CHARACTERIZATION OF AFLATOXINS AND TOXIGENIC *ASPERGILLUS* IN MAIZE AND SOIL FROM THE EASTERN REGION OF KENYA

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

This is my original work and has never been submitted in part or whole for an award in any institution.

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RECOMMENDATION

This thesis has been submitted with my approval as the supervisor for examination according to Egerton University regulations.

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ABSTRACT

Aflatoxin contamination is a major problem affecting cereal producers worldwide. *Aspergillus* species, which are known to produce these toxins, colonize cereals in the field, during post-harvest period through to storage. This study sought to establish variation in: (i) fungal species in maize and soil, (ii) their morphological diversity, (iii) the distribution of toxigenic *Aspergillus* species as well as, (iv) the seasonal variations of aflatoxins and (v) the predominant toxin type in Eastern Kenya. Maize and soil samples were collected from farmers' grain stores and fields respectively (in May and December 2013), which corresponded to two months following the first and second harvesting seasons. Fungi of the genus *Aspergillus* were isolated from maize and soil samples by direct plate and dilution plate techniques respectively on Czapek Dox Agar and thereafter sub-cultured and purified on Potato Dextrose Agar (PDA). The total aflatoxins were determined using solid phase direct competitive Enzyme-Linked Immunosorbent Assay (ELISA), whereas the aflatoxin types were determined using thin layer chromatography (TLC). *Aspergillus* isolates were identified to species level based on cultural and morphological characteristics. A total of 229 pure *Aspergillus* spp. cultures were obtained (55% from maize and the remaining 45% from soil). Eleven species of *Aspergillus* were isolated in the following order of abundance: *A. niger, A. flavus, A. clavatus, A. awamori, A. parasiticus, A. ochraceus, A. candidus, A. ustus, A. niveus, A. terreus*, and *A. wentii*. *Aspergillus niger* was the predominant fungus of genus *Aspergillus* in both seasons. The seasonal variations of the *Aspergillus* isolates was not significant (P=0.544). However, more aflatoxins were obtained in maize from the short-rains season (269.2 ppb) than from the long-rains season (142.4 ppb). The difference in seasonal distribution of the total aflatoxins was significant (P=0.019) whereas 16% and 44% of maize samples from the long-rains and short-rains seasons respectively exceeded the maximum allowed limit (10 ppb). The predominant aflatoxin type in both planting seasons was aflatoxin B1. However, the short-rains season recorded the production of more types of aflatoxins with the production of aflatoxins B1, B2 and G2. This meant that maize from Eastern Kenya was susceptible to aflatoxin poisoning especially in the short rain seasons and that measures to curb this deserve attention.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND ACRONYMS

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFPA	*Aspergillus flavus* and *Aspergillus parasiticus* differentiation Agar
CCA	Coconut Cream Agar
CFU	Colony Forming Unit
CZ	Czapek Dox Agar
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
HPLC	High Pressure Liquid Chromatography
ICA	Immunoaffinity Column Assay
LC-MS	Liquid chromatography-mass spectroscopy
MRBA	Modified Rose Bengal Agar
RIA	Radioimmunoassay
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Background information

Mycotoxins are poisonous substances of fungal origin and include compounds such as aflatoxins, sterigmatocystin, cyclopiazonic acid, kojic acid, β-nitropropionic acid, aspergillic acid, aflatrem, aspertoxin, gliotoxin, and fumonisin (Bluementhal, 2004). Aflatoxins are a collection of secondary metabolites formed by the fungus *Aspergillus flavus* and *Aspergillus parasiticus* (Williams *et al.,* 2004). These fungi can grow in a wide range of substrates and can be found in commodities such as rice, maize, cassava, peanuts, spices, chillies, and nuts. Four forms of aflatoxins B1, B2, G1, and G2 are the primary public health concern due to their central role in the incidence of primary liver cancer (Hedayati *et al.*, 2007). Aflatoxin B1 (AFB1) is the most commonly occurring mycotoxin, a genotoxin identified to be carcinogenic and teratogenic in humans and animals. It is one of the most potent naturally occurring carcinogenic substance ever characterized (Probst *et al.*, 2011).

When plants are exposed to fungi that produce aflatoxins, they become contaminated with aflatoxins. This may happen at any of these stages: pre-harvest or post-harvest, during processing and during storage. The United States Food and Drug Administration (FDA) has referred to aflatoxins as inevitable food contaminants hence has set the maximum allowable limit in human food products at 20 parts per billion. However, in the US, the levels are varied between 10 parts per billion for humans and 20 parts per billion for livestock (Williams *et al.*, 2004) while in Europe it is 4 ppb (Commission Regulation (EC) No 1881/2006, 2006). In contrast, a majority of the population in Africa relies on maize for their daily nutrition and subsistence (Probst *et al.*, 2010). These levels are not adhered to hence a large part of the population continues to consume maize with aflatoxin concentrations that exceed 10 ppb.

Aflatoxin contamination of foodstuffs occurs at temperatures between 24^{o}C and 35 ^{o}C whenever the moisture levels surpass 7% (Williams *et al.*, 2004). This implies that any food stored without proper drying is likely to be contaminated. As has been mentioned, fungal incursion and the subsequent infection by aflatoxins can take place prior to harvesting. In such cases where aflatoxin contamination occurs before harvest, the harvesting and processing conditions only serve to accelerate the growth of the fungi. Pre-harvest contamination is promoted by factors such as soil type, fungal genotype, climatic conditions such as drought and insect activity (Williams *et al.*, 2004). Therefore, it is imperative that crops undergo fast and sufficient drying after harvesting to minimize the chances of aflatoxin

contamination. This appears to be the only chance for management of these toxins yet it is largely out of reach of a vast majority of maize producers in not only Kenya but Africa as well. However, even adequately dried food can still provide favorable conditions for the growth of aflatoxin because of the presence of moisture produced from the respiration of insects (Williams *et al.*, 2004). Farmers in developed countries use extensive production, drying and storage facilities to prevent aflatoxin contamination of food. However, such measures are not attainable in developing countries and attempts to have them only serve to escalate the cost of production further pushing the cost of food beyond the reach of the bulk of the population.

Kenya is the only country in the world which has had recurrent episodes of human deaths caused by aflatoxicosis in the last four decades (Shephard, 2008). This poisoning continues to happen despite the World Health Organization setting standards of the highest limits of aflatoxin contamination in feeds meant for human and animal consumption. This is likely to suggest that farmers in Kenya may be playing a part in the rising cases of aflatoxin contamination of maize. Some parts of Kenya especially the Eastern region experience rampant aflatoxicosis cases than other areas. Therefore, it is necessary to establish the actual reasons underlying these occurrences to be able to mount an appropriate counter-measure for long-term management, which is part of what this project seeks to unravel.

It is widely believed that climate and weather patterns play a significant role in aflatoxin contamination (Cotty and Jaime-Garcia, 2007; Milani, 2013). The Eastern region of Kenya is semi-arid region that experiences annual rainfall of between 250mm 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 average temperatures in this region range from 23^{o}C to 34 ^{o}C (Funk, 2010). Production of aflatoxins occurs under specific conditions of moisture and temperature during pre-harvest, harvest and storage stages. Several studies have attempted to establish the relationship between moisture and temperature as depicted by planting seasons and the production of aflatoxins. However, these studies vary by design, type of crop and regions.

1.2 Statement of the problem

In many parts of the world, aflatoxin contamination is a major challenge to the production of good quality cereals such as maize, rice, groundnuts and many other crops. Maize is one of the most important and highly consumed food commodities in Kenya. Therefore, its regular consumption increases the risk of continued exposure to aflatoxicosis.

The consumption of contaminated maize presents a risk of human fatalities in acute incidences, but more importantly serious health conditions such as liver damage and depressed immunity in chronic cases. The United States Food and Drug Administration has set the maximum allowable limits of aflatoxins in human food as 10 ppb in human food and 20 ppb in animal feeds while Europe has set its limit at 4 ppb. Kenya, on the other hand, has set its limits at 10 parts per billion. However, the scenario in Kenya is complicated by the inability of the government to enforce similar regulations among small-scale farmers and maize handlers who continue to produce and circulate contaminated maize. Furthermore, little information is known about the seasonal variation in aflatoxin prevalence and types among the major maize producing regions in Kenya, which has currently necessitated increased research attention. A prerequisite is first to obtain information on the seasonal prevalence of the toxin-producing fungi types as well as the levels of aflatoxins in the two planting seasons before an intervention can be mounted. This information is of primal importance because currently the only attempt to control the quality of maize being consumed nationally is at the point of delivery to the major millers and other organizations like the National Cereals and Produce Board. This leaves out the small-scale producers. Therefore, there is need to obtain this information particularly in the traditionally problematic region of Eastern Kenya before the same can be ascertained in other maize growing regions of Kenya.

1.3 Objectives

1.3.1 General objective

To characterize aflatoxins and toxin producing *Aspergillus* species in maize and soil respectively from the Eastern region of Kenya

1.3.2 Specific objectives

1. To determine the predominant toxigenic *Aspergillus* species in maize and soil from Eastern Kenya
2. To determine the total aflatoxins in maize from Eastern Kenya by ELISA
3. To determine the predominant aflatoxin type in maize from Eastern Kenya by TLC

1.4 Hypotheses

1. There is no difference in the distribution of *Aspergillus* species in maize and soil from Eastern region of Kenya
2. There is no difference in total aflatoxins in maize from Eastern Kenya

3. There is no difference in the type of aflatoxin predominant in maize from Eastern Kenya

1.5 Justification

Maize is the staple food for Kenyans in most parts of the country. The bulk of maize harvest is consumed without processing. Maize processing allows for the determination of the level of contamination by aflatoxins through rigorous checks carried out by organizations such as the Kenya Bureau of Standards and the national Cereals Produce Board. Such testing prevents the distribution of aflatoxin-contaminated maize products to consumers. However, since the bulk of the maize consumed locally does not go through millers, it is likely that Kenyans continue to consume maize contaminated with aflatoxins. Aflatoxin contamination occurs either pre- or post-harvest of maize, handling and processing for storage. Therefore, it is necessary for the levels of aflatoxin in maize to be established to preclude the entrance of mycotoxins into the food chain and prevent further cases of aflatoxin poisoning.

The Eastern part of Kenya is the only region believed to be having recurrent incidences of aflatoxicosis over the years. Several possible contributing factors have been and continue to be investigated with a likely pointer to the various fungi as the main causative agents. Information as to the main factors driving contamination in the region is however lacking. Besides, it is still not known whether there are variations in the fungi type through all planting seasons and whether their potential to produce toxins is the same in both seasons. The scenario is further aggravated by the fact that information on the seasonal variation of levels, quantities and types of toxins putatively present in Eastern Kenya and other maize growing areas is lacking. This information is important in order to mount appropriate intervention. Therefore, this study aimed to fill this gap by providing information on the seasonal variation in the levels, quantity and types of toxins present in the Eastern region of Kenya. In the current study, data is provided on the seasonal variation in fungi types as well as quantities and types of aflatoxins present in the endemic region of Eastern Kenya. It is believed that this information will be useful in mounting interventions to prevent the entrance of aflatoxin contaminated maize into the food chain hence avoid additional cases of aflatoxicosis.

CHAPTER TWO
LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are a group of poisonous naturally occurring chemical substances produced by fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium*, which are commonly known as moulds. These fungi can grow on a variety of crops and foodstuffs including cereals, nuts, spices, and dried fruits kept under warm and humid conditions. One mold species may produce many different mycotoxins, and the same mycotoxin may be produced by several species. Examples of mycotoxins include aflatoxins, fumonisins, ochratoxins, patulins, trichothecenes (principally nivalenol, deoxynivalenol, T-2 and HT-2 toxin), Cyclopiazonic acid, zearalenone (Turner *et al.*, 2005). The presence of mycotoxins in foodstuffs may affect human and animal health as they may cause many different adverse health effects such as induction of cancer, mutagenicity and apoptosis of cells (Figure 1), as well as estrogenic, gastrointestinal and kidney disorders.

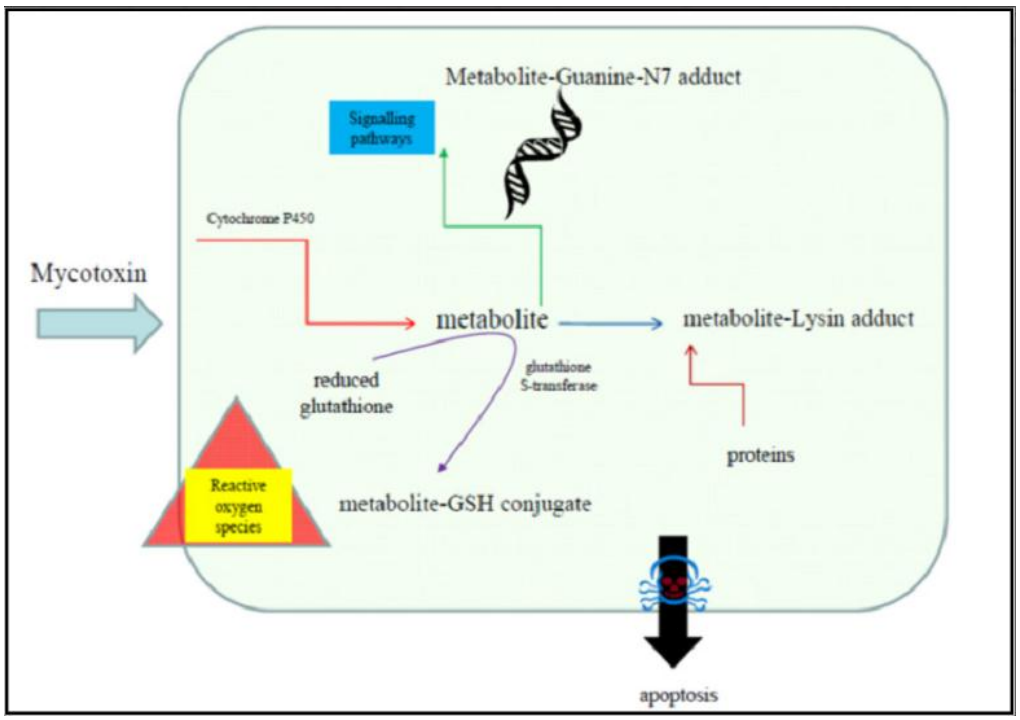

Figure 1: Mechanism of action of mycotoxins inducing apoptosis (Source: Omar, 2013).

Mycotoxins resist decomposition or being broken down during digestion. Therefore, they remain in the food chain in meat and dairy products. Temperature treatments, such as cooking and freezing, do not destroy mycotoxins (Strosnider *et al.*, 2006).

2.1.1 Aflatoxins

Aflatoxins are secondary metabolites produced by fungi of the genus *Aspergillus.* They are a group of polyketide-derived difuranocoumarins that are structurally related (Cavaliere *et al.*, 2007). Aflatoxins first hit the headlines in the 1960's when more than a 100,000 turkeys died in England because of liver necrosis and haemorrhages (Siller and Ostler, 1961). Investigation revealed that the illness was due to the presence of *Aspergillus flavus* in groundnut meal. Therefore, the toxin was named aflatoxin from *Aspergillus flavus* toxin (Nesbitt *et al.*, 1962). Aflatoxin poisoning occurs in humans through ingestion, inhalation and absorption via the skin. High-level (acute) exposure can lead to instant death, whereas long-term (chronic) exposure may cause cancer, nervous disorders and mutagenicity (Klich, 2007a).

Aflatoxins are mainly categorised into four broad structurally related compounds: B1, B2, G1, and G2. The letters B and G refer to the colour of their fluorescence under UV light (blue or green), whereas the numbers show their relative migration on TLC plates. Aflatoxin B1 is the most predominant and the most potent hepatocarcinogenic compound ever characterized (Gautam and Bhadauria, 2012). Aflatoxin B1 (AFB1) is metabolized by the hepatic microsomal mixed-function oxidase system, but it also can undergo several metabolic conversations depending on the animal species (Marsi *et al.*, 1974). Aflatoxin B1 undergoes hydroxylation in the gastrointestinal tract of some animals including cattle to form aflatoxin M1 and M2 (Figure 2 and Figure 3).

