected to Analysis of Variance (ANOVA) using the PROC ANOVA procedure of Genstat (Lawes Agricultural Trust Rothamsted Experimental Station, 1998, version 8) and differences among the treatment means compared using Fisher's Protected LSD test at 5% probability level.

RESULTS

The fungi genera isolated were *Fusarium*, *Aspergillus* and *Penicillium*. The maize kernels had an infection rate of up to 99%, the highest being in samples from agro-ecological zone UM2 (Table 1). *Fusarium* was more frequently isolated but only *Aspergillus* infection was significantly different ($p<0.05$) with an average infection rate of 24.7%. Samples from agro-ecological zones UM4 and LM4 had the highest *Aspergillus* infection at 50 and 41%, respectively. The *Aspergillus* species identified were *A. flavus*, *A. niger*, *A. terreus* and *A. versicolor* but only *A. flavus* and *A.niger* were most frequently isolated at 9.9 and 12.9%, respectively (Table 2). The agro-ecological zones with highest *A. flavus* isolation rates in decreasing frequency were UM4, UM2, LM4 and LM3.

Similar spectrum of fungi was isolated from soils and mill sweeping as from the maize kernels. *Fusarium* was most frequently isolated in soils collected from farms and in mill dust, while *Penicillium* was most frequently isolated in soils from under the stores. There were significant differences ($p = 0.05$) among the agro-ecological zones in amount of *Aspergillus* isolated, with agro-ecological zones LM3 and LM4 having up to 41.8×10^3 and 25.4×10^3 cfu g^{-1} soil collected from under the stores (Table 3). More *A. flavus* (117×10^3 cfu g^{-1}) of *Aspergillus* were isolated from mill dust than in soils from farms and under the stores. The main *Aspergillus* species isolated from soils and mill dust were *A. flavus*, *A. niger* and *A. terreus* (Table 4). Higher levels of up to 13×10^3 and $69.8\ 10^3$ cfu g^{-1} of *A. flavus* were isolated in soils from under the stores and mill dust, respectively, than in soils from farms.

Aflatoxin B1 was detected in most samples, with samples from regions with high *Aspergillus* isolation rate had higher levels of aflatoxin contamination (Table 5, 6). Whole grains had the highest aflatoxin concentration of up to 160 μg kg^{-1} compared to semi-processed grains and mill dust. About 80% of the mill dust were contaminated with aflatoxin B1 compared to 38.9 and 35.4% for whole

Table 1: Percentage isolation of fungi in maize kernels sampled from farms in different Agro Ecological Zones (AEZ) of Machakos district during 2007 harvest season

AEZ	Kernel infection	*Aspergillus*	*Fusarium*	*Pencillium*	Others
LH2	90.0	15.0	66.0	16.0	8.0
LH3	93.0	34.0	47.0	28.0	6.0
LM3	93.0	23.0	64.0	22.0	6.0
LM4	80.0	41.0	39.0	8.0	6.0
LM5	92.0	14.0	69.0	11.0	6.0
UM2	99.0	14.0	48.0	44.0	27.0
UM3	91.0	23.0	59.0	24.0	3.0
UM4	88.0	50.0	47.0	8.0	5.0
UM6	92.0	8.0	65.0	21.0	19.0
Mean	90.9	24.7	56.0	20.2	9.6
$LSD_{(p=0.05)}$	NS	23.7	NS	NS	NS

LH2: Lower highland zone 2; LH3: Lower highland zone 3; LM3: Lower midland zone 3; LM4: Lower midland zone 4; LM5: Lower midland zone 5; UM2: Upper midland zone 2; UM3: Upper midland zone 3; UM4: Upper midland zone 4; UM6: Upper midland zone 6; LSD: Least significant difference; NS: Not significant

Table 2: Percentage isolation of different *Aspergillus* species in maize kernels sampled from farms in different Agro Ecological Zones (AEZ) of Machakos district during 2007 harvest season

AEZ	*A. flavus*	*A. niger*	*A. terreus*	*A. versicolor*	Others
LH2	5.6	7.2	0.0	0.6	1.6
LH3	8.2	18.2	0.0	6.6	1.2
LM3	13.1	13.1	0.0	0.0	1.1
LM4	13.1	13.1	0.9	0.8	0.0
LM5	4.7	9.1	0.0	0.0	1.9
UM2	16.7	8.7	0.0	0.0	0.0
UM3	8.0	13.8	0.0	5.0	0.4
UM4	20.0	24.8	0.0	0.8	5.4
UM6	0.0	8.0	0.0	0.0	0.0
Mean	9.9	12.9	0.1	1.5	1.3
$LSD_{(p=0.05)}$	8.0	NS	NS	NS	NS

