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Vol. 10(6), pp. 172-184, 14 February, 2016
DOI: 10.5897/AJMR2015.7645
Article Number: 741DC9357109
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Diversity of putatively toxigenic *Aspergillus* species in maize and soil samples in an aflatoxicosis hotspot in Eastern Kenya

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Received 27 June, 2015; Accepted 12 November, 2015

Aflatoxin contamination impinges on grain quality worldwide. The causative agent, *Aspergillus* spp. colonizes grain in the field down to postharvest stages in storage where they may produce toxins. Kenya has experienced recurring cases of aflatoxicosis in Eastern region especially during periods of maize grain deficit. The risk of chronic exposure has not been widely studied. Therefore, seasonal variation in abundance and species composition of toxigenic *Aspergillus* in maize and soils of Eastern Kenya was investigated. Samples were obtained from farmers, two months after the first (May) and second (December) harvest seasons. *Aspergillus* spp. were isolated from maize and soil samples by direct and dilution plate techniques respectively on Czapek Dox Agar (CZ) and thereafter sub-cultured on potato dextrose agar (PDA). Positive identification was done using culture-morphological and microscopic characteristics in PDA media. The ammonium vapour test was used to screen for the putative toxigenic strains. A total of 229 *Aspergillus* spp. cultures were obtained (55% -maize, 45% -soil). Eleven *Aspergillus* sp. were identified: *Aspergillus niger, Aspergillus flavus, Aspergillus clavatus, Aspergillus awamori, Aspergillus parasiticus, Aspergillus ochraceus*, *Aspergillus candidus, Aspergillus ustus*, *Aspergillus niveus*, *Aspergillus terreus* and *Aspergillus wentii*. Of these 41 (18 %) were potentially toxigenic while the rest were putatively atoxigenic. Out of the 41 toxigenic isolates, 22 were from maize. The first season had 15 (68.2%) toxigenic maize isolates while 7 (31.8%) were from the second season. Generally, there were more fungal isolates in the first season (54.1%) than the second one (45.9%) while *Aspergillus niger* was the most abundant in both seasons. Such variation in fungal abundance supports the hypothesis that aflatoxin contamination of grain may vary seasonally but that remains to be unravelled and herein, a contrary opinion was presented.

Key words: Aflatoxin, mycotoxin, *Aspergillus*, maize, soil.

INTRODUCTION

Fungi belonging to the genus *Aspergillus* produce various toxins, which are of importance to human health. In particular, *Aspergillus flavus* and *Aspergillus parasiticus* mainly produce aflatoxins (Abbas et al., 2005) while other congeneric species: *Aspergillus* sections *Nidulantes, Versicolores, Usti, Circumdati* and *Nigri* produce various

metabolites related to aflatoxins which cause food poisoning (Blumenthal, 2004; Do and Choi, 2007). These fungi colonize grain in the field during planting and continue to do so during storage when they may produce toxins (Waliyar et al., 2015). Apart from aflatoxins, these fungi produce other metabolites such as ochratoxins and oxalic acid, which are hazardous to humans (Palencia et al., 2010). The effect of mycotoxin ingestion is dose-dependent, it may result in either death (acute) or other clinical complications (chronic). Chronic intake may lead to immunosuppression (Jiang et al., 2008), cancers, poor growth and abnormal foetal development (Gong et al., 2004; Probst et al., 2011).The United States Food and Drug Administration (FDA) has referred to aflatoxins as inevitable food contaminants, hence it has set the maximum allowable limit in human food products at 20 parts per billion. However, in the US, the levels vary between 10 parts per billion for humans and 20 parts per billion for livestock (Williams et al., 2004), while in Europe the limit is 4 ppb (Commission Regulation (EC) No 1881/2006, 2006). In Kenya, the limits are 10 parts per billion (Mutiga et al., 2014).

Over the last four decades, Kenya has experienced repeated cases of aflatoxicosis. In the year 2004, 317 people were hospitalised due to acute aflatoxin exposure 125 cases were fatal (CDC, 2004). It was hypothesised that there could be an underestimation of human aflatoxicosisburden in the country for four reasons: 1. Many cases may not be reported due to lack of infrastructure of capturing episodes in real time. 2. There is scanty clinical data to account for chronic sub-lethal exposure, 3. There is a systemic difficulty in enforcing residual limits policies among cereal traders and this ensures the circulation of cereals with high toxin levels. 4. There is a belief that aflatoxin contamination is a problem in only one region of the country yet it is more widespread covering other maize producing areas including Western and Rift Valley. Most of the human fatalities have been recorded in the Eastern region of Kenya, which is now considered a hot spot for aflatoxicosis (Probst et al., 2007; Muthomi et al., 2009). Fatalities common in the Eastern region is perhaps due to acute grain shortage, which force the populace to consume any available grain that is probably considered unfit for consumption. However, a survey by Mutiga et al. (2014) reported on high incidences of aflatoxin contamination in the Eastern region of Kenya following a bumper harvest in 2010. Preharvest drought and postharvest moisture are considered the most critical drivers of aflatoxin accumulation, which are factors that are largely influenced by cropping seasons. In Eastern Kenya, acute aflatoxicosis has been reported following periods of moisture stress during maize crop development, when rainfall occurs after the crop has attained physiological maturity and before it is harvested, and when harvests are stored incorrectly. For this reason, there has been renewed effort to study various aspects of the causal fungi and its interactions, which include intraspecific variations and competition, climatic and seasonal influence, toxigenicity (Probst et al., 2011), distribution in maize and soil (Muthomi et al., 2009).

Perhaps one of the most promising control efforts is in biological control whose success story has been reported in other countries like Nigeria (Fapohunda, 2009) and is undergoing initial stages of quarantined field trials (IITA, 2014). This approach uses non-toxigenic fungi to “competitively exclude” toxigenic ones from accessing maize kernels, thereby preventing toxin build-up (Cotty et al., 2007). As such, an inoculum of non-toxin producing fungi is introduced into the soil surface beneath the crop canopy 40-45 days after planting (two-to-three weeks before flowering) (Atehnkeng et al., 2014). However, considering the variations in both quantities and types of toxins produced by fungi of the genus *Aspergillus*, it is imperative to consider these variations alongside species composition, abundance and geographical distribution as would be affected by seasonal changes. This information would be important in understanding the possible toxin risks the population faces with each harvest and enable early preparedness. This study was conducted to determine the seasonal variation in the abundance and composition of putatively toxigenic *Aspergillus* species in maize and soil in Eastern Kenya. This has not been previously established in this region.