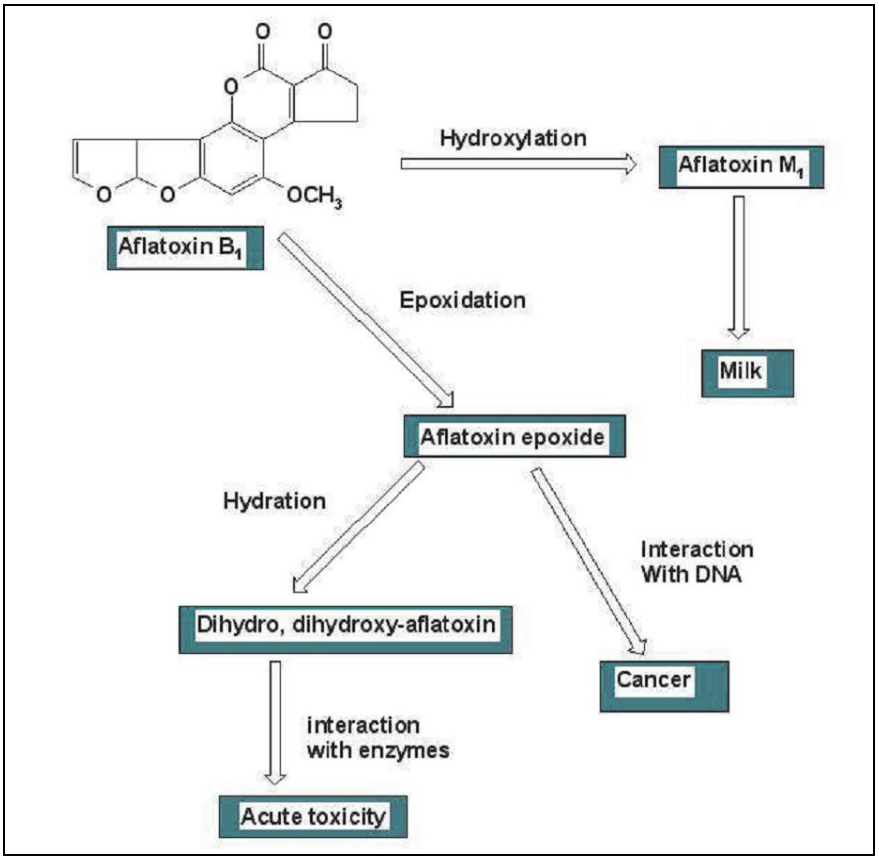

Figure 2: Some metabolic products of aflatoxin B1 (Source: Mohammad, 2011).

Aflatoxin B1 is converted to an epoxide or hydrolated metabolites by cytochrome P450 enzymes in the liver (Figure 1 and Figure 2). The epoxide can bind proteins resulting in toxicity (aflatoxicosis). It may also react with DNA resulting into cancer (hepatocellular carcinoma) or may form dihyrodiol and glutathione conjugates (Williams *et al.*, 2004). The set maximum allowable levels of aflatoxin in food are low enough to prevent acute aflatoxicosis and minimize losses to farmers. However, the long-term risk of exposure to aflatoxins remains. Young people are often more susceptible to poisoning than older people because the ability to detoxify aflatoxin increases with age (Williams *et al.*, 2004).

The amounts of aflatoxin M1 (AFM1) excreted in milk as a percentage of AFB1 averages 1-2%, varying from animal to animal, from day to day and from one milking to the other. The AFM1 could be detected in milk 12-24 h after the first AFB1 ingestion, reaching a high level after a few days. Consequently, cattle that consume feeds contaminated with aflatoxin B1 excrete aflatoxin M1 in their milk.

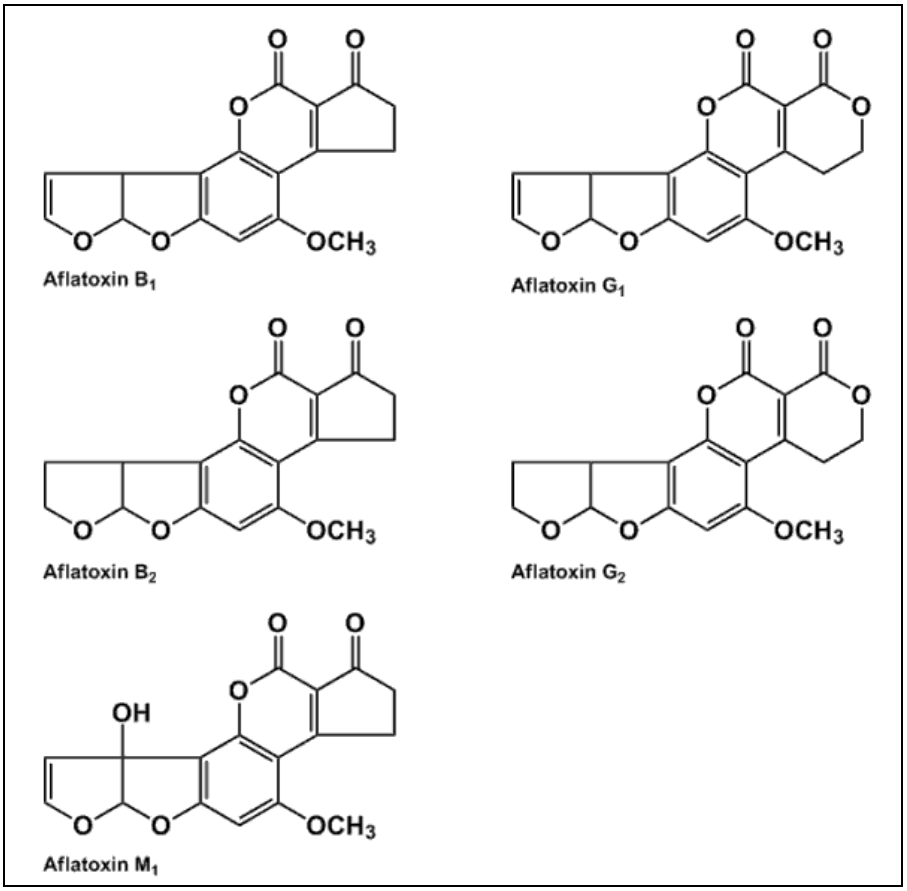

Figure 3: The molecular structures of aflatoxins (Source: U.S. Food and Drug Administration, 2009).

In the farms, aflatoxin contaminated maize appears just like normal maize without any outward sign of infection (Muthomi *et al.*, 2009). Therefore, it is difficult to distinguish clean maize from contaminated maize. Only thorough laboratory investigations can reveal the presence of aflatoxins, which is expensive and laborious especially in developing countries.

2.1.2 Health effects of aflatoxins

In humans, the amount of ingested aflatoxins determines the extent of the damage caused, which can be either chronic or acute. Aflatoxins lead to extensive liver damage (Plate 1). Chronic effects include complications such as the suppression of immunity (Cusumano *et al.*, 1996), cancers, poor child growth (Gong *et al.*, 2004), and abnormal development of fetuses. Consequently, the immunity of HIV positive individuals is further suppressed by the ingestion of aflatoxins (Jiang *et al.*, 2008). Acute effects in contrast include complications such as jaundice, hepatitis, distensions in the abdomen and death (Probst *et al.*, 2011).

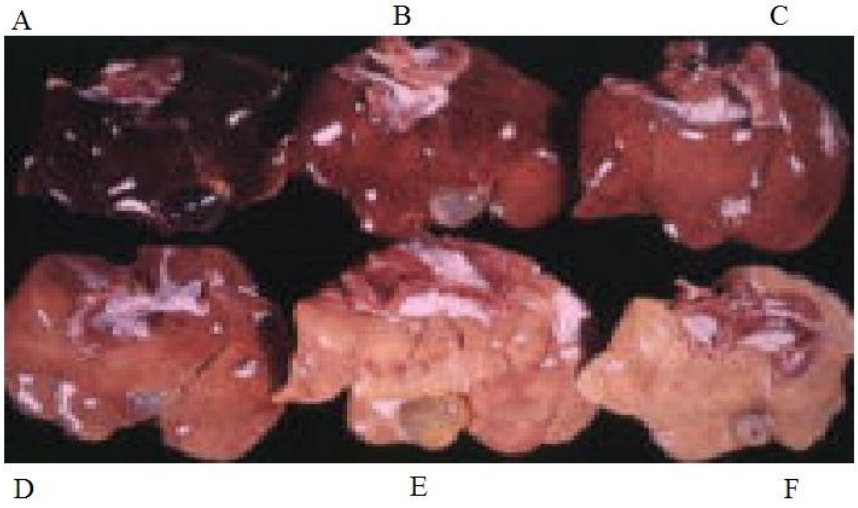

Plate 1: The effect of aflatoxin B1 on six rat livers injected with increasing doses of aflatoxin B1. Liver A received no aflatoxin (control), while liver F received the highest dose (Cornell University, 2014).

2.1.3 Aflatoxicosis in Kenya

Aflatoxicosis was first reported in Kenya in 1978 and has continued to recur until the worst outbreak of acute aflatoxicosis occurred in 2004. This happened to be the most severe episode of human aflatoxin poisoning in the world history with 317 cases being hospitalized by July 2004 accompanied by another 123 fatalities (CDC, 2004; Azziz-Baumgartner *et al.*, 2005; Lewis *et al.*, 2005; Probst *et al.*, 2007). A case fatality rate of 39% was recorded because of consuming aflatoxin-contaminated maize (Lewis *et al.*, 2005). Since then, several workers (Probst *et al.*, 2007; Muthomi *et al.*, 2009; Odhiambo *et al.*, 2013) have aspired to determine the relationships between aflatoxicosis, toxin production and fungi of the genus *Aspergillus*.

However, it is observed that most of these studies do not specifically mention the planting seasons in their sampling procedures. For example, Muthomi *et al.* (2009) obtained maize and soil samples from two consecutive years (2008 and 2009) from Machakos, Makueni, Kitui, Uasin Gishu and Trans Nzoia districts. However, it is not clear whether the sampling was done in the same or different seasons. Recently, Odhiambo *et al.* (2013) characterized/isolated toxigenic and atoxigenic *Aspergillus* species from Eastern region with a view of finding candidate biocontrol agents after the long rains in 2012. On the other hand, Probst *et al.* (2007) focused mainly on *Aspergillus flavus* species only with a view of establishing their production of aflatoxins by contrasting aflatoxin-producing fungi resident in the region with repeated outbreaks of lethal aflatoxicosis to those in regions without a

history of aflatoxicosis. They report that most aflatoxin-producing fungi belonged to *Aspergillus flavus*. In addition, the two major morphotypes of *A. flavus* varied greatly between provinces, with the S strain dominant in both soil and maize within aflatoxicosis outbreak regions and the L strain dominant in non-outbreak regions (Probst *et al.*, 2007). Previous studies also included maize samples from both farmers and traders (Muthomi *et al.*, 2009). Fungal contamination of maize diminishes its quality by causing discolouration, degrading the nutritional value as well as producing mycotoxins. It is estimated that the average daily intake of maize in Kenya is about 400g per person (EPZA, 2005; Muthomi *et al.*, 2009). Therefore, the high consumption of maize coupled with the high rates of fungal contamination in maize has seen the incidence of severe aflatoxin poisoning particularly in the Eastern region of Kenya.

2.2 Analyses of aflatoxins

There are various methods that have been established to analyze and quantify aflatoxins in human food and animal feed samples. These methods can be grouped into two broad categories based on the underlying principles as chromatographic and immunochemical techniques (Espinosa-Calderón *et al.*, 2011). Sampling and sample preparation remain a considerable source of error in the analytical identification of aflatoxins due to the uneven distribution of aflatoxins in cereals. Therefore, methodical approaches to sampling, sample preparation, and analysis are extremely necessary to determine aflatoxins at the parts-per-billion level. Incidentally, specific plans have been developed and tested rigorously for some commodities such as corn and peanuts. A common feature of all sampling plans is that the entire primary sample must be pulverized and mixed so that the analytical test portion has the same concentration of toxin as the original sample (Vincelli *et al.*, 2005).

2.2.1 Chromatographic techniques

Chromatographic techniques include Thin Layer Chromatography (TLC), Liquid Chromatography-Mass Spectroscopy (LC-MS), and High Pressure Liquid Chromatography (HPLC). These techniques involve prior extraction of toxins from food samples with aqueous solvent medium such as: methanol or acetonitrile in HPLC followed by clean up of the extract solution using immunoaffinity columns before the final analysis (Hussain, 2011).

Thin Layer Chromatography is among the oldest and commonly used methods in aflatoxin analysis. It has been used to identify and quantitate aflatoxins levels as low as 1 ppb. On a TLC plate, the B toxins fluoresce blue while the G toxins fluoresce green (Plate 2). Thin layer chromatography is a cheap and fast analytical technique that gives quantitative

approximations by visual inspection of fluorescence on TLC plates. However, it is laborious as well as time consuming and its low accuracy limits its use for research purposes (Espinosa-Calderón *et al.*, 2011).

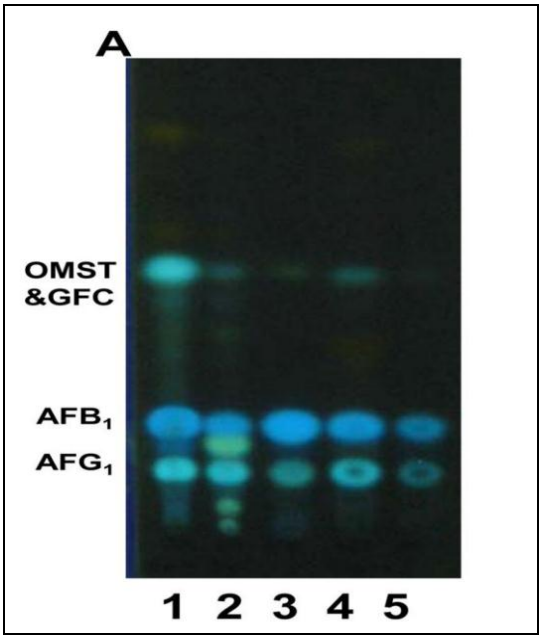

Plate 2: TLC image of aflatoxins exposed to UV light (Source: Erlich *et al.,* 2008).

Liquid chromatography (LC) is a chemical technique used to separate a sample into its individual parts. This separation occurs based on the interactions of the sample with the mobile and stationary phases. Many stationary and mobile phase combinations can be employed when separating a mixture. Consequently, several types of chromatography are classified based on the physical states of those phases. Liquid chromatography offers good sensitivity, high dynamic range, versatility and soft ionization conditions that permit access to the molecular mass of intact biological molecules. It is usually coupled to fluorescence detection stage (FLD), UV absorption and amperometric detection (Elizalde-González, 1998) with pre-column derivatization or post-column derivatization. Extraction and clean up procedures for aflatoxins analysis typically rely on solid phase extraction (SPE) with different absorbent materials such as immunoaffinity columns. On the other hand, LC, despite being similar to TLC in many aspects is considered backbreaking and time consuming. However, it is preferred to TLC in some cases because of its high selectivity and sensitivity (Cavaliere *et al.*, 2007).

Liquid Chromatography-Mass Spectrometry combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS) (Figure 4). LC-MS is a powerful technique that has very high sensitivity

and selectivity and is useful in many applications including the detection and quantification of several mycotoxins such as ochratoxins, deoxynivalenol, zearalenone, sterigmatocystin, cyclopiazonic acid, and fumonisins (Spanjer, Rensen & Scholten, 2008).

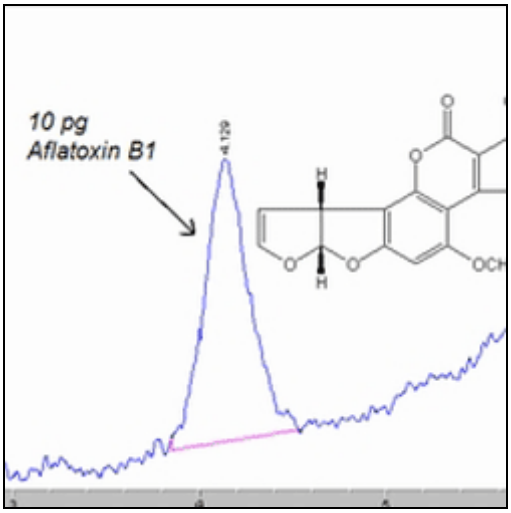

Figure 4: LC-MS output for aflatoxin B1 (Source: Prisna, 2012).

The other chromatographic technique is High Pressure Liquid Chromatography (HPLC), which is a chemistry-based means for quantifying the amount of a chemical compound in an assortment of chemicals. It is highly versatile and can be automated for the detection of target compounds in numerous samples simultaneously (Plate 3). Reverse phase HPLC is frequently used on C18 bonded phases where aflatoxins separate according to their hydrophobicity (Joshua, 1993). The peak height in combination with the resolution time is characteristic for the type of toxin whereas the area under the peak is proportional to the concentration of toxin (Figure 5). HPLC is extremely accurate and is currently used as the confirmatory test for aflatoxins. However, it requires expensive and sophisticated appliances as well as complex procedures.

Plate 3: A photograph of a HPLC system (Source: Hussain, 2011).