LH2: Lower highland zone 2; LH3: Lower highland zone 3; LM3: Lower midland zone 3; LM4: Lower midland zone 4; LM5: Lower midland zone 5; UM2: Upper midland zone 2; UM3: Upper midland zone 3; UM4: Upper midland zone 4; UM6: Upper midland zone 6; LSD: Least significant difference; NS: Not significant

Table 3: Isolation rate (10^3 CFU g^{-1}) of different fungi and *Aspergillus* species in soil from farms in different Agro Ecological Zones (AEZ) in Machakos district May 2007

	Soil from farms					
	Fungal genera			*Aspergillus* species		
AEZ	*Aspergillus*	*Fusarium*	*Penicillium*	*A. flavus*	*A. niger*	*A. terreus*
LH2	7.6	34.8	1.0	3.8	3.9	0.0
LH3	14.3	312.2	12.0	10.6	3.7	0.0
LM3	31.3	187.6	35.0	3.3	27.3	0.6
LM4	26.7	78.7	10.0	2.0	24.7	0.0
LM5	23.6	126.6	9.0	3.9	19.7	0.0
UM3	8.7	116.3	261.0	1.1	6.0	0.0
UM4	1.4	151.0	16.0	0.1	1.3	0.0
UM6	11.7	90.5	225.0	2.5	9.2	0.0
Mean	15.6	137.7	71.1	3.4	12.0	0.1
LSD	11.6	45.7	31.0	NS	8.8	NS
	Soil from under the stores					
	Fungal genera			*Aspergillus* species		
AEZ	*Aspergillus*	*Fusarium*	*Penicillium*	*A. flavus*	*A. niger*	*A. terreus*
LM3	41.8	137.0	332.0	41.2	0.7	0.0
LM4	25.4	134.0	21.0	0.1	25.3	0.0
LM5	8.9	245.0	83.0	2.0	6.9	0.0
UM4	10.0	173.0	970.0	10.0	0.0	0.0
Mean	21.5	172.3	351.5	13.3	8.2	0.0
LSD	NS	24.1	58.0	NS	4.4	

LH2: Lower highland zone 2; LH3: Lower highland zone 3; LM3: Lower midland zone 3; LM4: Lower midland zone 4; LM5: Lower midland zone 5; UM2: Upper midland zone 2; UM3: Upper midland zone 3; UM4: Upper midland zone 4; UM6: Upper midland zone 6; LSD: Least significant difference; NS: Not significant

Table 4: Isolation rate (10^3 cfu g^{-1}) of different fungi and *Aspergillus* species from mill dust in five administrative divisions of Machakos during 2007 harvest season

Division	*Aspergiilus*	*Fusarium*	*Penicillium*	*A. flavus*	*A. niger*	Others
Central	83.0	3,030.0	895.0	10.0	40.0	33.3
Katangi	40.0	2,108.0	485.0	39.0	1.0	0.0
Kathiani	106.0	5,930.0	530.0	86.0	20.0	0.0
Matungulu	320.0	7,030.0	375.0	190.0	130.0	0.0
Yathui	37.0	3,049.0	253.0	24.0	12.0	0.0
Mean	117.2	4,229.4	507.6	69.8	40.6	6.7
$LSD_{(p=0.05)}$	187.4	1605.0	NS	NS	NS	NS

Table 5: Aflatoxin content (μg kg^{-1}) in grains sampled from farmers in different Agro Ecological Zones (AEZ) in Machakos district May 2007

AEZS	Positive samples	Aflatoxin content	Range (μg kg^{-1})
LH2	40.0	27.0	0-70.0
LH3	20.0	0.4	0-2.0
LM3	66.7	15.0	0-100.0
LM4	0.0	0.0	-
LM5	0.0	0.0	-
UM2	66.7	55.7	0-160.0
UM3	20.0	16.6	0-80.0
UM4	57.1	19.6	0-66.4
UM6	0.0	0.0	-
Mean	30.1	14.9	0-160.0

LH2: Lower highland zone 2; LH3: Lower highland zone 3; LM3: Lower midland zone 3; LM4: Lower midland zone 4; LM5: Lower midland zone 5; UM2: Upper midland zone 2; UM3: Upper midland zone 3; UM4: Upper midland zone 4; UM6: Upper midland zone 6

grains and semi-processed grain, respectively (Table 6). The different agro-ecological zones showed great variation in the distribution and amounts of aflatoxin B1 contamination.