MATERIALS AND METHODS

Sampling

Maize and soil samples were collected in May and December 2013 two months after respective harvest seasons. The Eastern region of Kenya is a semi-arid region that experiences annual rainfall of between 250 and 500 mm (Freeman and Coe, 2002). The long rains start at the end of March and last until May while the short rains start in October to December. The minimum and maximum temperatures in this region range from 23 to 34°C (Funk, 2010). A transect was selected (along the main road cutting across the counties) from which sampling points were set every 5 km. At every sampling point, farmers were selected randomly on both sides of the road from whom half a kilogram of shelled maize was collected. Soil was collected from the open area where farmers dried their grain as well as under the storage facility. Samples were separately put in khaki bags, transported in a cool box to the laboratory, and stored at 4°C until analysis was done. During the second season, repeated sampling was undertaken in the same areas as the first one. In total, about 200 maize samples were collected during both seasons, but were segregated depending on whether the farmer had planted or purchased their grain. All samples obtained from farmers who had purchased maize during either of the seasons

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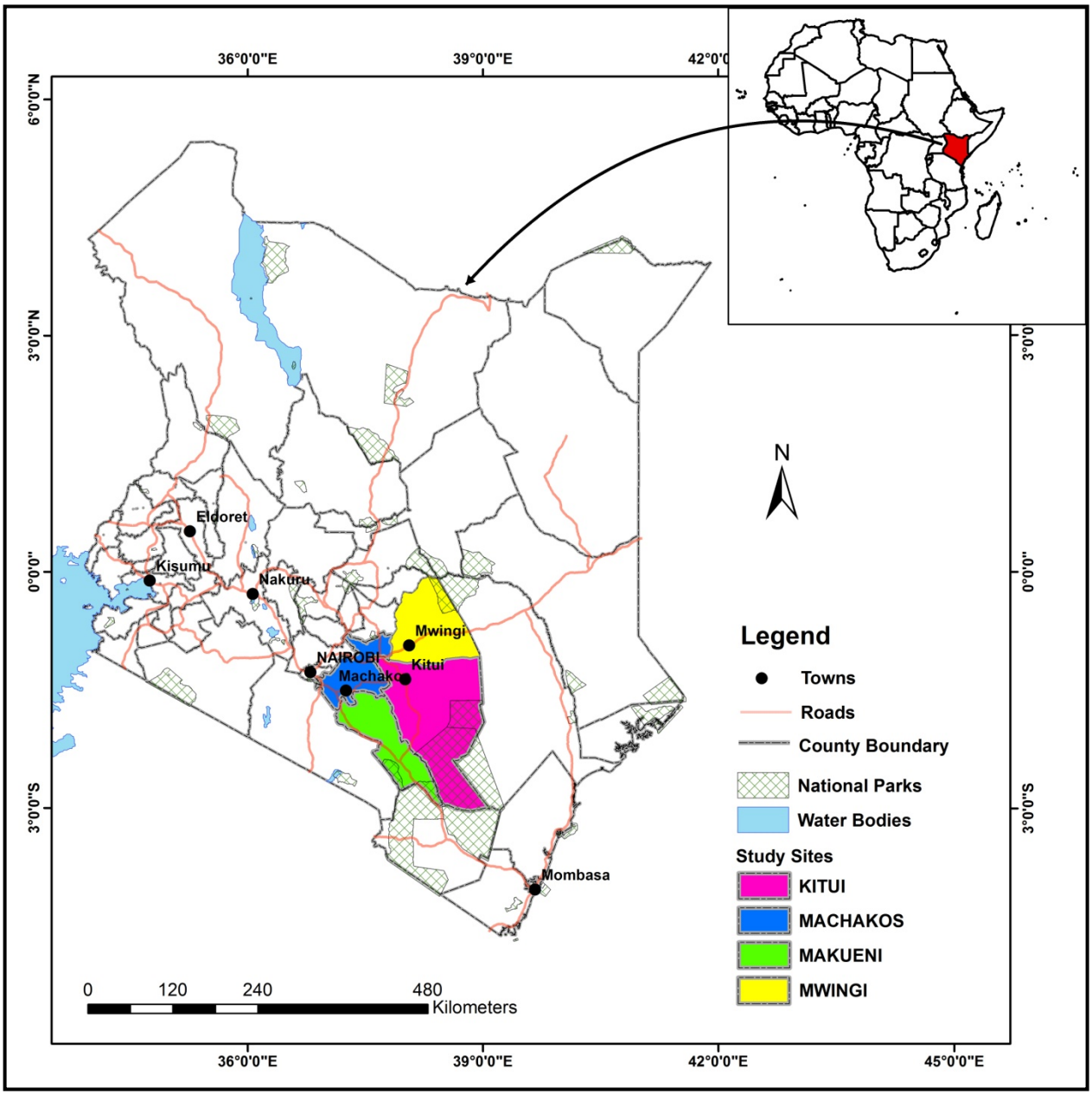


Figure 1. Map of the study area: Kitui, Machakos, Makueni and Mwingi. Source: Cartographer, Department of Geography, Egerton University (2015).

were excluded from the study. This procedure was guided by findings reported by Daniel et al. (2011) that home-grown maize was the major source of contamination compared to purchased maize. Owing to poor harvest that affected most of the region during 2013, most farmers sampled had bought maize for their daily use. Thus, only a total of 50 (maize with their respective soil) samples, which had been stored for two months were analysed further. These are as follows: location [Total samples = Season I + Season II]: Machakos [4=4+0], Makueni [16=16+0], Kitui [17=4+13] and Mwingi [13=1+12] (Figure 1).

Fungi isolation and identification

Fungi isolation from maize was carried out following the procedure described by Muthomi et al. (2009) after surface sterilisation of kernels in 2% NaOCl. Fifteen kernels were randomly picked from the khaki bags and introduced into a conical flask containing NaOCl and swirled gently for one minute. Afterwards, they were removed and rinsed thrice in sterile distilled water. Five kernels were plated about 2 cm apart on Petri dishes (90 × 15 mm) containing Czapek Dox Agar (HiMedia Laboratories Pvt. Ltd) amended with 50 mg each of streptomycin sulphate and penicillin (Zhonghuo Pharmaceutical Shijazhuang Co. Ltd., China) per litre of medium. The set up was replicated thrice for each sample. As for soil samples, dilution plate technique was used. Briefly, 1 g of soil was suspended in 9 ml sterile distilled water and serially diluted to 1 x 10^{-4}. One ml of 10^{-3} and 10^{-4} respectively were uniformly spread in duplicates in Czapek Dox Agar (amended as above). Afterwards, all

culture plates were incubated in a growth chamber at 28°C for 7 days. *Aspergillus* species were isolated from colonies in Petri dishes and dilution plates then sub-cultured in Potato Dextrose Agar (HiMedia Laboratories Pvt. Ltd, India) and incubated as above. Upon maturation, fungi were classified based on cultural and morphological features such as colony diameter, colony colour on agar, front and reverse and colony texture (Klich, 2002a; Rodrigues et al., 2007). This was followed by the preparation of slide cultures and incubation in moist chambers at 28°C for 5 days before observation under a light microscope. For microscopic characterisation, microscopic features studied included conidiophores, conidial shape, phialides and metulae, presence and shape of vesicles. These are the common features used to identify *Aspergillus* fungi to species level (Diba et al., 2007; Klich, 2002a).

Screening for toxigenic *Aspergillus* species

Toxin producing ability of the fungi was tested following the ammonium hydroxide (NH_4OH) vapour test (Kumar et al., 2007). A single fungal colony was grown in the centre of a Petri dish containing yeast extract-sucrose medium for 5 days at 28°C. Then 2 drops of concentrated (27%) NH_4OH solution were added to the inverted lid of the Petri dish and allowed (30 min) to react. Toxin production (positive test) was evidenced by formation of a pink to plum-red colour on the underside of the fungal colony while negative tests had no observable colour changes (Zrari, 2013).

Data analysis

The data on the abundance of the fungi in each region were represented as a percentage of score total count. Analysis of variance (ANOVA) was performed to determine whether the distribution of the *Aspergillus* isolates in the four sites was significantly different. Student t-test was performed to determine whether the distribution of the species between the two seasons was statistically significant. SPSS (version 20.0) was used in the analysis of data.