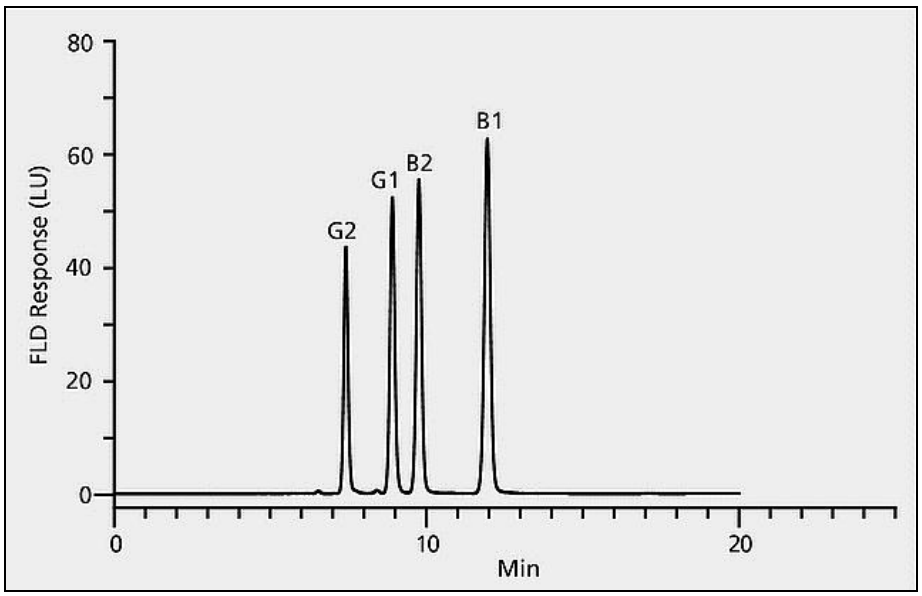

Figure 5: HPLC output for aflatoxins on C18 bonded phase: The retention time and peak heights are identical for a specific type of toxin (Source: Sigma Aldrich, 2015).

2.2.2 Immunochemical methods

Immunochemical methods are based on the affinities of the monoclonal or polyclonal antibodies for aflatoxins. Due to advancements in biotechnology, highly specific antibody-based tests are now commercially available for measuring aflatoxins in foods in less than ten minutes. The three forms of immunochemical techniques are radioimmunoassay (RIA), Immunoaffinity Column Assay (ICA) and Enzyme-Linked Immunosorbent Assay (ELISA). One of the advantages of immunochemical techniques is that they are fast because

crude extracts can be used with such techniques to determine the total aflatoxins (Pirestani, 2011). Consequently, they are referred to as lateral flows or rapid tests. There are two major requirements for immunological methods. First requirement is high quality antibodies and second is methodology to use the antibodies for the estimation of aflatoxins. Being low molecular weight molecules, aflatoxins cannot stimulate the immune system to produce antibodies. Such molecules of low molecular weight, which cannot evoke the immune system, are called haptens. Therefore, before immunization, aflatoxins must be conjugated to a carrier molecule, which is a larger molecule like proteins. Bovine serum albumin (BSA) is most commonly used as a carrier protein, and a hapten is conjugated with it.

The main disadvantage, however, is that immunochemical methods are more costly than TLC. Enzyme-Linked Immunosorbent Assay is commonly used for aflatoxin analysis because it is simple, fast, and extremely sensitive (Beley *et al.*, 2013). However, false positive results can be produced. Therefore, for the purposes of accuracy, it is important to confirm such results using confirmatory techniques such as HPLC. For this reason, ELISA functions very well in an environment that needs routine screening as a basis of rejection or acceptance of commodities.

2.3 The Genus *Aspergillus*

The genus *Aspergillus* consists of a group of ubiquitous filamentous fungi that comprises approximately 200 species and belongs to the class Ascomycota (Klich, 2002). Several *Aspergillus* species have become of importance because of their impact in human health. Some members of genus *Aspergillus* are of medical importance as they are opportunistic pathogens in humans causing aspergillosis (Bertout *et al.*, 2001; Balagee *et al.*, 2007) while others are of economic importance because of their immense economic costs especially to farmers. The genus can be categorized into two groups; the aflatoxin producers (toxigenic), which include members of *A. flavus*, *A. parasiticus*, and *A. nomius* and the atoxigenic (non-toxigenic) species such as *A. oryzae*, *A. sojae* and *A. tamari* (Kumeda and Asao, 2001).

Approximately 20 members of genus *Aspergillus* are responsible for detrimental infections in humans and animals with the most common species being *Aspergillus flavus*. In addition, *A. flavus* infests crops such as corn, peanuts and cotton and contaminate them with aflatoxins resulting in reduced quality of yields. *Aspergillus* species can only colonize plants especially those that have wounds. However, they also have some degree of parasitic capacity and can at times infest seeds and inhabit living tissues. This is attributed to a wide array of

proteinases and proteases produced by *Aspergillus* spp. since resistance to *A. flavus* in corn kernels arises from a protease inhibitor (Chen *et al.*, 1998). Insects, however, play a significant role in influencing crop colonization by *Aspergillus* spp. by creating kernel damage sites (Chen *et al.*, 1998).

2.3.1 Biology of *Aspergillus* species

Members of genus *Aspergillus* are saprophytic fungi with the ability to thrive on any organic nutrient sources such as plant debris, leaves, rotting wood, compost piles, stored grains, as well as live animals (Klich, 1998). The plant body of *Aspergillus* consists of branched separate mycelium, which bears bright or pale hyphae lying on substratum. Their hyphae are much branched and separate. Each cell is multi nucleate and has mass of granular protoplasm and oil globules. Reproduction takes place by vegetative, asexual and sexual methods. Vegetative reproduction takes place by fragmentation while asexual reproduction occurs during favourable conditions of growth and nutrition by conidiospores. Conidiospores arise from mycelium, and their free ends swell up into vesicles. Many small and bottle shaped hyphal outgrowths bud out from the vesicle and cover their entire surfaces. Each conidium or conidiospore in a small, oval, greenish uni or multi nucleate structure. Once mature, the conidia or conidiospore are distributed by air to the substratum where the spores germinate to form hyphae, which develop into fresh mycelia. Sexual reproduction is rare and takes place in favourable conditions. Sex organs are produced on the same mycelia that produce conidia (Klich, 1998).

The life cycle of *Aspergillus* spp. in agricultural fields can be classified into two stages: a) colonization of plant debris in the soil and b) invasion of seeds and grain in actively growing plants (Figure 6).

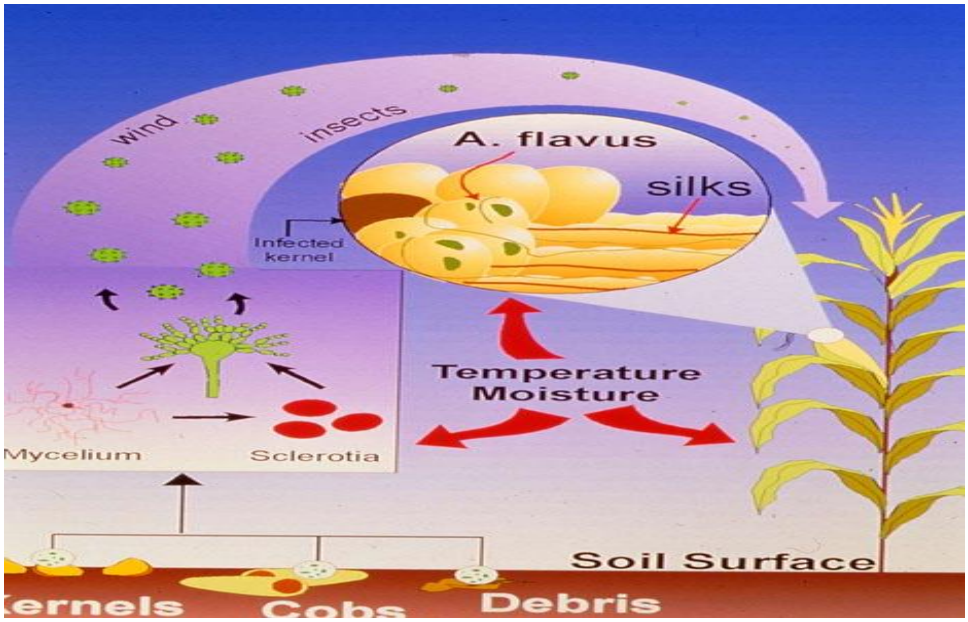

Figure 6: Life cycle of *A. flavus* and *A. parasiticus* in agricultural ecosystems (Source: Robertson, 2006)

Under unfavourable environmental conditions such as dry and poor nutrition, the mycelia assemble and form resilient edifices known as Sclerotia (Yu *et al.*, 2005). Sclerotia are coloured compact masses of hyphae that can withstand hostile environmental conditions with the ability to remain dormant for lengthy durations (Klich, 2007). When conditions become favourable, the sclerotia germinate to yield extra hyphae or conidia (asexual spores), which can be further dispersed in soil and air (Plate 4).

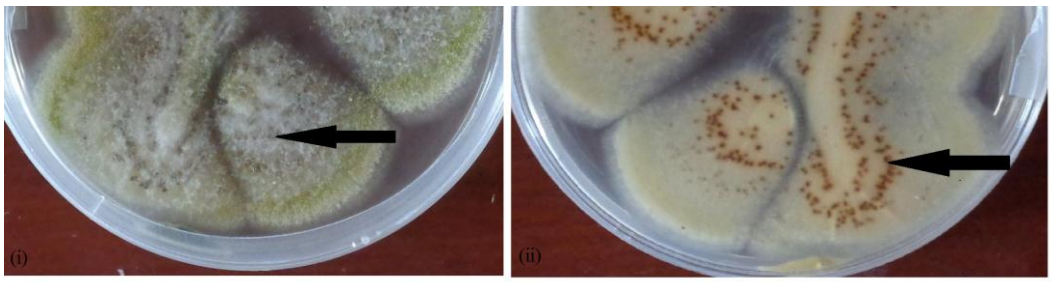

Plate 4: Images of sclerotia as seen on the colony surface (i) and reverse (ii) of *A. flavus* colonies.

There are two distinct phases of aflatoxin infection; i.e. the infection of the developing crop in the initial phase and escalation in contamination following maturation in the second stage. Wounding of developing crops mechanically by birds, insects and mammals or by drought stress and high temperatures promotes the contamination of plants by *A. flavus* and *A. parasiticus* (Guo *et al.*, 2002). The fungi can consequently attack seeds of monocots and dicots found above and below the ground (Yu *et al.*, 2005). The second phase of

infection entails the growth and development of the fungi to maturity until they start producing toxins (Guo *et al.*, 2002).

2.3.2 Identification of *Aspergillus* spp.

Conventional approaches for species identification rely on morphological traits such as the diameter of colonies, colour, size and texture of conidia and conidiophores as well as the production of exudates and soluble pigments (Klich, 2002). Previous studies reveal that the genus *Aspergillus* comprises nine species and two varieties according to the colour and ornamentation of conidial heads, therefore, the identification of members of this genus is usually achieved by the recognition of characteristic conidiophores (Klich, 2002). The main feature of distinction between *A. flavus* and *A. parasiticus* is the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only for (uniseriate conidial head) *A. parasiticus* (Figure 7). Currently, the ornamentation of the conidial wall is the key diagnostic trait for the distinction of these two species. Conidia of *A. flavus* have thick walls, which are lightly roughened and their shape ranges from spherical to elliptical while *A. parasiticus* has more spherical and noticeably echinulate conidia (Hedayati *et al.*, 2007).

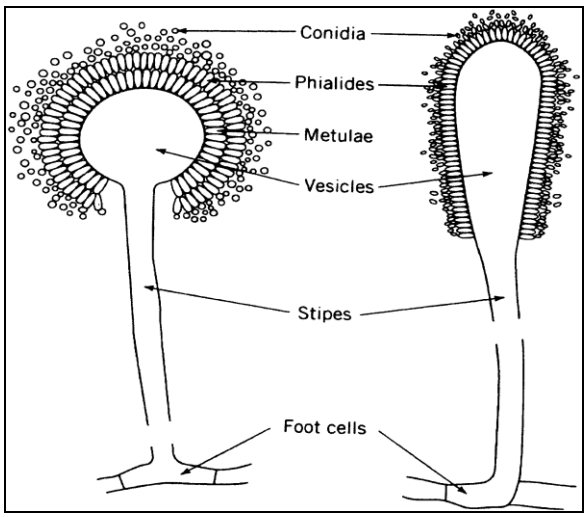

Figure 7: Characteristic conidiophores of *Aspergillus* (Source: Klich, 2007a)

2.3.4 Toxigenic species within *Aspergillus* section *Flavi*

There is reported variability in the production of aflatoxins by various isolates of *Aspergillus flavus*. Only a half of *A. flavus* strains produce aflatoxins, but they produce more than 106 µg/kg (Cotty, 1994). *Aspergillus flavus* has two morphotypes namely the L and S strain. The L-strains have sclerotia greater than 400µm in diameter, whereas the S strains have sclerotia that are less than 400µm in diameter (Probst *et al.*, 2011). The S strain has a

variant known as the S_{BG} strain that was first isolated in West Africa (Cotty and Cardwell, 1999). It has been shown that the S-type *A. flavus* is toxigenic and produces large quantities of B aflatoxins while the L-type produces less B aflatoxins than the S strain. *Aspergillus parasiticus* and the S_{BG} strain produce high levels of aflatoxins and are, therefore, typically toxigenic. *Aspergillus flavus* only produces aflatoxins B1 and B2 due to a 0.8- to 1.5-kb deletion in the 28-gene aflatoxin biosynthesis cluster (Probst *et al.*, 2007), whereas the S_{BG} strains produce all the four toxins (Cotty, 1994).

A number of selected media such as *Aspergillus flavus* and *Aspergillus parasiticus* differentiation agar (AFPA), coconut cream agar (CCA) and Czapex dox agar (CZ) can also be used to distinguish between the species. AFPA identifies *A. flavus* strains based on the development of an orange colour on the back of the plates (Plate 5) (Cotty, 1994a).

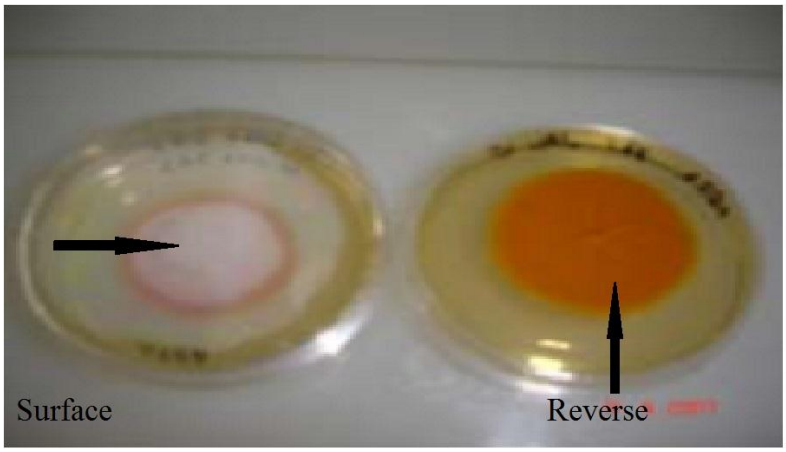

Plate 5: *A. flavus* in AFPA medium. Note the development of an orange colour on the reverse of the colony (Source: Rodriguez, 2007).

On the other hand, coconut cream agar can distinguish aflatoxin producer strains by the observation of a blue fluorescence when exposed to ultraviolet light (Davis *et al.*, 1987). The two species can also be identified by growing them on CZ where colonies of *A. flavus* are usually yellow-green while those of *A. parasiticus* are darker green. Table 1 below gives the distinguishing characteristics of *Aspergillus flavus* and *Aspergillus parasiticus*. The distinction of the two species based on conidia and cultural features are illustrated (Plates 6 and 7).