DISCUSSION

The fungal genera predominantly isolated from maize, mill dust and soils were *Fusarium*, *Aspergillus* and *Penicillium*. *Fusarium* had the highest isolation in all sample types indicating possible contamination of maize with *Fusarium* mycotoxins like fumonisins, deoxynivalenol and zearalenone. The results are in agreement with those of Almeida *et al*. (2000) and Cvetnić *et al*. (2005) that *Fusarium* is a common contaminant of grains, especially under field conditions. Semi processed grains had lower isolation frequency of *Fusarium* and *Aspergillus* compared to whole grain, which concurs with the findings of Pietri *et al*. (2009), who found that maize processing reduces mycotoxin levels and that aflatoxins are concentrated in bran and germ, while fumonisins affect inner layers of the kernel. The traditional method of processing maize involves removal of the

Table 6: Aflatoxin content (μg kg^{-1}) in whole grains, semi-processed grain and mill dust sampled from traders in six divisions in Machakos district May 2007

Whole grain			
Division	Percentage positive	Aflatoxin content	Range
Central	25.0	16.0	0-11
Katangi	0.0	0.0	-
Kathiani	25.0	12.5	0-50
Matungulu	33.3	25.3	0-150
Ndithini	100.0	16.0	3-29
Yathui	50.0	13.0	0-26
Mean	38.9	13.8	0-150
Semi processed grains (Muthokoi)			
Division	Percentage positive	Aflatoxin content	Range
Central	16.7	1.0	0-6
Kathiani	75.0	17.9	0-63.5
Matungulu	0.0	0.0	-
Yathui	50.0	1.5	0-3
Mean	35.4	5.1	0-63.5
Mill dust			
Division	Percentage positive	Aflatoxin content	Range
Central	50	4.5	0-9
Katangi	100	3.7	3.7
Kathiani	100	44.0	8-80
Matungulu	50	14.5	0-29
Yathui	100	25.0	25
Mean	80	18.3	0-80

pericarp and the germ, which could imply removal of fungi and the associated mycotoxins (Fandohan *et al*., 2005). *Aspergillus flavus* was isolated in the grains, soils and mill dust and was distributed across all the agro-ecological zones.

Aspergillus flavus was isolated in soils from farms previously planted with maize and from under the stores and was distributed in all the agro-ecological zones. This indicates that high levels of of aflatoxin-producing *A. flavus* inoculum are prevalent in Machakos and, therefore, risk of aflatoxin poisoning in event that favourable conditions occur. This agrees with reports by Scheidegger and Payne (2003) that conidia of *Aspergillus flavus* are the major source of primary inoculum in maize fields The fungus forms sclerotia in insect-damaged kernels before harvest. These sclerotia are dispersed into the soil during harvest. The sclerotia survive in soil and produce conidiophores and conidia during the following season to infect the crop via silk (Marsh and Payne, 1984; Scheidegger and Payne, 2003). Machakos is prone to drought, which stresses plants and render them susceptible to contamination by *Aspergillus* sp. (Holbrook *et al*., 2004). Other factors that favour aflatoxin contamination are late harvesting of crops, high ambient humidity, inadequate drying and poor storage practices (Bhat and Vasanthi, 2003).

The study showed that many samples had more than the maximum 20 μg kg^{-1} aflatoxin B1 allowed in Kenya. The highest aflatoxin B1 levels were found in the whole grain, which had up to 160 μg kg^{-1}, suggesting chronic poisoning to the consumers. Mill dust had the highest aflatoxin contamination, which could be attributed to dehulling and constant presence of maize products, which are a good substrates of *A. flavus*. Semi processed grains had lowest aflatoxin contamination, which could be due to removal of contaminated fraction. Processing maize into traditional products has been found to significantly reduce levels of aflatoxin and Fumonisin by up to 93% (Fandohan *et al*., 2005; Pietri *et al*., 2009).

The results indicate that there is a need to effect management strategies to reduce aflatoxins to levels below the regulatory limits. Different countries have established regulations to protect the consumer from the harmful effects of these mycotoxins (FAO, 1996; FDA, 2004; Fellinger, 2006; Barug *et al*., 2003; Van Egmond, 2002; Wu, 2006). Human foods are allowed 4-30 ppb aflatoxin, depending on the country involved. The European Union has set 4 μg kg^{-1} total aflatoxin in food for human consumption are the maximum acceptable limits, the strictest standard worldwide (EC, 2006; Wu, 2006). This has great implications on food trade and therefore, continued nationwide surveillance, increased food and feed inspections and awareness creation would be a long-term intervention strategy to ensure food safety (WHO, 2006). However, limited laboratory capacity and efficient, cost-effective sampling and analytical methods are a major problem in detection and management of mycotoxins in most developing countries (WHO, 2006).

The results of this study indicated that *A. flavus* is the major causative factor in aflatoxin elaboration in Eastern Kenya. *Aspergillus* was isolated more frequently in soils where maize had been planted and under stores therefore, suggesting that removal and destruction of debris of the previous harvest and cleaning stores before loading new produce would help reduced aflatoxin levels (Hell *et al*., 2000). However, the most promising long-term strategy would be through breeding resistant maize lines. Sources of resistance to *Aspergillus flavus* and *Fusarium* sp. have been identified and incorporated into breeding programs (Hell *et al*., 2005; Mulé *et al*., 2005; Munkvold, 2003).

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