RESULTS

Aspergillus species isolates from maize and soil

The morphological and cultural features of the *Aspergillus* isolates are presented in Table 1. In total (from both seasons), 229 isolates were obtained from maize and soil samples. The identification process resulted into 11 *Aspergillus* species (Plate 1 and 2a – k).

The species and their order of abundance (%) were as follows: *A. niger* (47.6), *Aspergillus flavus* (22.3), *Aspergillus clavatus* (12.2), *Aspergillus awamori* (4.4), *Aspergillus parasiticus* (3.9), *Aspergillus ochraceus* (2.2), *Aspergillus candidus* (1.7), *Aspergillus ustus* (1.7), *Aspergillus niveus* (1.7), *Aspergillus terreus* (1.3) and *Aspergillus wentii* (0.9). There was consistency in species identity as individual isolates (across the four study sites) belonging to a single species showed same morphological traits on PDA. Conversely, *A. parasiticus* showed similar cultural and morphological traits to *A. flavus* but were segregated on the basis of conidia colour since *A. parasiticus* (conifer green) differed from *A. flavus* (dark green) (Plate 2 d(i) and h(i) respectively).

There were some species with somewhat similar cultural traits, but were segregated microscopically. For example, *A. candidus* was distinguished from *A. niveus* by a fertile region of the vesicle and colony diameter. Isolates of *A. niveus* were fertile on the top one-to-two thirds of the vesicle while *A. candidus* were fertile on the entire vesicle with colony diameters not exceeding 35 mm in PDA (Klich, 2002a).

Aspergillus species abundance and composition

Due to the sampling procedure and the choice of only analysing the farmer planted maize samples and excluding the bought maize samples from this study, it was not possible to reliably compare the study sites in Eastern region and respective seasons but rather consider the entire region as a block. This could partly explain why there were some observable differences in distribution of fungi in the two sampling seasons but they did not appear significant (Table 2).

In general, there were more *Aspergillus* species isolated from maize (54.6%) than from soil samples (45.4%). This was consistent in each location except Makueni where there were more isolates from soil than from maize (Table 3).

The percentage proportion of fungal isolates of maize to soil in each location was as follows: Kitui (57:43), Makueni (47:53), Mwingi (62:38) and Machakos (52:48). In addition, the abundance (%) of fungi appeared to correlate positively with the number of samples collected from each location as follows: Kitui (34.9), Makueni (31.9), Mwingi (23.1) and Machakos (10).

In terms of species composition, three species (*A. clavatus, A. niveus*and *A. wentii*) were isolated strictly from soil, but not from maize (Figure 2). All species obtained from maize were present in soil, which was consistent with the expectations. Five species (*A. flavus, A. niger, A. parasiticus, A. ochraceus* and *A. ustus*) were more abundant in maize than in soil samples. In contrast, two species (*A. terreus* and *A. awamori*) were more abundant in soil than maize samples (Figure 3).

Toxigenic *Aspergillus* species from maize and soil

The colony reverse of the toxigenic species changed from pale yellow to pink or plum-red (Plate 3). In general, of the 229 *Aspergillus* isolates, 41 (18%) were toxigenic while the rest were non-toxigenic, 22 (53.7%) of the toxigenic isolates were from maize while 19 (46.3%) from soil. 15 (68.2%) of the isolates from maize were from first season while 7 (31.8%) were from the second season. For the soil isolates, 11 (57.9%) were from season one while 8 (42.1%) were from the second season (Table 4).

Table 1. Cultural, morphological and microscopic features of the identified *Aspergillus* species.

Species name	Cultural and morphological features on PDA			Microscopic features			
	Colony diameter (mm)	Surface colour	Reverse colour	Shape of vesicle	Conidial head	Seriation	Conidiophore
A. awamori	58±2	Dark brown to black conidia with white to yellow mycelia arranged in alternating concentric rings	Cream to dull yellow reverse with a wrinkled centre	Globose	Radiate	Biseriate	Colourless, long and smooth
A. candidus	26±2	Pure white conidia with dense white mycelia	Light yellow wrinkled reverse	Globose	Radiate	Biseriate	Colourless, short and finely roughened
A. clavatus	32±2	Bluish green conidia, white mycelia with a white margin	Brown centre with alternating yellow and brown concentric rings	Clavate	Radiate	Uniseriate	Brownish, short and finely roughened
A. flavus	40±2	Deep green conidia or olive green conidia with white margin Presence of white mycelia	Cream to light brown reverse with a smooth texture	Globose	Columnar	Biseriate	Colourless, relatively short roughened conidiophores
A. niger	62±2	Dark brown to black densely packed conidia, inconspicuous white mycelia, thin white to cream margin	Yellow to dull brown reverse with a wrinkled texture	Globose	Radiate	Biseriate	Brownish, relatively long conidiophores with smooth surfaces
A. niveus	22±2	Dull orange-white conidia with white mycelia	Yellow-gold reverse with star-shaped striations and concentric ring patterns	Columnar	Radiate	Biseriate	Colourless, short and finely roughened
A. ochraceus	45±2	Wheat-coloured conidia, purplish sclerotia with yellowish exudates	Yellow to light brown wrinkled reverse	Globose	Radiate	Biseriate	Colourless relatively long roughened conidiophores
A. parasiticus	Full plate	Conifer green conidia with white mycelia and white margin	Cream reverse with slightly wrinkled centre	Globose	Columnar	Uniseriate	Colourless short and finely roughened
A. terreus	29±2	Brownish orange conidia with white mycelia	Yellow to gold reverse with star-shaped striations and concentric ring patterns	Sub-globose	Columnar	Biseriate	Colourless short smooth-walled conidiophores

Table 1. Contd.

A. ustus	50±2	Light brown to greyish conidia, with white to greyish mycelia	Cream reverse with yellowish wrinkled centres	Pyriform	Columnar	Biseriate	Brownish, long, smooth-walled conidiophores
A. wentii	28±2	Greyish-yellow to olive-brown conidia	Yellow to pale brown reverse	Globose	Radiate	Biseriate	Colourless and relatively long conidiophores with smooth walls