Table 1: Morphological separation of *A. flavus* and *A. parasiticus* (Source: Rodrigues *et al.*, 2007)

	Colony colour	Seriation	Conidia shape	Texture
A. flavus	Yellow/green	b/u	gl/el	sm/fr
A. parasiticus	Ivy green	u/b	Gl	R

u= uniseriate; b= biseriate; gl= globose; e= elliptical sm= smooth; fr= finely roughened; r= rough

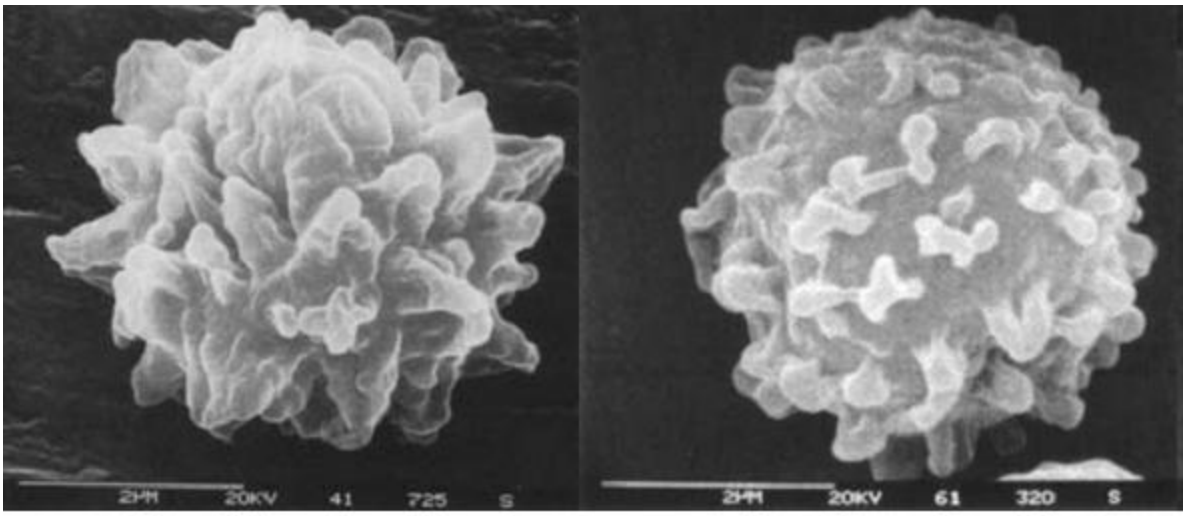

Plate 6: Scanning electron micrographs that distinguish the spores of *A. parasiticus* (a) from those of *A. flavus* (b). Note the appearance of the conidial surfaces in the two species (Source: Rodriguez *et al.*, 2007).

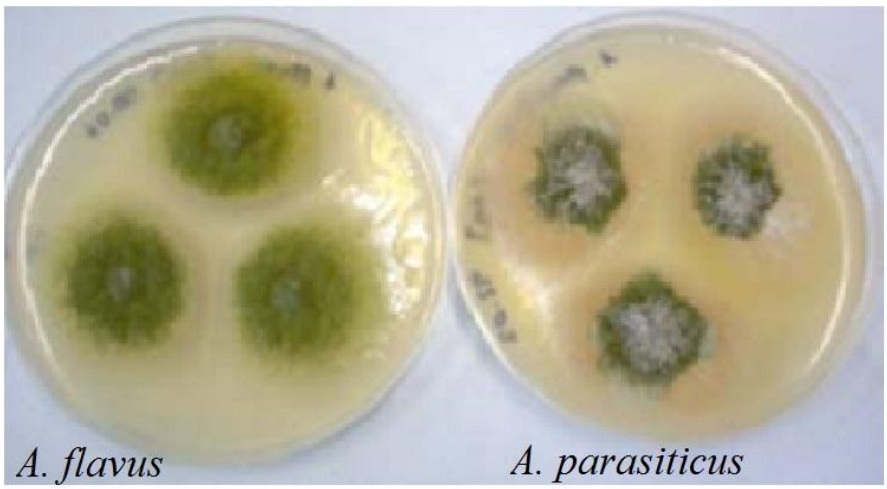

Plate 7: *A. flavus* and *A. parasiticus* growing on CZ medium. A. *flavus* has bright green spores while *A. parasiticus* has dark green spores (Source: Rodriquez *et al.*, 2007).

2.4 Management of aflatoxin contamination

Aflatoxin contamination can be minimized or controlled when the crop is still in the farm, during harvest and post-harvest. In the farm, biological control of *Aspergillus* species is commonly used. Potential biocontrol agents include bacteria such as *Bacillus subtilis*, *Lactobacilli* species, *Pseudomonas* species, and *Burkolderia* species, which show the ability to inhibit fungal growth and aflatoxin producing ability of *Aspergillus* species (Yin *et al.*, 2008). *Bacillus pumilis* has been shown to prevent aflatoxin production by *Aspergillus parasiticus* NRRL 2999 in yeast extract sucrose broth. This inhibition has been proven in laboratory conditions, but its efficacy in the fields has not been established due to the challenge of bringing bacterial cells to the affected plants (Munimbazi and Bullerman, 1998). Saprophytic yeasts such as *Candida krusei* and *Pichia anomala* also exhibit inhibition of *A. flavus* under laboratory conditions. Immense success in biological control of aflatoxins has been achieved using atoxigenic strains of *Aspergillus flavus* in the field (Pitt and Hocking, 2006; Kimatu *et al.*, 2012). These atoxigenic strains are applied to crops in the field. Significant reductions of contamination in the range of 70% -90% have been achieved in peanut and cotton fields in the United States (Dorner, 2004; Dorner, 2008; Klich, 2007).

There are numerous public health interventions to control aflatoxin contamination of foodstuffs and these are broadly categorized into three: i) agricultural, ii) dietary, and iii) clinical. Agricultural interventions include technologies that can be applied in the field before harvest or post-harvest during drying, storage and transportation. Such measures are regarded as prime interventions as they directly lower aflatoxin in food and can include biocontrol of *Aspergillus* strains as already mentioned. Inoculating corn with atoxigenic strains of *A. flavus* such as AF36 (Cotty, 1994) reduces aflatoxin contamination. Afla-Guard™ is a commercially available product for aflatoxin biocontrol in the US where pearl barley grains are coated with conidia of an atoxigenic strain of *A. flavus* (Abbas *et al.*, 2006). These grains are applied to groundnut fields and provide competitive exclusion of toxigenic strains. Studies by Wu *et al.* (2008) reveal that the use of Afla-Guard™ and AF36 is cost effective since market benefits of yields outweigh the cost of the commodities. The ability of two non-aflatoxigenic *Aspergillus flavus* Link isolates (CT3 and K49) to reduce aflatoxin contamination of corn has been demonstrated in a 4-year field study in the US (2001 to 2004) by Abbas *et al.* (2005). In a separate study in Nigeria by Atehnkeng *et al.* (2008), maize grains in vials or ears at mid-silking stage in field plots were inoculated with the toxigenic isolate (La3228) or atoxigenic isolate alone and co-inoculation of each atoxigenic isolate and

La3328. It was noted that the coinoculation of atoxigenic and toxigenic *Aspergillus flavus* led to the reduction of aflatoxin production in both field and laboratory conditions.

A large proportion of food contamination occurs during post-harvest storage compared to pre-harvest conditions. Interventions to reduce exposure to aflatoxins can occur at various stages of food production and preparation. Interventions vary in their cost, labour intensiveness, applicability, and effectiveness in preventing aflatoxin development. The appropriate intervention or combination of interventions depends on the crop and the country. Therefore, further evaluation is needed with consideration on the sustainability, cultural acceptability, economic feasibility, ethical implication, and overall effectiveness of potential interventions.

The work of Turner *et al.* (2005) describes a postharvest intervention package in peanut that includes five elements including (i) hand sorting of nuts, ii) the use of natural-fibre mats for drying the nuts, iii) natural-fibre bags for storage, iii) proper sun-drying, iv) wooden pallets for storage of bags, and v) the application of insecticides on the floor of the storage facility under the wooden pallets. In their work, they recommend the separation of kernels based on visible damage (Plate 8). This study yielded a 57.2% lower aflatoxin-albumin concentration (8pg/mg) in the blood of individuals who received the intervention package compared to 18.7 pg/mg in the blood of the controls (Turner *et al.*, 2005).

Plate 8: a) shows an image of maize cob affected by *Aspergillus flavus* while b) shows insect damage on maize kernels and the effect on invasion by *Aspergillus spp.*

Dietary and clinical interventions are secondary interventions because they cannot lessen the real aflatoxin content in food but can reduce aflatoxin-related disorders. These can be achieved by diminishing the bioavailability of aflatoxins in the body through enterosorption or by reducing aflatoxin-induced injury by the stimulation of phase II enzymes that detoxify the aflatoxin-8,9-epoxide (Strosnider *et al.*, 2006). Enterosorption is the use of clay, such as NovaSil®, processed calcium montmorillonite clay with a high affinity for

aflatoxins (Wang *et al.,* 2005). Clay has been used as an anti-caking additive in animal feed and has been shown to protect animals from ingested aflatoxins. Chemoprotection is the use of chemicals such as chlorophylin or dietary intervention e.g., eating foods rich in antioxidants to alter the body's susceptibility to carcinogens (Wang *et al.,* 1999).

Interventions during food preparation or consumption involve removing contaminated portions of food, diluting contaminated food with uncontaminated food, or neutralizing aflatoxins present in food. Simple food preparation methods such as sorting, washing, crushing, and dehulling may reduce aflatoxin levels (Fandohan *et al.,* 2005). Aflatoxins are not largely affected by routine cooking temperatures, but traditional methods of cooking food with alkaline compounds (i.e., nixtamalization) have been used to reduce aflatoxin exposure. Although the chemical reaction may temporarily inactivate aflatoxins, the reaction may then reverse in the gastric acid of the stomach (Fandohan *et al.*, 2005).

Other mechanisms that reduce aflatoxin contamination include reducing plant stress by using the recommended production practices. These include the right techniques to control weeds, insects and diseases. Irrigation during pollination may diminish predisposition of the crop to aflatoxin. Pre-harvest interventions include choosing crops with resistance to drought, disease, and pests and choosing varieties that are genetically more resistant to the growth of the fungus and the production of aflatoxins (Chen *et al.* 2001; Cleveland *et al.* 2003; Cotty and Bhatnagar 1994). Elimination of inoculums sources, such as infected debris from the previous harvest may prevent infection of the crop (Olanya *et al.* 1997). Early harvesting of corn when it is above the 20 percent moisture content level, and drying it within 24 to 48 hours to a moisture content less than 14% significantly reduces infection, growth and toxin production by *Aspergillus* (Cassel *et al.*, 2001; Kimatu *et al.*, 2012). Damaged kernels ought to be avoided as they are likely to be contaminated.

CHAPTER THREE

SEASONAL VARIATION IN DISTRIBUTION OF PUTATIVELY TOXIGENIC *ASPERGILLUS* SPECIES IN THE EASTERN REGION OF KENYA

3.1 Abstract

Aflatoxin contamination is a major problem affecting cereal producers worldwide. *Aspergillus* species, which are known to produce these toxins, colonize cereals in the field, during post-harvest period through to storage. This study sought to establish the seasonal variation in the incidence, species diversity and distribution of toxigenic *Aspergillus* species in maize and soils of Eastern Kenya- a region of the country historically known to have the worst incidences of aflatoxicosis. Maize and soil samples were collected from farmers' fields in May and December 2013, which corresponded to two months following the first and second harvesting seasons. Fungi of the genus *Aspergillus* were isolated from maize and soil samples by direct plate and dilution plate techniques respectively on Czapek Dox Agar and thereafter sub-cultured and purified on Potato Dextrose Agar (PDA). A total of 229 pure *Aspergillus* spp. cultures were obtained (55% from maize). *Aspergillus* isolates were identified to species level based on cultural and morphological characteristics. Eleven species of *Aspergillus* were isolated. The species in order of abundance were *A. niger, A. flavus, A. clavatus, A. awamori, A. parasiticus, A. ochraceus, A. candidus, A. ustus, A. niveus, A. terreus,* and *A. wentii*. *Aspergillus niger* was the most predominant fungus of genus *Aspergillus* in both seasons. The seasonal variations of the total isolates from maize and soil and the seasonal variation of isolates from maize were not significant (P=0.286 and P=0.853). However, the seasonal variation in the isolates from soil was significant (P=0.044).

3.2 Introduction

Fungi of the genus *Aspergillus* are known to produce various mycotoxins, which are of human health importance. *A. flavus* and *A. parasiticus* are the two main producers of aflatoxins (Abbas *et al.,* 2005). Apart from aflatoxins, other metabolites such as ochratoxins and oxalic acid are potentially hazardous to humans if ingested (Palencia *et al.*, 2010). In addition, other *Aspergillus* species such as *Aspergillus* sections *Nidulantes, Versicolores, Usti, Circumdati,* and *Nigri* produce other secondary metabolites such as sterigmatocystin (Blumenthal *et al.*, 2004), which is an intermediate in the biosynthesis of aflatoxins and may contribute to food poisoning (Do and Choi, 2007).

There are studies that have looked at the influence of climate and seasons on the distribution of *Aspergillus* species. For example, a study by Gandipilli *et al.* (2013) looked at

the seasonal variation of *Aspergillus* and *Penicillium* species in black cotton soils in Viziyanagaram District in India. The term black cotton soils in this context does not literally refer to black soils, but rather soils dominated by smectitic clays, which maybe red or black in colour. These soils are collectively known as Vertisols and are common in semi-arid environments due to their capacity to hold water. Such soils are found in the Eastern region of Kenya, which is characterized as semi-arid due to its annual rainfall of between 250mm and 500 mm (Freeman and Coe, 2002). Gandipilli *et al.* (2013) collected soil from three different seasons namely summer (March 2011 June 2011), Kharif (July 2011-October 2011) and Rabi (November 2011 – February 2012). However, Gandipilli *et al.* (2013) did not subject the soils from the dry season to detailed analysis. In addition, the soil samples were obtained from farms as growing a variety of crops such as maize, rice, sugarcane, groundnuts, red grams, black grams etc. and not only maize as is the case in this study. It is also impossible to make a direct comparison of the seasons in India with those experienced in the Eastern region of Kenya. Additionally, there is little information regarding the effect of climate and seasonal distribution of *Aspergillus* species in Kenya particular the aflatoxicosis-prone Eastern region. Therefore, the objective of this study was to establish the seasonal variability and predominant fungal strains in maize and soil from the Eastern region of Kenya.

3.3 Materials and Methods

3.3.1 Field survey locations

Sampling of maize and soil was carried out in two stages to include maize samples from two planting seasons. The sampling was performed in May 2013 and December 2013, which were two months after the March and December harvests since significant aflatoxin contamination is known to occur during storage. 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 were analysed further. These are as follows: location [Total samples=Season I + Season II] as follows: Machakos [4=4+0], Makueni [16=16+0], Kitui [17=4+13], Mwingi [13=1+12].

3.3.2 Sampling/Survey methods

Samples were selected by random sampling from smallholder farms within the Eastern region of Kenya including the counties of Kitui (17 farms), Makueni (16 farms), Mwingi (13 farms), and Machakos (4 farms). Fewer samples were collected from Machakos County since a previous study by Muthomi *et al.* (2009) had extensively characterized

Aspergillus species from maize and soil from that county. From each farm, a soil sample was taken at random using a sterile trowel from the top 4-6 cm of soil. A half a kilogram of shelled maize grain was also collected from each farmer. The soil and maize samples were each put in khaki bags, labelled and transported in a cool box to the laboratory where they were stored at 4^{o}C awaiting analysis.

3.3.3 Isolation of *Aspergillus* section *Flavi* from maize and soil samples

A solution of 2% sodium hypochlorite was used to sterilize the surface of the maize grains for one minute after which the grains were rinsed three times in sterile distilled water. In total, 15 kernels were selected at random from each sample and five kernels per plate were placed approximately 2 cm apart on plastic petri dishes (90×15 mm) containing Czapek Dox Agar medium modified with 50 mg of streptomycin and 50 mg of penicillin. Dilution plate technique was used for the soil samples. One gram of the soil was suspended in 9 millilitres of sterile distilled water and serially diluted to 10^{-4}. One millilitre of the 10^{-3} and 10^{-4} dilutions was plated in duplicate in Czapek Dox Agar. The plates were then placed in a growth chamber for 7 days at a temperature of 30 ^{o}C. The number of grains presenting growth of *Aspergillus* species in each petri dish was counted. The number of *Aspergillus* colonies was also counted for the dilution plates. *Aspergillus* colonies were sub-cultured on potato dextrose agar and incubated at 30 ^{o}C for 7 days.

3.3.4 Identification and characterization of *Aspergillus* isolates

The resulting *Aspergillus* pure cultures were identified to species level based on cultural and morphological characteristics such as colony diameter, colony color on agar and reverse, colony texture and zonation (Rodriguez *et al.*, 2007). Morphological features were studied under the microscope while taking into consideration the key outstanding microscopic features such as conidiophores, conidial shape, phialides and metulae, presence and shape of vesicles (Diba *et al.*, 2007). Contemporary diagnosis of the *Aspergillus* species was based on the descriptions and keys by Klich (2002).