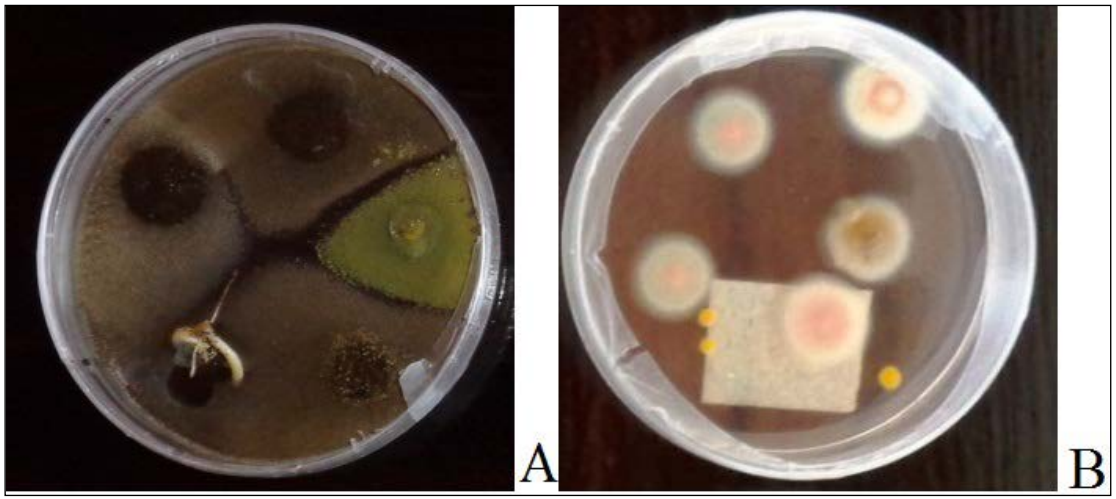
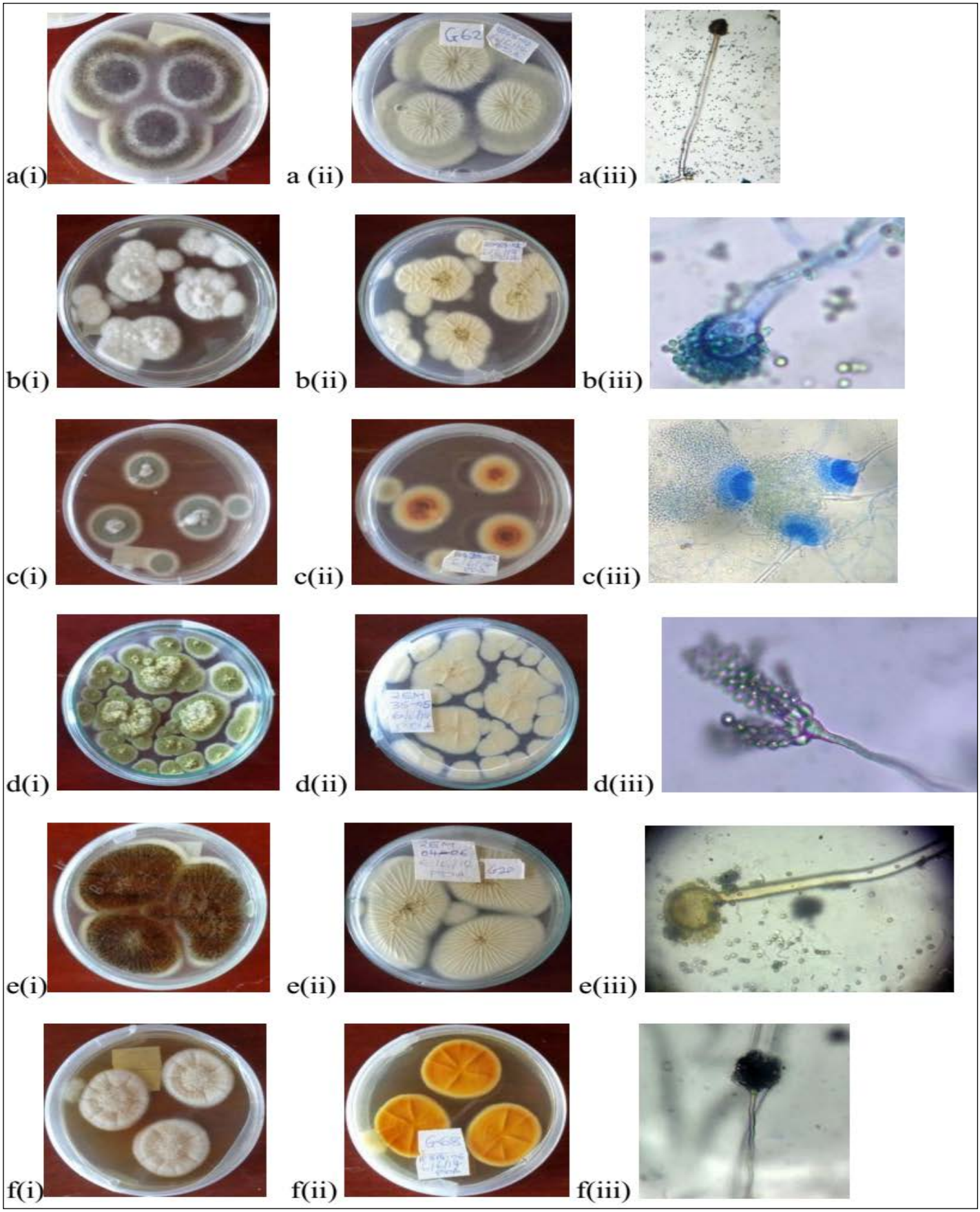


Plate 1. (A) Growth of *Aspergillus niger* and *Aspergillus flavus* on maize kernels in CZ media after 7 days of incubation at 28°C and (B) colonies of Aspergillus species from soil serial dilutions on CZ media after 7 days of incubation at 28°C.

The toxigenic *Aspergillus* isolates were of the species *A. flavus* (24), *A. parasiticus* (3), *A. ochraceous* (3), *A. clavatus* (8), *A. ustus* (1), *A. niveus* (1) and *A. wentii* (1).

DISCUSSION

It is important to determine whether the abundance and composition of putatively toxigenic *Aspergillus* species in maize and soil of Eastern Kenya are affected by the climatic changes in the two planting seasons. In the current study, *A. niger* was the most abundant amongst the eleven species isolated in both seasons while *A. flavus* was the second most abundant. *A. niger* was formerly believed to be harmless and non-toxigenic (Blumenthal, 2004), but recent studies present it as potentially being toxigenic, producing fumonisins (Palencia et al., 2010). *A. flavus* is known to produce aflatoxins (Probst et al., 2007; Rodrigues et al., 2007). The current findings of the abundance of *A. niger*in semi- arid soils contradicts the propositions of Klich (2002b) that this black *Aspergillus* species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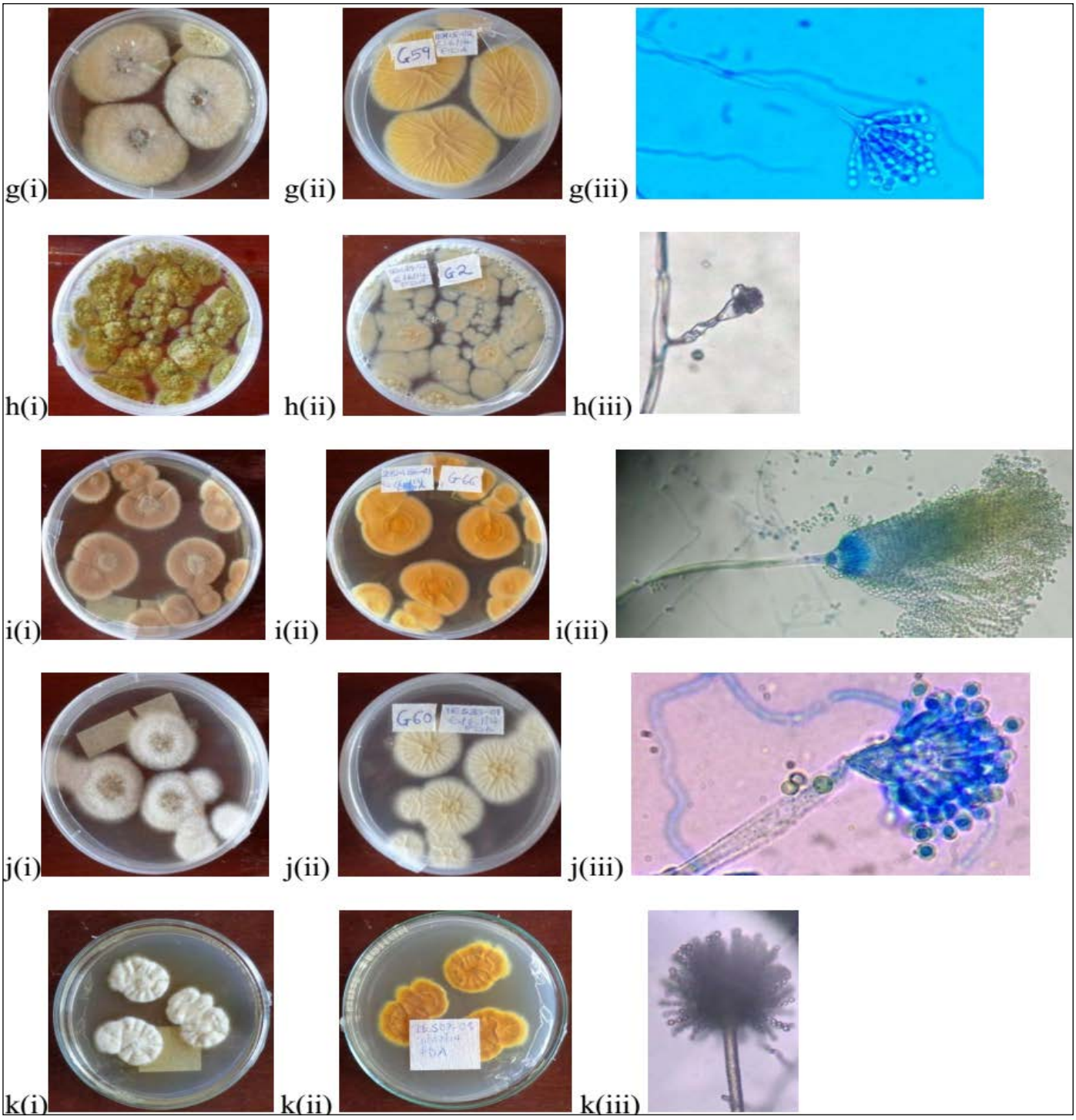

Plate 2. The cultural and morphological traits of the 11 *Aspergillus* species growing in PDA after 7 days of incubation. a(i) *A. awamori* surface, a(ii) *A. awamori* reverse and a(iii) colourless, long, and smooth conidiophore of *A. awamori* (Mg=400×); b(i) *A. candidus* surface, b(ii) *A. candidus* reverse and b(iii) globose vesicle in *A. candidus* (Mg=400×); c(i) *A. clavatus* surface, c(ii) *A. clavatus* reverse and c(iii) clavate vesicle in *A. clavatus* (Mg=1000×); d(i) *A. flavus* surface, d(ii) *A. flavus* reverse and d(iii) a biseriate conidial head with a globose vesicle of *A. flavus* (Mg400×); e(i) *A. niger* surface, e(ii) *A. niger* reverse and e(iii) brownish, relatively long and smooth conidiophore of *A. niger* (Mg=400×); f(i) *A. niveus* surface, f(ii) *A. niveus* reverse and f(iii) short, finely roughened stipe in *A. niveus* (Mg=400×); g(i) *A. ochraceus* surface, g(ii) *A. ochraceus* reverse and g(iii) globose vesicle, colourless relatively long conidiophore of *A. ochraceus* as observed under the microscope (Mg=400×). h(i) *A. parasiticus* surface, h(ii) *A. parasiticus* reverse and h(iii) short stipe of *A. parasiticus* as observed under the microscope (Mg=400×); i(i) *A. terreus* surface, i(ii) *A. terreus* reverse and i(iii) columnar conidial ornamentation in *A. terreus* (Mg=1000×) j(i) *A. ustus* surface and j(ii) *A. ustus* reverse and j(iii) biseriate conidial head in *A. ustus* (Mg=×1000); k(i) *A. wentii* surface, k(ii) *A. wentii* reverse and k(iii) radiate conidial head with roughened stipe of *A. wentii* (Mg=400×).

Table 2. The distribution of *Aspergillus* sp. in the two planting seasons.

Aspergillus sp.	Season				
	I		II		Total
	Count	%	Count	%	Count
A. awamori	5	50.0	5	50.0	10
A. candidus	1	25.0	3	75.0	4
A. clavatus	23	82.1	5	17.9	28
A. flavus	26	51.0	25	49.0	51
A. niger	55	50.5	54	49.5	109
A. niveus	2	50.0	2	50.0	4
A. ochraceous	3	60.0	2	40.0	5
A. parasiticus	5	55.6	4	44.4	9
A. terreus	1	33.3	2	66.7	3
A. ustus	3	75.0	1	25.0	4

Table 3. The incidence (%) and distribution of *Aspergillus* species across the four study sites.

Aspergillus sp.	Site			
	Kitui (N=17)	Machakos (N=4)	Makueni (N=16)	Mwingi (N=13)
A. awamori	50.0	0.0	40.0%	10.0
A. candidus	50.0	0.0	0.0	50.0
A. clavatus	32.1	17.9	35.7	14.3
A. flavus	37.3	11.8	25.5	25.5
A. niger	33.9	7.3	35.8	22.9
A. niveus	25.0	0.0	50.0	25.0
A. ochraceus	40.0	20.0	40.0	0.0
A. parasiticus	11.1	22.2	11.1	55.6
A. terreus	66.7	33.3	0.0	0.0
A. ustus	50.0	0.0	50.0	0.0
A. wentii	0.0	0.0	0.0	100.0
Total	**34.9%**	**10.0%**	**31.9%**	**23.1%**

is more dominant in forests and well-cultivated soils than in dry regions. The sampled region in this study is largely characterized by semi-arid dry climatic conditions. However, they postulated that the black *Aspergillus* species (Section *Nigri*) and *A. flavus* are highly likely to be found in areas of latitudes ranging (26 - 35°), which is close to our sampled areas in the latitude range (Mwingi 38.05°- Machakos 37.26°). The fact that *A. flavus* was the second most abundant species in both seasons implies that the risk of exposure to aflatoxin occurs all year round and not during any particular cropping season.

The findings of this study differ from those of Muthomi et al. (2009), who reported *A. flavus* as being the most abundant in the same region in two consecutive years (2008-2009). While there seem to be some differences, they could be explained by differences in collection times, sampling strategy and even the season of collection. In addition, Muthomi et al. (2009) reported seven species (*A. flavus, A. niger, A. terreus, A. ochraceus, A. fumigatus, A. clavatus* and *A. versicolor*). While the current study reported eleven, five of which matched the earlier study except for *A. versicolor* and *A. fumigatus,* which were not isolated in this study. We report five *Aspergillus* species that were not reported by Muthomi et al. (2009) including *A. parasiticus, A. ustus, A. candidus, A. niveus, A. awamori* and *A. wentii*, out of which *A. parasiticus* is known to be toxigenic.