3.3.5 Screening for atoxigenic and aflatoxigenic strains

Production of aflatoxin was detected based on the ammonium hydroxide method by (Kumar *et al.*, 2007). A single colony was grown in the centre of a Petri dish containing yeast extract-sucrose medium for five days at 28^{o}C. The dish was inverted and 2 drops of concentrated ammonium hydroxide solution was placed on the inside of the lid. The undersides of aflatoxin-producing colonies were expected to turn plum red after the bottom of

the Petri dish is inverted over the lid containing the ammonium hydroxide. Conversely, no colour change is expected to occur on the undersides of atoxigenic colonies (Zrari, 2013).

3.3.6 Data Analysis

One way analysis of variance (ANOVA) was performed to determine whether the distribution of the *Aspergillus* isolates in the four study sites was statistically significant. Student t-test was performed to determine whether the distribution of the fungi between the two seasons was statistically significant. SPSS (version 20.0) was used in the analysis of data.

3.4 Results

3.4.1 Cultural and morphological identification and characterization of the *Aspergillus* spp. isolates

A total of 229 *Aspergillus* isolates were obtained. The morphological and cultural features of the *Aspergillus* isolates (Plate 9) were studied and used to identify the isolates (Table 2). Eleven *Aspergillus* species were identified using distinct cultural features (Plates 10 to 20). Isolates of the same species from the four counties showed similar morphological traits in PDA. *A. parasiticus* showed similar cultural and morphological traits to *A. flavus*. However, the color of the colonies was one important feature that enabled the distinction of the two species as *A. parasiticus* was distinguished by the presence of conifer green conidia. It was also difficult to determine the precise diameters of the colonies produced by *A. parasiticus* due to the growth pattern of the species that resulted in the dispersal of spores throughout the petri dishes. Some species with somehow similar cultural traits were distinguished using morphological features under the microscope. For example, *A. candidus* was distinguished from *A. niveus* by the 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* isolates were fertile on the entire vesicle and the colony diameters did not exceed 35 mm (Klich, 2002).

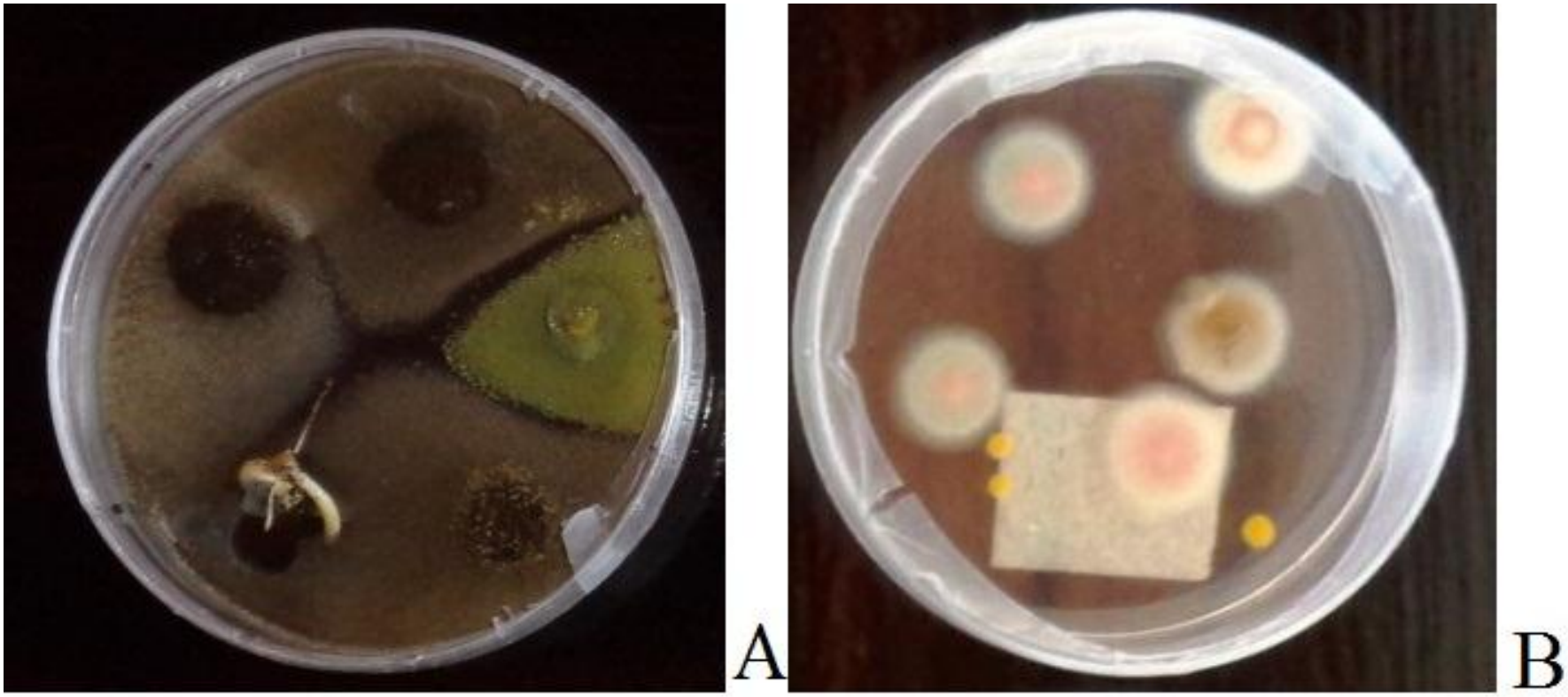

Plate 9: Colonies of *Aspergillus* from maize and soil. (A) Growth of *Aspergillus niger* and *Aspergillus flavus* on maize kernels in CZ media and (B) colonies of *Aspergillus* species from soil serial dilutions on CZ media.

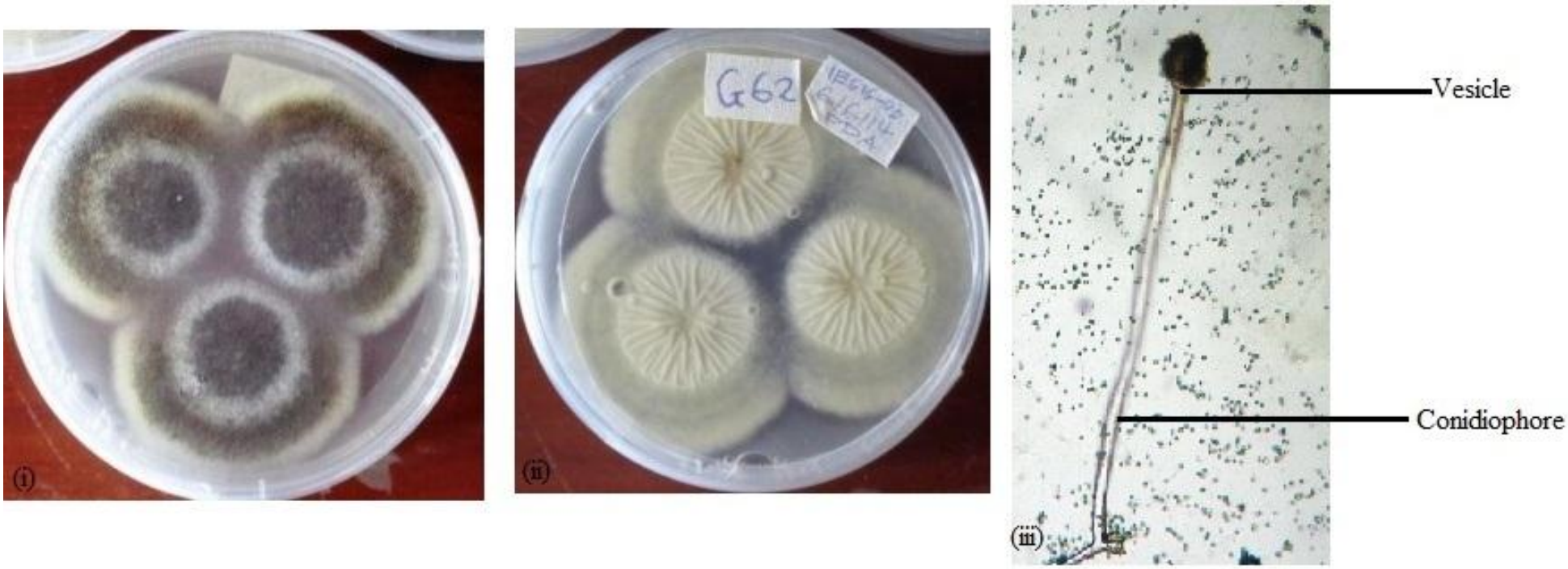

Plate 10: (i) *A. awamori* surface, (ii) *A. awamori* reverse and (iii) morphological features of *A. awamori* as observed under the microscope (Mg=×400).

Plate 11: (i) *A. candidus* surface, (ii) *A. candidus* reverse and (iii) morphological features of *A. candidus* as observed under the microscope (Mg=×400).

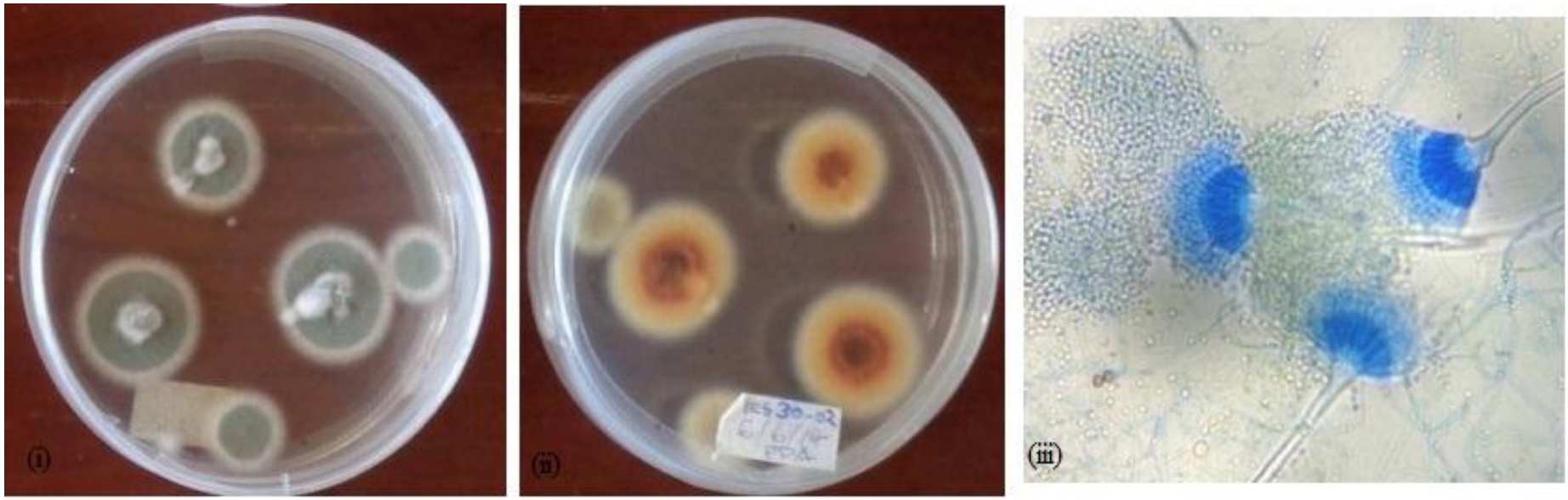

Plate 12: (i) *A. clavatus* surface, (ii) *A. clavatus* reverse and (iii) morphological features of *A. clavatus* as observed under the microscope (Mg=×1000).

Plate 13: (i) *A. flavus* surface, (ii) *A. flavus* reverse and (iii) a biseriate conidial head with a globose vesicle of *A. flavus* (Mg=×400).

Plate 14: (i) *A. niger* surface, e(ii) *A. niger* reverse and e(iii) morphological features of *A. niger* as observed under the microscope (Mg=×400).

Plate 15: (i) *A. niveus* surface, (ii) *A. niveus* reverse and (iii) morphological features of *A. niveus* as observed under the microscope (Mg=×400).

Plate 16: (i) *A. ochraceus* surface, (ii) *A. ochraceus* reverse and (iii) morphological features of *A. ochraceus* as observed under the microscope (Mg=×400).

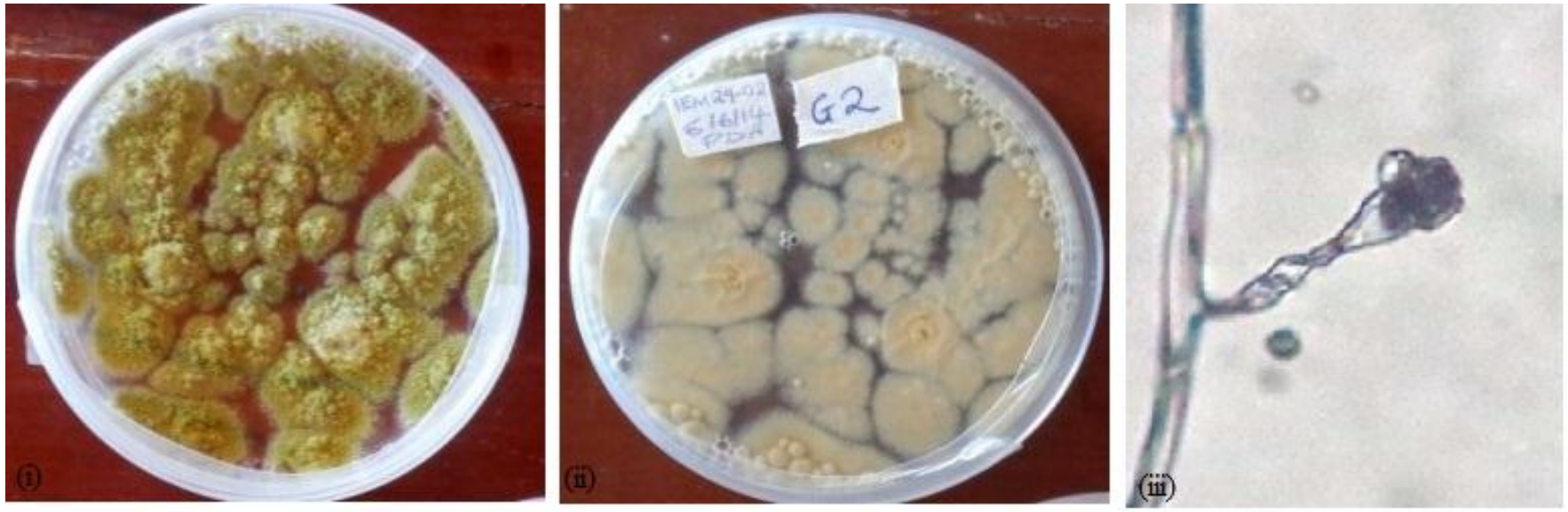

Plate 17: (i) *A. parasiticus* surface, (ii) *A. parasiticus* reverse and (iii) morphological features of *A. parasiticus* as observed under the microscope (Mg=×400).

Plate 18: (i) *A. terreus* surface, (ii) *A. terreus* reverse and (iii) morphological features of *A. terreus* as observed under the microscope (Mg=×1000).

Plate 19: (i) *A. ustus* surface and (ii) *A. ustus* reverse and (iii) morphological features of *A. ustus* as observed under the microscope (Mg=×1000).

Plate 20: (i) *A. wentii* surface, (ii) *A. wentii* reverse and (iii) radiate conidial head with roughened stipe of *A. wentii* (Mg=×400).

Table 2: Cultural and morphological features of *Aspergillus* species isolated from maize and soil in the Eastern region of Kenya

Serial no	Species name	Cultural and morphological features on PDA			Microscopic features			
		Colony diameter (mm)	Surface	Reverse	Shape of vesicle	Conidial head	Seriation	Conidiophore
1	*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
2	*A. candidus*	26±2	Pure white conidia with dense white mycelia	Light yellow wrinkled reverse	Globose	Radiate	Biseriate	Colourless, short and finely roughened
3	*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
4	*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
5	*A. niger*	62±2	Dark brown to black	Yellow to dull	Globose	Radiate	Biseriate	Brownish,

			densely packed conidia, inconspicuous white mycelia, thin white to cream margin	brown reverse with a wrinkled texture				relatively long conidiophores with smooth surfaces
6	*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	Colorless, short and finely roughened
7	*A. ochraceus*	45±2	Cream to brown conidia, purplish sclerotia with yellowish exudates	Yellow to light brown wrinkled reverse	Globose	Radiate	Biseriate	Colorless relatively long roughened conidiophores
8	*A. parasiticus*	Full plate	Conifer green conidia with white mycelia and white margin	Cream reverse with slightly wrinkled center	Globose	Columnar	Uniseriate	Colorless short and finely roughened
9	*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

10	*A. ustus*	50±2	Light brown to greyish conidia, with white to greyish mycelia	Cream reverse with yellowish wrinkled centers	Pyriform	Columnar	Biseriate	Brownish, long, smooth-walled conidiophores
11	*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

3.4.2 Isolates from maize and soil and their incidence across the four counties

The *Aspergillus* isolates were identified and characterized to species level (Table 2). The species (in order of prevalence) were *A. niger* (47.6%), *A. flavus* (22.3%), *A. clavatus* (12.2%), *A. awamori* (4.4%), *A. parasiticus* (3.9%), *A. ochraceus* (2.2%), *A. candidus* (1.7%), *A. ustus* (1.7%), *A. niveus* (1.7%), *A. terreus* (1.3%), and *A. wentii* (0.9%). The species were further grouped according to their county of origin (Figure 8). Out of the 229 isolates, 34.9% was obtained from Kitui County, 10% from Machakos, 23.1% from Mwingi and 31.9% from Makueni County (Table 3). *A. niger* was the most abundant isolate in all the four counties. Three *Aspergillus* species such as *A. clavatus, A. niveus* and *A. wentii* were only isolated from soil and not in maize (Figure 9). The incidences of *A. flavus, A. niger, A. parasiticus A. ochraceus* and *A. ustus* was higher in maize samples than in soil (Figure 9). On the other hand, the incidence of *A. terreus* and *A. awamori* was higher in soil than in maize. Analysis of variance showed that the distribution of the various species in the four counties was not statistically significant at 0.05 level.