Recently, Odhiambo et al. (2013) while prospecting for candidates for biological control in the same region reported the dominance of *A. flavus* but absence of *A. niger* from Makueni. In addition, four other species (*A. glaucus, A. sydowii, A. nidulans* and *A. fumigatus*) reported in the same study were not reported in the current one. Here, the reverse was reported, *A. niger* dominating (39 isolates) followed by *A. flavus* (13 isolates). Such variations can be due to differences in the sampling strategies (McHugh et al., 2014) or even species overlap as seasons change, hence the time of sampling is critical (Kennedy et al., 2006). In their work, Odhiambo et al. (2013) sampled 2 weeks after harvest as opposed to 2 months in this study. This could explain changes in fungal community structure in the course of storage with selection forces favouring more adapted species. The choice of sampling time in this study was informed by the observation that toxin build up approaches peak in store at 6-8 weeks post-harvest. The findings of this study could inform the development of a sampling protocol towards assurance of food safety as knowledge of abundance and composition of toxigenic species helps in early warning systems. This is more important, considering that the maize sampled is what was being consumed. A striking finding corroborated in the two studies is the low density of *A. parasiticus* isolated from Makueni County. This is attributable to geographic factors such as the latitudinal position of the area, besides competition and other limiting factors like water and nutrients Klich (2002b).

In India, Venkataramana et al. (2013) determined the mould incidence and mycotoxin contamination in 150 freshly harvested maize samples and obtained 288 fungal isolates consisting of *Fusarium*, *Aspergillus* and *Penicillium* species. *A. flavus* was the predominant *Aspergillus* species as opposed to *A. niger* in the current study. In this study, 229 *Aspergillus* isolates were obtained from 50 maize and soil samples thus implying a higher fungal burden in the Kenyan samples. One possibly contributing factor could be the climatic differences during sampling as well as the samples. Venkataramana et al. (2013) collected freshly harvested samples in winter, which is non-existent in Kenya. In this study, the samples were collected 2 months postharvest. The hot climatic conditions of Eastern Kenya as well as the postharvest (storage) conditions of the samples in

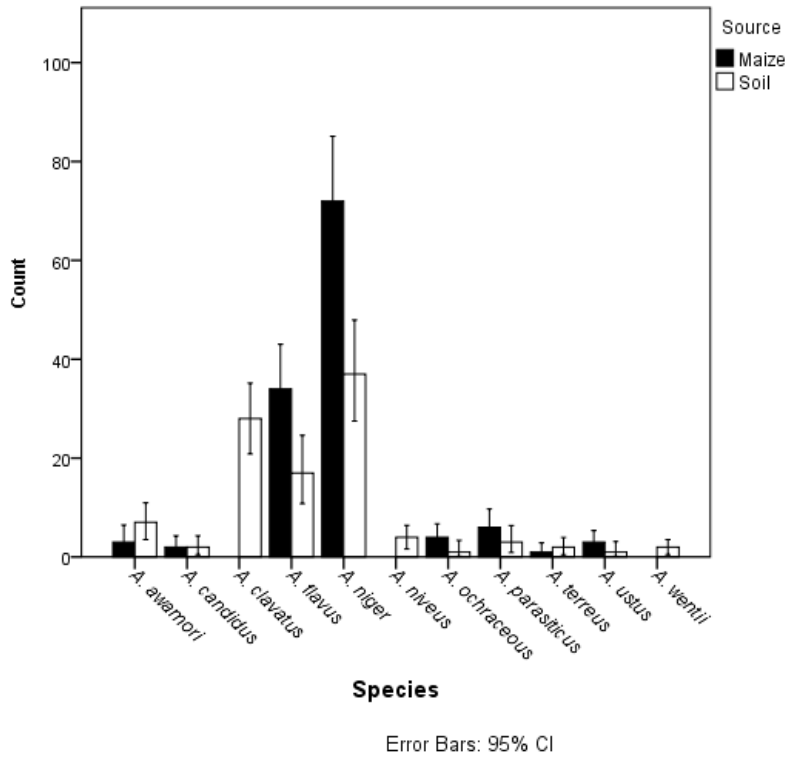

Figure 2. The incidence of the eleven *Aspergillus* species in maize and soil from the Eastern region of Kenya.

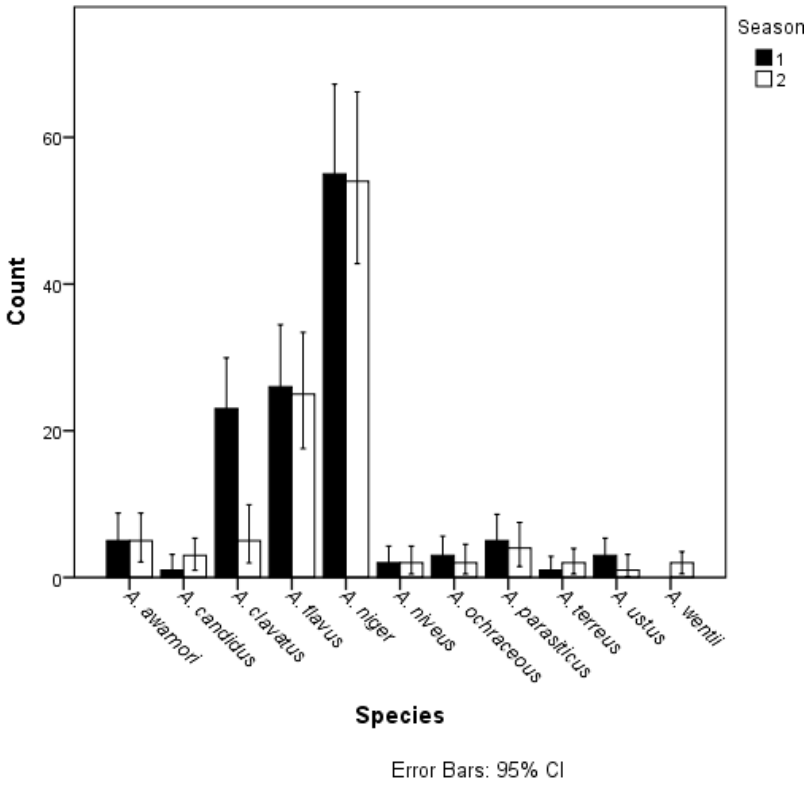

Figure 3. The distribution of *Aspergillus* spp. in the two planting seasons.

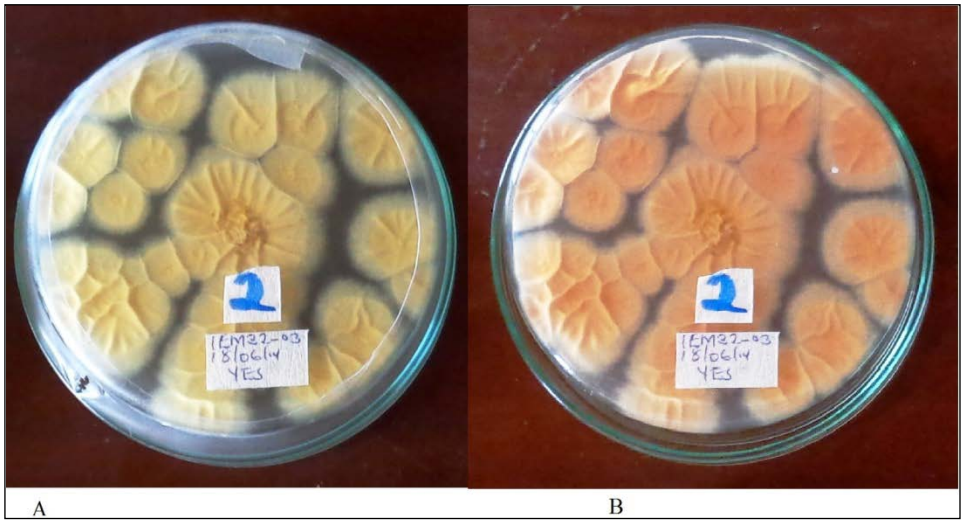

Plate 3. Toxigenic *Aspergillus* species. A and B show the colony reverse of *Aspergillus flavus* isolate from maize in yeast extract sucrose media before and after exposure to NH_4OH. The colony reverse turned from pale yellow to pink to indicate the production of toxins.