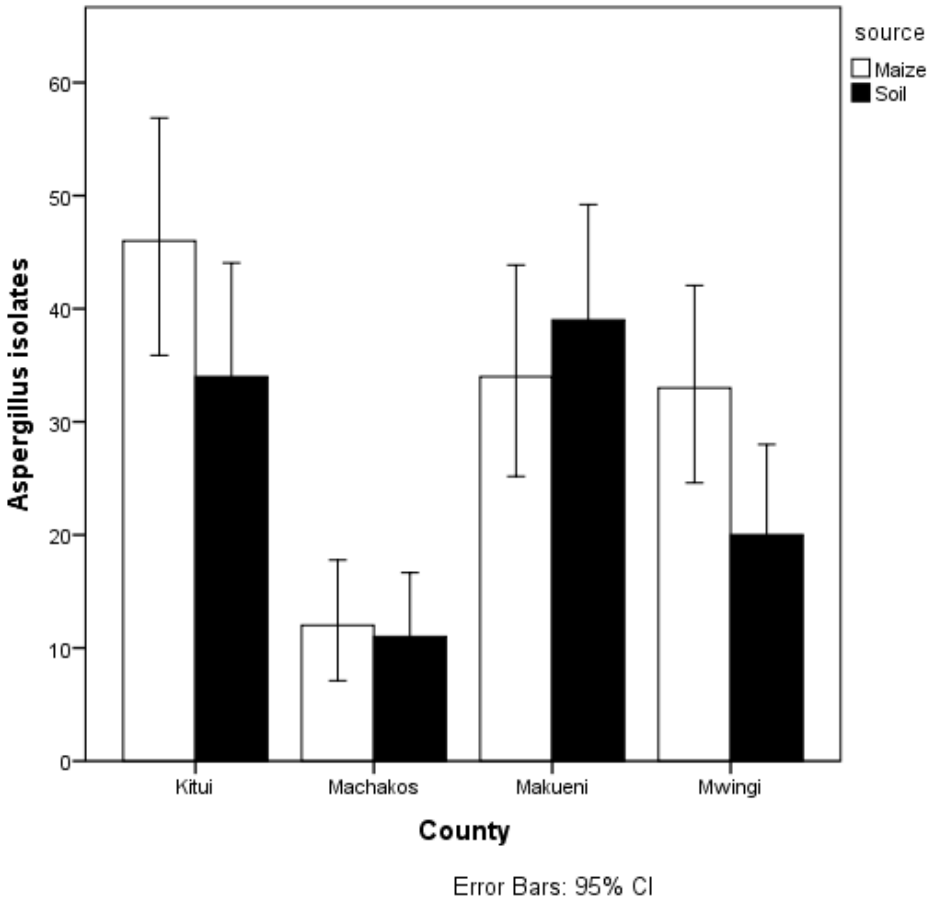

Figure 8: The distribution of *Aspergillus* isolates from maize and soil in the four counties

Table 3: Incidence and distribution of *Aspergillus* species across the four counties

	Kitui		Machakos		Makueni		Mwingi	
Species	Count	%	Count	%	Count	%	Count	%
A. awamori	5	50.0	0	0.0	4	40.0	1	10.0
A. candidus	2	50.0	0	0.0	0	0.0	2	50.0
A. clavatus	9	32.1	5	17.9	10	35.7	4	14.3
A. flavus	19	37.3	6	11.8	13	25.5	13	25.5
A. niger	37	33.9	8	7.3	39	35.8	25	22.9
A. niveus	1	25.0	0	0.0	2	50.0	1	25.0
A. ochraceus	2	40.0	1	20.0	2	40.0	0	0.0
A. parasiticus	1	11.1	2	22.2	1	11.1	5	55.6
A. terreus	2	66.7	1	33.3	0	0.0	0	0.0
A. ustus	2	50.0	0	0.0	2	50.0	0	0.0
A.wentii	0	0.0	0	0.0	0	0.0	2	100
Total	80	34.9	23	10.0	73	31.9	53	23.1

The number of isolates of each species was calculated as a percentage of the total number of species isolated. The *Aspergillus* counts constitute the incidence, whereas the percentages constitute the distribution of the species in the four counties.

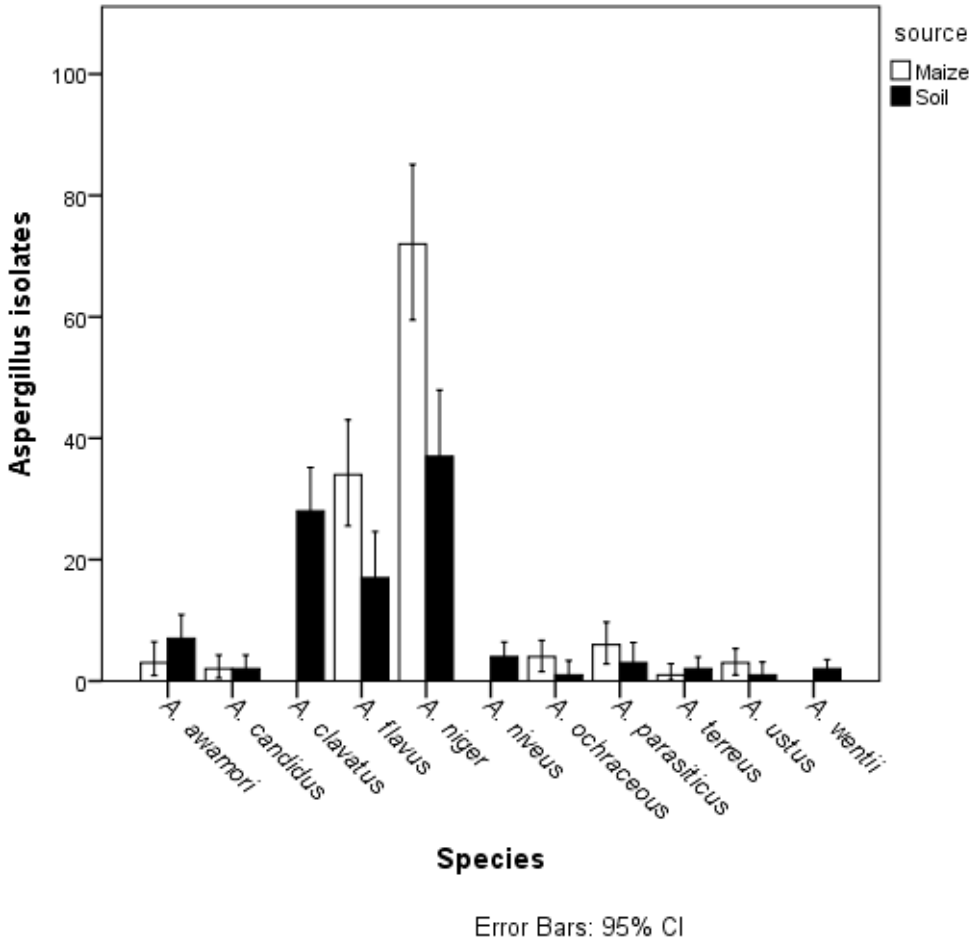

Figure 9: The incidence of *Aspergillus* species in maize and soil

There were more *Aspergillus* isolates from maize (54.6%) than from soil (45.4%). The percentage ratio of isolates from maize to those from soil in each county was as follows: Kitui 57.5%: 42.5%, Machakos 52.2%:47.8%, Makueni 46.6%:53.4% and Mwingi 62.3%:37.7%. It was observed that whereas the isolates from maize were higher than those from soil in most of the counties, in Makueni County there were more from soil than maize.

3.4.3 Seasonal distribution of the various *Aspergillus* spp.

There were more *Aspergillus* isolates from the first planting season (124) than the second season (105) (Table 4). There were slight differences in the distribution of *Aspergillus* species between the two planting seasons. There were more *A. clavatus, A. flavus, A. niger, A. ochraceus, A. parasiticus,* and *A. ustus* isolates in the first (long rains) season than in the second (short rains) season. The numbers of *A. awamori* and *A. niveus* were equal in both seasons. Conversely, there were more *A. candidus* and *A. terreus* isolates from the second

season than from the first. Furthermore, *A. wentii* was only isolated in the short rains season (Table 4). The distribution of the *Aspergillus* species in the two seasons was not statistically significant (P=0.405). Isolates from maize were considerably more than from soil in the short rain season (Figure 10). Conversely, the isolates from soil were slightly more than the isolates from maize in the long rain season.

Table 4: The distribution of *Aspergillus* spp. in the two planting seasons

	Season					
	I		II		Total	
Species	Count	%	Count	%	Count	%
A. awamori	5	50.0	5	50.0	10	100.0
A. candidus	1	25.0	3	75.0	4	100.0
A. clavatus	23	82.1	5	17.9	28	100.0
A. flavus	26	51.0	25	49.0	51	100.0
A. niger	55	50.5	54	49.5	109	100.0
A. niveus	2	50.0	2	50.0	4	100.0
A. ochraceous	3	60.0	2	40.0	5	100.0
A. parasiticus	5	55.6	4	44.4	9	100.0
A. terreus	1	33.3	2	66.7	3	100.0
A. ustus	3	75.0	1	25.0	4	100.0
A. wentii	0	0.0	2	100.0	2	100.0
Total	124	54.1	105	45.9	229	100.0

3.4.4 Toxigenic *Aspergillus* species from maize and soil from Eastern Kenya

The colony reverse of the toxigenic *Aspergillus* isolates turned pink to plum-red following exposure of colonies to concentrated ammonia solution for 30 minutes (Plate 21).

A B

Plate 21: Ammonia test for toxigenic species of *Aspergillus*.

21a shows the colony reverse of an *Aspergillus flavus* isolate from maize in yeast extract sucrose before exposure to concentrated ammonium hydroxide. Plate 21 b shows the colony reverse of the same species following exposure to concentrated ammonium hydroxide for 30 minutes. The colony reverse turned from pale yellow to pink to indicate the production of aflatoxin.

Of the 229 *Aspergillus* isolates, 41 were toxigenic whereas the remaining 188 were non-toxigenic as determined by the ammonia vapour test (Table 5).

Table 5: 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	100.0	58	89.2	7	10.8	65	100.0
Soil	53	82.8	11	17.2	64	100.0	32	80.0	8	20.0	40	100.0
Total	98	79.0	26	21.0	124	100.0	90	85.7	15	14.3	105	100.0

3.5 Discussion

Aspergillus spp. are known to spend a large part of their lives growing as saprophytes in the soil where they act as nutrient recycler, supported by plant and animal debris (Scheidegger and Payne, 2003). The fungi are known to out-compete other organisms for substrates (Hedayati *et al.*, 2007). However, it was observed that there were more *Aspergillus* isolates from maize than from soil, which could be attributed to post-harvest farmer practices where maize is dried on the ground hence exposing it to fungi. Additionally, maize contains carbon, which is an important nutrient for the growth of fungi (Al-Gabr *et al.*, 2013). It was noted that the predominant *Aspergillus* species in Eastern Kenya was *A. niger*. The results of this study differed from those reported by Muthomi *et al.* (2009), which had revealed that *A. flavus* was the most predominant *Aspergillus* species in the Eastern region of Kenya. In addition, Muthomi *et al.* (2009) isolated seven *Aspergillus* species namely *A. flavus, A. niger, A. terreus, A. ochraceus, A. fumigatus, A. clavatus* and *A. versicolor* while in this study, eleven species were isolated. Out of these, five species matched those isolated by Muthomi *et al.* (2009). However, *A. versicolor* and *A. fumigatus* were not isolated in this study. Muthomi *et al.* (2009) also observed that Kitui County had the highest number of *A. flavus* isolates, which is consistent with our findings. Furthermore, the authors observed that there was no *A. flavus* isolate from Machakos County, whereas in this study six *A. flavus* isolates were obtained from Machakos County. It is difficult to account for these differences because Muthomi *et al.* (2009) do not specify the season from which the maize and soil were sampled.

A separate study by Odhiambo *et al.* (2013), which included Makueni County from Eastern Kenya, indicated that there was no single isolate of *Aspergillus niger* from Makueni County and that *A. flavus* was the predominant fungi in the county. However, in this study, *A. niger* was the predominant species in Makueni County (39 isolates) followed by *A. flavus* (13 isolates). Four different *Aspergillus* species *A. glaucus, A. sydowii, A. nidulans* and *A. fumigatus* were also isolated from Makueni County by Odhiambo *et al.* (2013). These species were not isolated in this study. However, one similarity between these two studies is that only one isolate of *A. parasiticus* was obtained from Makueni County.

A previous study indicated that the black *Aspergillus* species (*A. niger*) were more predominant in forests and well-cultivated soils than in dry regions. However, this was contrary to the observations as the Eastern region of Kenya is characterized by semi-arid climatic conditions (Klich, 2002a).

The second most abundant species of *Aspergillus* was *A. flavus*, which is known to produce aflatoxins. This finding indicates that maize from this region is likely to be contaminated by aflatoxins. Five isolates of *Aspergillus ochraceus* were also isolated from the region (2 from Kitui County, 2 from Makueni County and 1 isolate from Machakos County) signifying a possibility of ochratoxin A contamination in the maize (Bayman *et al.*, 2002). By linking the fungi types to toxin, it enables more reliable testing of toxins such as ochratoxin A, which would otherwise be missed.

There were slight variations in the incidences of the various *Aspergillus* species between the two seasons. However, the differences were not statistically significant indicating that seasons did not influence the growth and distribution of *Aspergillus* species in the Eastern region of Kenya. These findings could be attributed to the ubiquitous nature of *Aspergillus* species that makes them widely distributed in nature. *Aspergillus* species have a worldwide distribution due to the production of numerous airborne conidia, which easily disperse by air movements and possibly by insects. The growth of the conidia is greatly influenced by atmospheric conditions especially humidity, which has a great impact on mould growth (Gibson *et al.,* 1994). *Aspergillus* spp. grow better with water activity (aw) between 0.86 and 0.96 and optimum temperatures that range from 12 to 48 ^{o}C with optimum growth being experienced at 37 ^{o}C (Vujanovic *et al.,* 2001).

Quality control bodies in Kenya such as Government Chemist focus primarily in screening maize and other food products for the presence of four aflatoxins. However, taking into account the predominance of *Aspergillus niger* and other *Aspergillus* species suggests that maize from the Eastern region of Kenya is likely to be contaminated by other mycotoxins apart from aflatoxins. Due to presence of other mycotoxin producing fungi in the region, other studies could focus on testing for ochratoxins and fumonisins in maize produced in the area. An extensive survey across seasons on a few selected hot spots in a long time and tied to grain sampling for levels of toxins should be done so the two may be related.

3.6 References

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CHAPTER FOUR
SEASONAL VARIATION OF AFLATOXINS IN MAIZE IN THE EASTERN REGION OF KENYA

4.1 Abstract

Aflatoxin contamination is a major constraint to sustained high quality cereal production worldwide. The causative organisms, *Aspergillus* species colonize cereals in the field during planting and growth, and continue to do so post-harvest when they produce toxins. This study sought to establish the seasonal variation in the total and types of aflatoxins in maize from the Eastern region of Kenya, which is known to have the worst incidence of aflatoxicosis. Maize samples were collected from farmers' fields in May and December 2013, which corresponded to two months following the long-rains and short-rains harvesting seasons. The total aflatoxins were determined using solid phase direct competitive Enzyme-Linked Immunosorbent Assay, whereas the aflatoxin types were determined using Thin Layer Chromatography. There were more aflatoxins in short-rains season (269.2 ppb) than from the long-rains season (142.4 ppb). 16% and 44% of maize samples from the long-rains and short-rains seasons respectively exceeded the maximum limit (10 ppb). The difference in the seasonal distribution of the aflatoxins was significant (P=0.019) following a student t-test. The predominant aflatoxin type in both planting seasons was aflatoxin B1. However, the short-rains season recorded the production of more types of aflatoxins with the production of aflatoxins B1, B2 and G2.

4.2 Introduction

In the last four decades, Kenya has become the leading country in terms of severity of exposure to aflatoxins. In 2004, there were 317 cases of hospitalisation accompanied by 125 human fatalities (Probst *et al.*, 2007). The majority of these cases were from the Eastern region of Kenya. Therefore, this region of the country is regarded as a hot spot for aflatoxin poisoning. Several factors such as biological factors, harvesting, storage and processing conditions interact and cause aflatoxin contamination of foodstuffs. However, climate and weather patterns are thought to play a significant role in aflatoxin contamination (Cotty and Jaime-Garcia, 2007; Milani, 2013). The Eastern region of Kenya is semi-arid region that experiences annual rainfall of between 250mm 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 average temperatures in this region range from 23^{o}C to 34 ^{o}C. The production

of aflatoxins occurs under specific conditions of moisture and temperature during pre-harvest, harvest and storage conditions.

A number of studies have tried to establish the relationship between moisture and temperature as depicted by planting seasons and the production of aflatoxins. However, these studies vary by design, type of crop and regions. For example, Okonkwo and Obionu (1981) observed that aflatoxin B1 levels were higher in food products obtained from the peak rainy season, compared to the short rain season in Nigeria. In a separate study, Gautam, Bhatta and Bhandary (2008) established that the highest levels of contamination were present in chilli, maize and groundnut samples harvested between January and February in Kathmandu Valley in Nepal.

A different study by Jonsyn-Ellis (2001) has gone as far as trying to establish the seasonal variation of the effects of aflatoxin consumption by measuring the concentrations of aflatoxins and ochratoxins in the urine of children in Sierra Leone. Jonsyn-Ellis (2001) observed that the urine of all but one child had detectable levels of aflatoxins B1, B2 and ochratoxins in the dry season, whereas in the rainy season only four children were not exposed to the mycotoxins. To the best of our knowledge there is no information regarding the seasonal distribution of aflatoxins in the Eastern region of Kenya, which is the "hot spot" for aflatoxin contamination in Kenya as established by recent incidences of aflatoxicosis. Determining the precise relationship between seasons and toxin production is vital in enabling the quantification of pre-harvest aflatoxin hazard and its possible management. Therefore, this study sought to understand the seasonal variation of aflatoxin in the Eastern region of Kenya by quantifying the total aflatoxins in maize from the region by ELISA as well as determining the predominant type of aflatoxin by TLC.

4.3 Materials and Methods

4.3.1 Field survey locations

A total of 50 maize samples were collected from the Eastern region of Kenya as described in the previous chapter. The samples were put in khaki bags, labelled and transported in a cool box to the laboratory where they were stored in the khaki bags at 4^{o}C until analysis.

4.3.2 Quantification of Aflatoxins by ELISA

Maize samples were ground to a fine powder using a sample mill grinder. 20 grams of the ground maize sample were weighed and mixed with 100 ml of extraction solvent consisting of 70 % methanol in a 250 ml conical flask. The flask containing the mixture was

covered with aluminium foil and vortexed for 2 minutes. The particulate substances were allowed to settle, and the extract was filtered through a Whatman filter paper no. 1. The first 10 ml of the filtrate was collected ready for testing. The total aflatoxin in the maize samples was determined using a solid phase direct competitive Enzyme-Linked Immunoassay (HELICA Biosystems Inc). All the reagents were brought to room temperature (approximately 26 ^{o}C) before use. 200 µl of the conjugate was placed in dilution wells in a microwell holder for all the samples and six standards. 100 µl of each standard and sample were added to the wells containing the conjugate. The two were mixed by aspirating three times with the pipette. The six standards that were used had concentrations of 0.0 ng/ml, 0.2ng/ml, 0.5ng/ml, 1.0ng/ml, 2.0ng/ml and 4.0 ng/ml. These concentrations corresponded to 0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 parts per billion.

100µl of the conjugate-sample mixture and conjugate-standard mixtures were added to corresponding antibody-coated microtiter wells and incubated at room temperature for 15 minutes. The colour of the wells containing the conjugate and samples as well as the standards was inversely proportional to the concentration of the aflatoxins (Plate 22). The first three wells on the left correspond to standards of 0, 1 and 4 ng/ml. It was observed that the higher the concentration of aflatoxin the lower the colour intensity. Therefore, the absorbance was inversely proportional to the concentration of the total aflatoxins

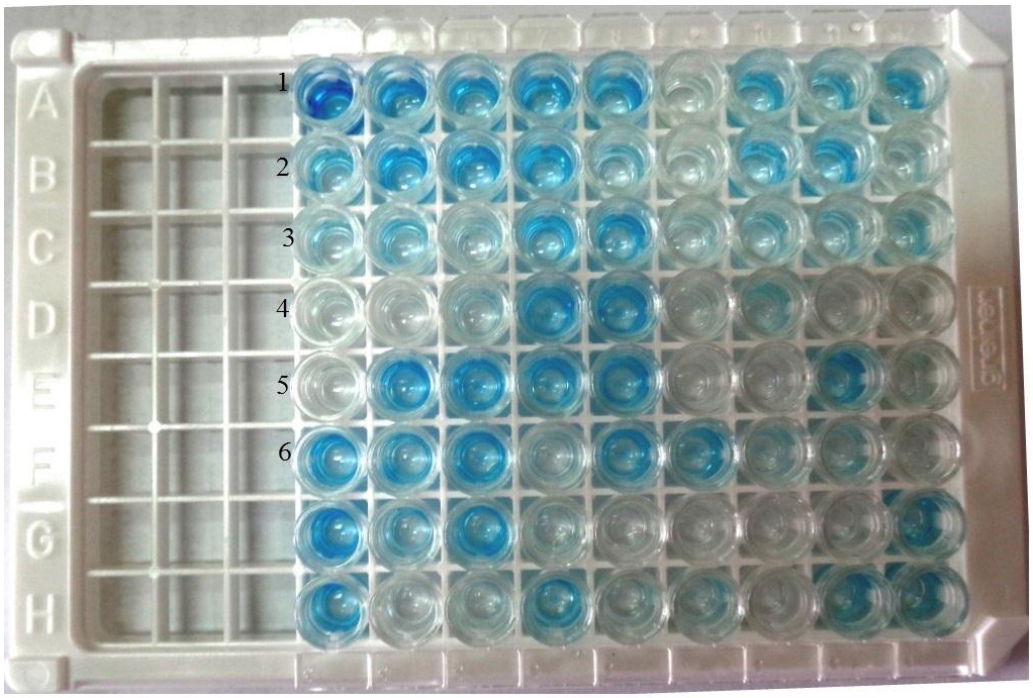

Plate 22: Antibody-coated wells containing the conjugate, samples and standards. The colour intensity is inversely proportional to the concentration of aflatoxins.

The contents of the wells were discarded followed by washing the plate five times using distilled water. The microwells were tapped on a layer of absorbent towels to remove residual water. 100µl of the substrate was added to each of the wells, which were then incubated for 5 minutes at room temperature followed by the addition of 100µl of the stop solution in the same order that the substrate was added. The colour of the wells changed from blue to yellow following the addition of the substrate, which was attributed to the formation of a yellow-coloured product (Plate 23).

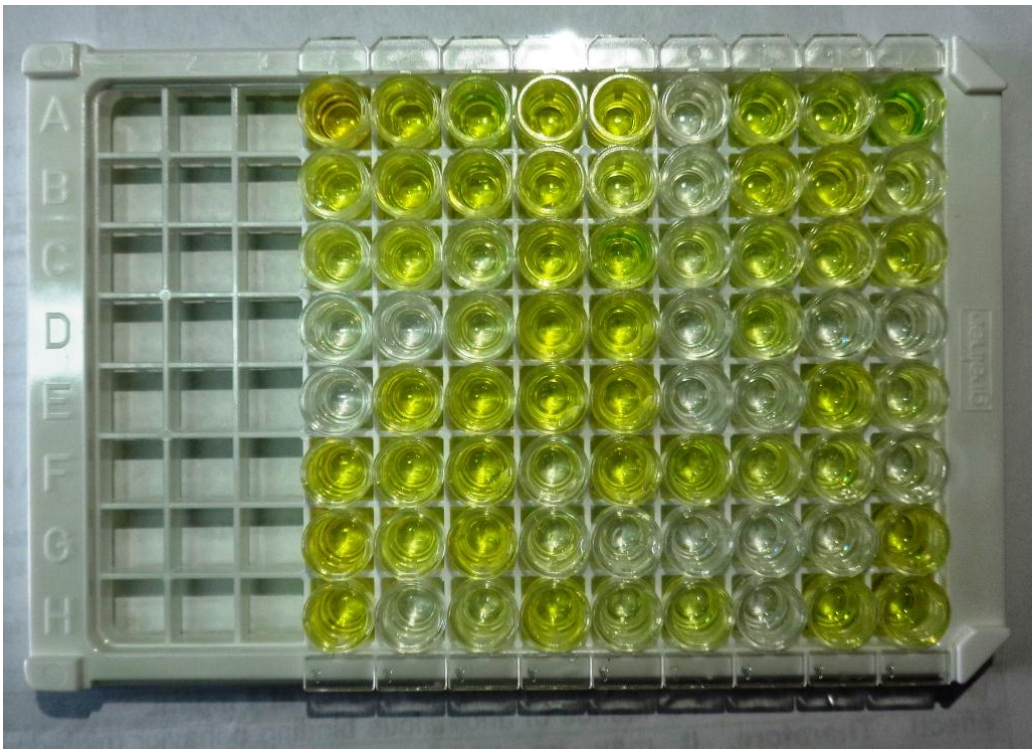

Plate 23: Antibody-coated wells containing the conjugate, samples and standards following the addition of the substrate. The enzyme-substrate reaction led to the formation of a yellow substrate.

The absorbance of each well was read at 450 nm using a microplate reader (ThermoScientific). A standard curve was made using the absorbance and the known concentrations of the six standards. The concentrations of the samples were extrapolated from the standard curve using GraphPad Prism version 6.0.5.0.

4.3.3 Quantification of aflatoxins by TLC

Approximately 20g of ground maize sample was added to a 500 ml flat-bottomed flask. 25 g of hyflo super cel 200 ml chloroform and 20ml H_2O were then added to the flask, which was then covered and shaken on a mechanical shaker for 30 minutes. The mixture was filtered into a 100 ml measuring cylinder. The first 100 ml was collected and transferred to a round-bottomed flask and concentrated to near dryness at 40 ^oC on a rotary evaporator. Two-thirds of a column chromatography tube was filled column with chloroform after which 5g of Na_2SO_4 was added through a sieve to avoid the formation of air bubbles. 10g of silica gel slurry was transferred into the column until it all settled on top of the Na_2SO_4. The setup was allowed to stand for 15 minutes after which 15g of Na_2SO_4 was added again through a sieve.
A piece of cotton wool was placed on top of the Na_2SO_4 layer, and the tap was opened to drain the chloroform. The sample extract was transferred into the column containing the adsorbent silica gel using several washings of chloroform not exceeding 20 ml.

100ml of hexane was then added to the column and drained to the level of the cotton wool after which 100ml of diethyl ether was added and drained. The toxin was eluted using 100ml of chloroform methanol mixture in the ratio of 145.5:4.5 and collected into a round-bottomed flask. The eluate was concentrated on a rotary evaporator to near dryness. 1 ml of chloroform was added to the concentrate ready for thin layer chromatography. TLC Silica gel 60 F_{254} plates measuring 20 ×20 cm were prepared by marking the solvent front at a distance of 100 mm. Using a capillary tube, the extract and the standard were spotted on the TLC plates. The plate was allowed to dry before developing in a developing tank comprising chloroform, ethyl-acetate, toluene and formic acid in the ratio of 30:25:35:10 to make 100 ml to form a 1cm layer in the unsaturated tank. The plate was inserted into the tank and developed until the mobile phase (the solvent) reached the limit line. The plate was removed and dried in a fume hood. The chromatogram was examined by use of UV goggles under UV light at 360 nm by placing the plate 10cm from the lamp to observe the pattern of fluorescence of the sample and the standard. B1 and B2 fluoresced blue while G1 and G2 fluoresced green. The identity of the aflatoxins in the sample extract was confirmed by comparing the Rf values of the sample spots with those of the standard.

4.3.4 Data Analysis

Student t-test was used to determine whether the total aflatoxins in the two planting seasons were significantly different. All data was analyzed using SPSS version 20.

4.4 Results

4.4.1 Total aflatoxin concentrations in maize as determined by ELISA

The colour intensity of the assay was indirectly proportional to the concentration of aflatoxins. As a result, the absorbance was inversely proportional to the concentration of the aflatoxins as shown in (Plate 24). The absorbance values of all the maize samples that were tested are shown in table 6.

Plate 24: An image of the ELISA readout of absorbance values. Column 1 A to F shows the absorbance values corresponding to aflatoxin standards of concentrations 0.0, 0.2, 0.5, 1.0, 2.0 and 4.0 ng/ml, which corresponded to concentrations of 0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 parts per billion.

4.4.2 Seasonal variation of total aflatoxins

It was observed that the total aflatoxins in the long-rain season were less than the total aflatoxins in the short-rains season (Table 6). The total aflatoxin in maize from the long-rain season was 142.4 ppb while the total aflatoxin in maize from the short-rains period was 269.2 ppb (Table 6). The difference in the variation of the total toxin was significant (P=0.019).

Table 6: The distribution and seasonal variation of total aflatoxins (ppb) and toxin type in the short-rain reason and long-rain season in Eastern Kenya in 2013.

Long-rain season (Season 1)				Short-rain season (Season 2)				
Sample Code	Absorbance value	Total Toxins by ELISA (ppb)	Toxin Type by TLC	Sample Code	Absorbance value	Total Toxins by ELISA (ppb)	Toxin Type by TLC	Residual Moisture Content (%)
1EM14	0.968	3.6	B1	2EM02	0.163	30.0	B1, G1	16.4
1EM15	0.961	3.6	B1	2EM03	1.803	1.4	B1	11.2
1EM18	1.090	3.0	B1	2EM04	0.563	8.0	B1	11.7
1EM19	1.068	3.1	B1	2EM05	1.274	2.5	B1	13.1
1EM20	0.667	6.0	B1	2EM06	0.445	12.7	B1	12.1
1EM21	1.354	2.2	B1	2EM07	0.393	15.4	B2	12.7
1EM23	1.377	2.2	B1	2EM09	1.182	2.7	G1	11.7
1EM24	0.292	21.3	B1	2EM11	1.380	2.2	B1	11.2
1EM25	1.356	2.2	B1	2EM12	0.395	15.2	B2	16.3
1EM26	0.432	13.3	B1	2EM22	1.909	1.2	B1	13.6
1EM27	0.816	4.5	B1	2EM30	1.039	3.2	B1	12.2
1EM28	1.277	2.4	B1	2EM34	1.117	2.9	B1, G1	12.4
1EM29	1.893	1.2	B1	2EM35	0.245	24.3	G1	13.2
1EM30	0.883	4.0	B1	2EM42	1.469	2.0	B1	11.6

1EM32	0.970	3.5	B1	2EM43	1.718	1.5	B1	12.0
1EM33	0.340	18.4	B1	2EM51	1.674	1.6	B1	12.2
1EM35	1.491	1.9	B1	2EM52	1.057	3.2	B1	11.7
1EM36	0.743	5.1	B1	2EM54	0.327	19.2	B1	11.5
1EM41	1.233	2.6	B1	2EM55	0.505	10.0	G1	10.9
1EM42	0.254	23.7	B1	2EM61	1.235	2.6	B1	11.7
1EM45	1.162	2.8	B1	2EM63	0.205	27.0	G1	11.2
1EM46	0.831	4.3	B1	2EM66	0.316	19.8	B1	11.8
1EM47	1.089	3.0	B1	2EM86	0.226	25.6	B1, G1	12.9
1EM48	1.168	2.8	B1	2EM87	0.179	28.9	G2	11.4
1EM49	1.832	1.3	B1	2EM92	0.652	6.2	B1	11.2
Total		**142.3**				**269.2**		

4.4.3 The predominant aflatoxin type in maize from Eastern Kenya

Thin layer chromatography of aflatoxins enabled the identification of three main types of toxins namely B1, B2, and G1. Plate 25 below shows an image of the blue fluorescence of aflatoxin B1 in maize samples.