Table 4. Abundance of toxigenic and non-toxigenic *Aspergillus* species from maize and soil.

	Season									
	I					II				
	Negative		Positive		Total	Negative		Positive		Total
Maize	45	75.0	15	25.0	60	58	89.2	7	10.8	65
Soil	53	82.8	11	17.2	64	32	80.0	8	20.0	40
Total	98	79.0	26	21.0	124	90	85.7	15	14.3	105

this study were likely to have influenced the abundance and diversity of fungal isolates observed. This is in line with the observation that successful colonization postharvest as influenced by storage conditions may influence the growth of mycotoxigenic fungi and the subsequent production of toxins (Chulze, 2010). Priyanka et al. (2014) studied the molecular diversity of toxigenic *Aspergillus* species from food samples grown in high-rainfall regions in India where 200 isolates were recovered from 320 grain samples. This study also points to a higher fungal burden in Kenya as compared to India. The areas sampled in this study have temperate climatic conditions and experience high rainfall, which is a contrast to eastern Kenya. Even though the authors do not compare the incidence of the fungi by seasons, it is evident that the predominant *Aspergillus* species in both continents are *A. flavus* and *A. parasiticus*.

In the current study, seven putatively toxigenic species (*A. flavus*, *A. parasiticus*, *A. ochraceous*, *A. clavatus,A. ustus*, *A. niveus* and *A. wentii*) were reported. This corroborates earlier reports of toxin production by *Aspergillus* species other than *A. flavus* for example; *A. parasiticus* produces aflatoxins (Abbas et al., 2005); *A. ochraceous* produce ochratoxin A (Montessinos et al., 2015) while *A. wentii* produces the mycotoxins emodin (Pitt and Hocking, 2009). A polyisoprenoid toxin referred to as Austin was reported to be produced by *A. ustus* (Chexal et al., 1976) whereas *A. niveus* has been reported to produce fumonisins (Storari et al., 2012) and aspochalasin Z (Gebhardt et al., 2004). *Aspergillus clavatus* was isolated from soil only and was not isolated from maize, it was classified as toxigenic in this study. Since it was not isolated from maize, human risks appear to be reduced. Earlier studies have reported this species to produce a variety of secondary metabolites (SM) such as patulin, pseurotin A and cytochalasin E (Zutz et al., 2013). On the other hand, other species which did not produce toxin in this study have been reported to be toxigenic in earlier studies for example; *A. candidus* was reported to produce a mycotoxin known as AcT1 (Chattopadhyay et al., 1987), whereas *A. awamori* has been reported to produce fumonisins (Storari et al., 2012) and aspochalamins A-D (Gebhardt et al., 2004). *Aspergillus terreus* has been reported to produce territrems in bakery products and grains (El-Sayed Abdalla et al., 1998). The ammonium test was used to

test the production of toxin in YES medium. It is a reliable method that has been published by Kumar et al. (2007). Another method is to include beta cyclodextrins in the culture medium, which would enhance the natural fluorescence of aflatoxins under ultra violet light (Fente, 2001; Yazdani, 2010). So far, three species of *Aspergillus* namely *A. flavus*, *A. parasiticus* and *A. nomius* have been reported to secrete aflatoxins (Varga et al., 2011). In this paper, additional putatively toxigenic species that may also be producing aflatoxins or its precursors are reported. For these reasons, there is need to confirm the toxigenicity of these fungi through analytical procedures such as HPLC, or through the use of molecular markers to confirm presence of toxin coding sequences in the various *Aspergillus* isolates' genome. Follow-up studies such as molecular studies to confirm the species of the fungi reported herein is also recommended.

The findings of this study are vital in understanding the possible toxin risks the population faces with each harvest and enable early preparedness. There is very little information on the seasonal variations of toxigenic *Aspergillus* species in Kenya, particularly in the Eastern region where repeated cases of aflatoxicosis have been reported. Therefore, our findings contribute to new knowledge with respect to the Eastern region of Kenya.

The wide variety of toxigenic fungi reported in this study points out that the burden of mycotoxin exposure in Eastern Kenya is likely higher than previously thought. This is supported by the fact that the sampled maize grain was what was being consumed at the local household. The situation is further aggravated by lack of regulatory testing at that level. It is also possible that fatalities reported in Kenya could be due to cases of acute exposure to a cocktail of toxins.

In Kenya, the routine tests of toxins by the regulatory agencies (The Government Chemist and The Kenya Bureau of Standards) mainly aimed at aflatoxins (B1, B2, G1 and G2). While, this is proper due to the abundance of aflatoxins (Probst, 2007), we hold the view that consideration to include Ochratoxins and Fumonisins should be made since isolates of *Aspergillus* in this and previous studies point to higher risks of exposure (Bayman et al., 2002).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge funding from the Bill and Melinda Gates Foundation (Grant ID# OPP1060246) from which reagents to carry out the analysis were purchased. Sampling was carried out with support from The National Commission for Science and Technology Foundation (NACOSTI/RCD/ST&I 5th Call MSc 085). The authors express their heartfelt gratitude to the farmers in Eastern Kenya for their co-operation in this work. They are grateful to Collins Omondi (Egerton University) for technical support in microbiology work.

REFERENCES

Abbas HK, Zablotowicz RM, Bruns HA, Abel CA (2006). Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillusflavus* isolates. Biocontrol Sci. Technol. 16:437-449.

El-Sayed Abdalla A, Zeinab Kheiralla M, Sahab A, Hathout A (1998). *Aspergillusterreus* and its toxic metabolites as a food contaminant in some Egyptian bakery products and grains. Mycotoxin Res. 14:83-91.

Atehnkeng J, Ojiambo PS, Cotty PJ, Bandyopadhyay R (2014). Field efficacy of a mixture of atoxigenic*Aspergillusflavus* Link: Fr vegetative compatibility groups in preventing aflatoxin contamination in maize (*Zea mays* L.). Biol. Control 72:62-70.

Bayman P, Baker JL, Doster MA, Michailides TJ, Mahoney NE (2002). Ochratoxin production by the *Aspergillusochraceus* group and *Aspergillusalliaceus.* Appl. Environ. Microbiol. 8(5):2326-2329.

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillusniger, Aspergillusoryzae,* and *Trichodermareesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharm. 39:214-228.

CDC (2004). Outbreak of aflatoxin poisoning – Eastern and Central provinces, Kenya, January–July 2004.Morb. Mortal Wkly. Rep. 53:790-793.

Chattopadhyay SK, Nandi B, Ghosh P, Thakur S (1987). A new mycotoxin from *Aspergilluscandidus* Link isolated from rough rice. Mycopathologia 98(1):21-26.

Chexal KK, Springer JP, Clardy J, Cole RJ, Kirksey JW, Dorner JW, Cutler HG, Strawter BJ(1976). Austin, a novel polyisoprenoidmycotoxin from *Aspergillus ustus*. J. Am. Chem. Soc. 98(21):6748-6750.

Chulze SN (2010). Strategies to reduce mycotoxin levels in maize duringstorage: A review. Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess. 27:651-657.

Commission Regulation (EC) No 1881/2006 (2006). Setting maximum levels for certain contaminants in foodstuffs.http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1419348957971anduri=CELEX:02006R1881-20140701Accessed 5 Oct. 2015.

Cotty PJ, Antilla L, Wakelyn PJ (2007). Competitive exclusion of aflatoxin producers: Farmer-driven research and development. In: Vincent C, Goettel MS, Lazarovits (eds). Biological control: A global perspective. New York, CAB International, pp.241-253.

Daniel JH, Lewis LW, Redwood YA, Kieszak S, Breiman RF, Flanders WD, Bell C, Mwihia J, Ogana G, Likimani S, Straetemans S, McGeehin MA (2011). Comprehensive assessment of maize aflatoxin levels in Eastern Kenya, 2005-2007. Environ. Health Persp. 119:1794-1799.

Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M (2007).Identification of *Aspergillus* species using morphological characteristics. Pak. J. Med. Sci. 23:867-872.

Do JH, Choi D-K (2007). Aflatoxins: detection, toxicity, and biosynthesis. Biotechnol. Bioprocess Eng. 12:585-593.

Fapohunda SO (2009). Impact of Mycotoxins on Sub-Saharan Africa: Nigeria as a Case Study. In European Mycotoxins Awareness Network. 2009. http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=71 Accessed 8 Jan. 2015.

Freeman AH, Coe R (2002). Smallholder farmers use of integrated nutrient management strategies: Patterns and possibilities in Machakos District of Eastern Kenya. In: Barrett CB, Place F, Aboud AA (eds) Natural resource management in African agriculture. New York, CABI, pp. 143-154.

Funk C (2010). A climate trend analysis of Kenya—August 2010. US

Geological Survey Fact Sheet 2010-3074.http://pubs.usgs.gov/fs/2010/3074/pdf/fs2010-3074.pdf Accessed 2nd October 2015.
Gebhardt K, Schimana J, Höltzel A, Dettner K, Draeger S, Beil W, Rheinheime J, Fiedler HP (2004). Aspochalamins A-D and aspochalasin Z produced by the endosymbiotic Fungus *Aspergillusniveus* LU 9575. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot. (Tokyo). 57(110):707-714.
Gong Y, Hounsa A, Egal S, Turner PC, Sutcliffe AE, Hall AJ, Cardwell K, Wild CP (2004). Post weaning exposure to aflatoxin results in impaired child growth: A longitudinal study in Benin, West Africa. Environ. Health Persp. 112(3):1334-1338.
IITA, aflasafe™ (2014). Development and commercialization of biological control of aflatoxins in Kenya and Nigeria. http://www.iita.org/agriculture-health-project-asset;jsessionid=24A51C026D2BD1614D1023ED50C3F3A7. Accessed 10th April 2015.
Jiang Y, Jolly PE, Preko P, Wang J-S, Ellis WO, Phillips TD, Williams, JH (2008). Aflatoxin-related immune dysfunction in health and in Human Immunodeficiency Virus Disease. Clin. Dev. Immunol. vol. 2008, Article ID 790309, 12 pages, 2008.
Kennedy N, Brodie E, Connolly J, Clipson N (2006). Seasonal influences on fungal community structure in unimproved and improved upland grassland soils. Can. J. Microbiol. 52:689-694.
Klich MA (2002). Identification of common Aspergillus species 1stedn. Utrecht, The Netherlands, The Centraal bureau Voor Schimmelcultures.
Klich MA (2002). Biogeography of *Aspergillus* species in soil and litter. Mycologia 94(1):21-27.
Kumar S, Shekhar M, Ali KA, Sharma P (2007). A rapid technique for detection of toxigenic and non-toxigenic strain of *Aspergillus flavus* from maize grain. Indian Phytopathol. 1:31-34.
McHugh TA, Koch GW, Schwartz E (2014). Minor changes in soil bacterial and fungal community composition occur in response to monsoon precipitation in a semiarid grassland. Microb. Ecol. 68(2):370-378.
Montessinos E, Frances J, Badosa E, Bonaterra A (2015). Postharvest control. In: Lugtenberg B (ed) Principles of plant-microbe interactions. New York, Springer, pp. 193-202.
Muthomi JW, Mureithi BK, Chemining'wa GN, Gathumbi JK, Mutitu EW (2009). *Aspergillus* and aflatoxin B1 contamination of maize and maize products from Eastern and North-rift regions of Kenya. http://www.fao.org. Accessed 110 Jan. 2015.
Mutiga, SK, Were V, Hoffmann V, Harvey, JW, Milgroom MG, Nelson, RJ (2014).Extent and drivers of mycotoxin contamination: Inferences from a survey of Kenyan maize mills. Phytopathology 104:1221-1231.
Odhiambo BO, Murage H, Wagara IN (2013). Isolation and characterisation of aflatoxigenic *Aspergillus* species from maize and soil samples from selected counties of Kenya. Afr. J. Microbiol. Res. 7(35):4379-4388.
Palencia ER, Hinton DM, Bacon C W (2010). The black *Aspergillus* species of maize and peanuts and their potential for mycotoxin production. Toxins 20:399-416.
Pitt JI, Hocking AD (2009). Fungi and food spoilage, 3rd ed. New York, Springer Science & Business Media.
Priyanka SR, Venkataramana M, Kumar GP, Rao KV, Muralia HCS, Batra HV (2014). Occurrence and molecular detection of toxigenic *Aspergillus*species in food grain samples from India. J. Sci. Food Agric. 94:537-543.
Probst C, Bandyopadhyay R, Price LE, Cotty PJ (2011). Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. Plant Dis. 95(2):212-218.
Probst C, Njapau H, Cotty PJ (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. Appl. Environ. Microbiol. 73(8):2762-2764.
Rodrigues P, Soares C, Kozakiewics Z, Paterson RRM, Lima N, Venancio A (2007). Identification and characterization of *Aspergillusflavus* and aflatoxins. In: A. Méndez-Vilas (ed). Communicating current research and educational topics and trends in applied microbiology. Spain, Formatex Research Centre pp. 527-534.
Storari M, Dennert FG, Bigler L, Gessler C, Broggini GAL (2012). Isolation of mycotoxins producing black *Aspergilli* in herbal teas available on the Swiss market. Food Control 26:157-161.
Varga J, Frisvad JC, Samson RA (2011).Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi.* Stud. Mycol. 69(1):57-80.
Venkataramana M, Siddaih CN, Nagesh M, Garapati P, Kumar KN, Sreepathi H, Murali HCS, Mattilad TY, Batra HV (2014). Mould incidence and mycotoxin contamination in freshly harvested maize kernels originated from India. J. Sci. Food Agric. 94(13):2674-2683.
Waliyar F, Osiru M, Ntare BR, Kumar VKK, Sudini H, Traore A, Diarra B (2015). Post-harvest management of aflatoxin contamination in groundnut. World Mycotoxin J. 8(2):245-252.
Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004). Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr.80:1106-1122.
Zrari TJO (2013). Detection of aflatoxin from some *Aspergillus* sp. isolated from wheat seeds. J. Life Sci. 7:1041-1047.
Zutz C, Gacek A, Sulyok M, Wagner M, Strauss J, Rychli K (2013). Small chemical chromatin effectors alter secondary metabolite production in *Aspergillusclavatus*. Toxins 5:1723-1741.