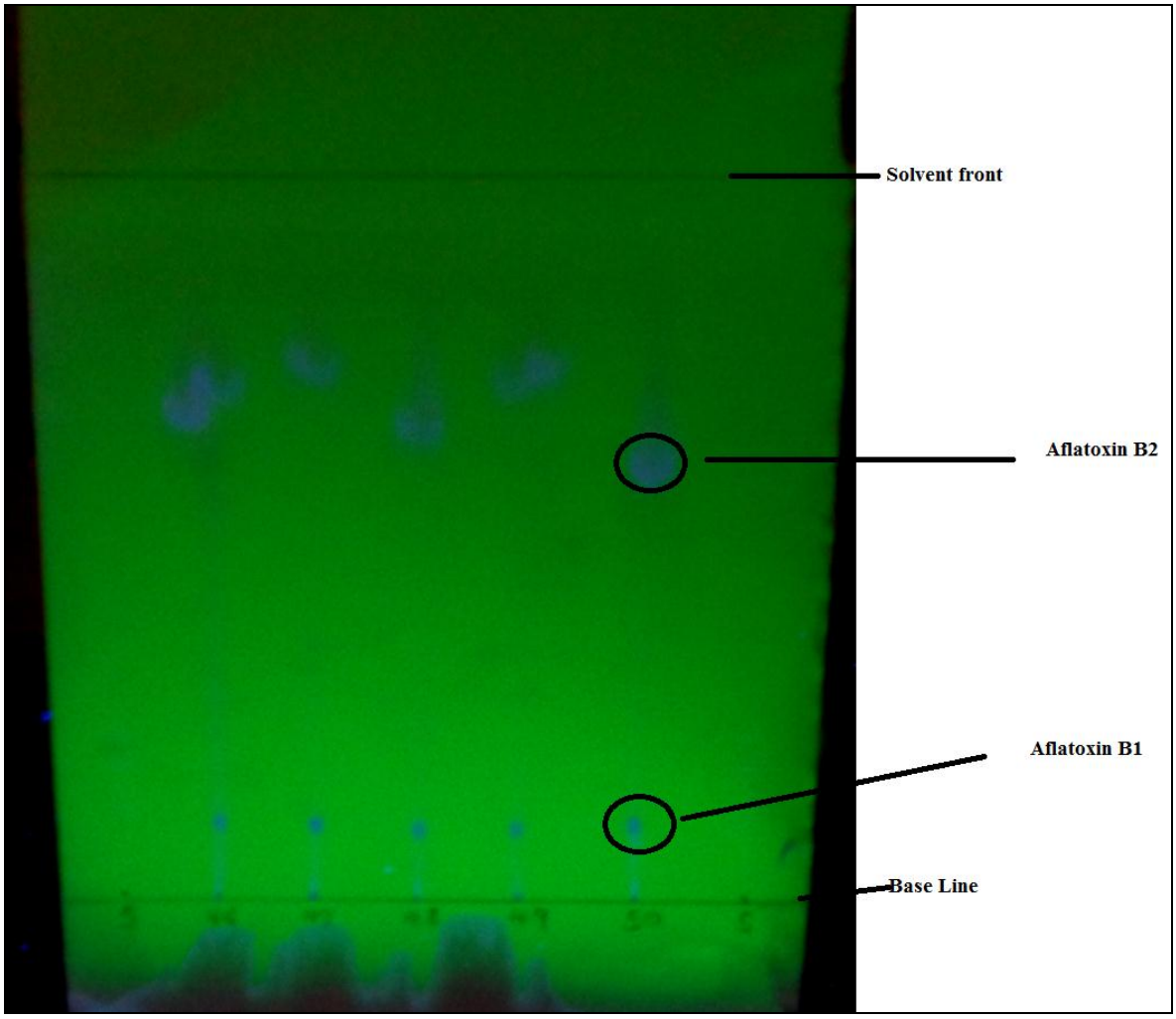

Plate 25: Image of the fluorescence of aflatoxin B1 as observed on a TLC plate illuminated with UV light.

It was observed that aflatoxin B1 was the predominant aflatoxin in maize from both seasons. However, maize from the long-rain season produced only aflatoxin B1, whereas maize from the short-rain reason produced a variety of aflatoxins including aflatoxin B1, B2, and G1. Table 6 provides a summary of the total aflatoxins in the maize samples as well as the toxin type as obtained by TLC.

4.5 Discussion

The Kenya Bureau of Standards stipulates that the maximum allowable content of aflatoxins in maize meant for human consumption should be 10 ppb. Going by these limits, 4 (16%) out of 25 maize samples from the long-rains season were unfit for human consumption (>10 ppb), whereas 11 (44%) out of 25 samples from the short-rain season were not suitable for human consumption. The findings of this study were in agreement with the view that climate influences aflatoxin contamination (Cotty and Jaime-Garcia, 2007). It was observed that the short-rains season produced maize with higher aflatoxin levels compared with maize from the long-rain season. However, in the recent aflatoxicosis cases in Eastern Kenya, a large number of aflatoxin poisoning was reported between May and June 2004 (Anonymous, 2004). Stuckey *et al.* (1984) report that the conditions that favour the production of aflatoxins in maize include temperatures ranging from 80 to 100 ^{o}F, kernel moisture content that surpasses 18% and relative humidity of between 85 and 100%. In addition, it is proposed that the development of the fungi that produce aflatoxins halts when the grain moisture content is less than 12% and the temperatures drop to 55^{o}F, (12.78 ^{o}C) (Stuckey *et al.*, 1984).

However, in this study, aflatoxin levels were as high as 10 parts per billion in maize samples whose moisture content was less than 11%. This suggests that moisture levels alone do not influence aflatoxin contamination of maize and other factors must have contributed to the observations. Cotty and Jaime-Garcia (2007), on the other hand, report that drought plays a significant role in aflatoxin contamination and that the presence of rain near the harvesting time leads to unacceptable levels of contamination in many predisposed crops including maize. The presence of rain during harvest times is likely to have led to the high levels of aflatoxin contamination that was recorded in this study.

In conclusion, seasonal variations in climatic conditions influence the aflatoxin content of maize. More maize samples from the short-rain season than in the long rain season was unfit for human consumption. However, all the maize samples were contaminated with aflatoxins even at low levels. These findings imply that the risk of chronic exposure to aflatoxins remains through all planting seasons. Therefore, a cocktail of integrated approaches are necessary in preventing chronic and acute aflatoxin exposure in eastern Kenya.

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CHAPTER FIVE
GENERAL DISCUSSION

5.1 The predominant toxigenic *Aspergillus* species in maize and soil from Eastern Kenya

It was established that *A. niger* was the predominant *Aspergillus* species in Eastern Kenya in both the short-rain and long-rain seasons. In a previous study, Blumenthal (2004) indicated that *Aspergillus niger* is harmless and is in fact used in fermentation processes by food industries to produce aspergillic acid. However, it is worth noting that the presence of *A. niger* in food does not rule out the possibility of mycotoxin poisoning as this species has been found to produce appreciable quantities of toxic metabolites such as malformins, Naphtho-r-pyrones, Aurasperone D, Nigerazine B, Nigragillin, Ochratoxin A, and Oxalic acid (Blumenthal, 2004).

Some of the compounds have only been established to be toxic to plants and animals other than humans. For example, nigragillin has been found to be toxic to silkworm larvae while malformins cause deformations in plants (Riemenschneider *et al.*, 2005). However, a compound such as oxalic acid has been found to cause hypocalcemia due to the formation of calcium oxalate. The subsequent accumulation of calcium oxalate blocks the renal tubules and the surrounding blood vessels leading to renal failure (Dassanayake and Gnanathasan, 2012). Naphthopyrones, on the other hand, affect the central nervous system of mice and rats and ultimately cause death when ingested in large quantities intraperitoneally (Cardellina *et al.*, 2012). It is possible that such effects can be replicated in humans if the compound is ingested in large quantities. Naphthopyrones have been reported to undo drug resistance in KB cells from humans (Ikeda *et al.*, 1990). Ochratoxin A is the most potent ochratoxin whose effects on human as well as animal health have already been covered in detail (Abarca *et al.*, 2001; Bondy and Pestka, 2000). Evidence by Palencia *et al.* (2010) showed that *A. niger* also produced fumonisins, which were previously thought to be produced by *Fusarium* species only. This points to an alarming scenario as the regulatory agencies in Kenya only test for aflatoxins. This could indicate that the toxin burden in the country is grossly underestimated.

5.2 The total aflatoxins in maize from Eastern Kenya

The Eastern region of Kenya is characterized by semi-arid to arid climatic conditions and warm temperatures throughout. In addition, the recent years have seen deviations of the rain seasons from the expected durations as well as quantity of rainfall, which is attributed to incidences of acute aflatoxicoses and death (Lewis *et al.*, 2005). The contamination of crops by aflatoxins can be divided into two main phases, which include contamination during the

crop-development stage and infection post-maturation during harvesting (Cotty, 2001). The weather has variable influences on these two phases. The maize samples from the long-rain season were collected, in May 2013, which corresponded to two months post-harvest, implying that the long rains began just before the crop was harvested. Similarly, the short-rain maize samples were collected in December 2013, which also corresponded to two months post-harvest. Therefore, the short rains reached their climax after the maize had been harvested. The temperatures in Eastern Kenya always range from 23 ^{o}C to 34 ^{o}C with an average of 28.5 ^{o}C being experienced on a daily basis (Freeman and Coe, 2002). The fact that the maize was harvested during the peak of the rains implied that the maize was exposed to high levels of humidity, which made the grains susceptible to contamination. As reported earlier, temperatures of 26.6 ^{o}C favour the production of toxin, which could be attributed to the high incidences of poisoning seen during the short-rain season.

Delayed harvest is another probable cause of the high levels of contamination following the short-rains season. Jaime-Garcia and Cotty (2003) report that delayed harvesting leads to severe incidences of aflatoxin contamination especially if the rains catch up with the crop during harvest. Climate also influences the populations of aflatoxin – producing fungi. Temperatures greater than 25 ^{o}C favour the growth of *Aspergillus flavus* hence increasing their presence in soils, the air and crop surfaces. Although other aflatoxin-producing fungi such as *A. nomious* and *A. parasiticus* are not as common as *A. flavus*, the influence of climate may also favour their existence (Cotty and Bhatnagar, 1994).

The fungal populations themselves may be responsible for the observed results. *A. flavus*, which is known to be the chief producer of aflatoxins, exists in multifaceted communities where numerous genetically distinct groups interrelate. The S and L trains are the two main morphotypes of *A. flavus*, out of which the S strain produces substantially higher quantities of aflatoxins than the L strains (Cotty and Bhatnagar, 1994). According to Cardwell and Cotty (2002), hot and dry agroecological regions particularly in Africa are known for the predominance of certain aflatoxin-producers. In addition, latitudinal changes in climate affect the structure of the fungal populations as well as their capacity to produce aflatoxins. It is likely that these factors interacted and increased the aflatoxin-producing potential of the fungi hence leading to the high levels of aflatoxins that were recorded in this study.

Another predisposing factor is the presence of toxigenic inoculums in the soil. According to Jaime-Garcia and Cotty (2004), the rate of decomposition of organic matter in

soil also determines the extent of aflatoxin contamination. *A. flavus* typically lives in the soil as saprophytes, which depend on organic matter for their survival. Crop residues including maize cobs are the key sources of organic matter in the soil. However, maize cobs take a long time to decompose compared to other organic matter in the soil. Harvest and cultivation practices determine how long crop residues remain in the soil. Additionally, factors such as moisture and warmth determine the rate of decomposition of organic matter. During the long-rains season, there is a likelihood of sufficient moisture levels to encourage the decomposition of organic matter compared to the short-rains season. Therefore, it is likely that more organic matter decomposes in the long-rain season, which provides less favourable environment for the survival and propagation of aflatoxin producing fungi and the subsequent contamination of maize. As a result, higher levels of aflatoxin contamination of maize are experienced during the short-rains season due to inadequate moisture levels to promote the decomposition of organic matter in the soil, which tends to favour the growth and propagation of aflatoxin-producing fungi (Jaime-Garcia and Cotty, 2004).

5.3 The predominant aflatoxin type in maize from Eastern Kenya

As explained earlier, *A. flavus* produces mainly aflatoxin B1 while *A. parasiticus* and *A. nomious* produce the B and G toxins. The results of the study showed that aflatoxin B1 was the only toxin produced in the long-rain season while B1, B2 and G1 were present in the short-rain season. A plausible explanation for this observation is the possibility of the presence of other aflatoxin-producing fungi such as *A. parasiticus*, which produces both B and G aflatoxins (Probst, Njapau and Cotty, 2007). However, five isolates of *A. parasiticus* were isolated in the long-rain season whereas four *A. parasiticus* were isolated in the short rain season yet there was no production of G aflatoxins in the long-rain season.

It has been shown that the S-type *A. flavus* is toxigenic and produces large quantities of B aflatoxins while the L-type produces less B aflatoxins than the S strain (Cotty and Cardwell, 1999). Though this study did not look at the classification of *A. flavus* strains into L and S strains, the high levels of B toxins obtained from the samples suggest the predominance of S strains of *A. flavus*. It is also possible that the climatic conditions in the short-rain season favoured the growth of the S strains of *Aspergillus flavus* hence the production of more toxins.

It was observed that *A. niger* was the predominant *Aspergillus* species in maize and soil from both rain seasons followed by *A. flavus.* These findings were in agreement with those reported by Klich (2007) that *A. flavus* and *A. niger* often occur concurrently in the

field. It has been reported that *A. niger* inhibits the production of aflatoxins in sterilized maize inoculated with *A. flavus* NRRL6412 (Klich 2007). Under culture conditions, *A. niger* also lowers the production of aflatoxin by approximately 90% in media such as malt extract agar and Czapek-Dox broth. Unfortunately, a study by Wicklow *et al.* (1987) earlier reported that corn ears coinoculated with *A. niger* and *A. flavus* 21 days after silking, reduced, but did not eliminate, aflatoxin production. Additionally, the presence of *A. niger* also did not eliminate aflatoxin production in stored corn naturally contaminated with *A. flavus* (Seitz *et al.* 1982). Therefore, the presence of aflatoxins at variable levels in all the maize samples confirm the idea that *A. niger* does not inhibit the production of toxin in stored maize that is naturally contaminated with *A. flavus.*Advances to reduce the incidence of aflatoxin contamination suggest the biological control of *A. flavus* by using of non-toxigenic strains to outcompete the toxigenic strains (Yin *et al.*, 2008). Success rates of biological control of toxigenic *A. flavus* have been reported by Cotty and Bhatnagar (1994) as well as Ehrlich and Cotty (2004). A recent study by Odhiambo *et al.* (2013) also reported the isolation of certain non-toxigenic *A. flavus* species that were speculated to have the potential to inhibit the growth and toxin production of toxigenic *A. flavus*. The findings of this study show the predominance of non-toxigenic *Aspergillus* species over toxigenic species. However, the corresponding aflatoxin content does not seem to support the findings that non-toxigenic *A. flavus* prevents the growth and production of aflatoxins since all the maize samples tested positive for aflatoxins at various concentrations. There are two possible explanations for this observation. A) The variations could be attributed to the experimental procedure. During the isolation of fungi, the five maize grains that were plated in CZ medium were selected at random from each packet of maize sample. It is known that the distribution of *Aspergillus* is not uniform on maize cobs as well as in a sack of shelled maize (Klich, 2007a). Therefore, it is likely that the maize grains that were selected for fungal isolation did not contain toxigenic *Aspergillus* species, but the maize that was ground and assayed for aflatoxins contained the toxigenic *Aspergillus*, which led to toxin production.

Another possible explanation is that B) the non-toxigenic *Aspergillus* species did not out compete the toxigenic species and prevent the formation of aflatoxins as reported in earlier studies by Cotty *et al.* (2007). A study by Damann (2014) compiles evidence that suggests that a direct touch-based inhibition is responsible for the inhibition of aflatoxin synthesis by toxigenic *Aspergillus* species. Damann (2014) proposes that a form of intraspecific aflatoxin inhibition requiring growth of the competing strains together during

the infection process in such a way that hyphae physically interact or touch is the trigger for preventing induction of aflatoxin synthesis. Therefore, the growth of toxigenic and non-toxigenic *Aspergillus flavus* strains in the same field without the making direct contact does not prevent the formation of aflatoxins, which explains why more atoxigenic *Aspergillus* strains were isolated from the maize samples yet all of them were contaminated with aflatoxins.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the study, it was concluded that *Aspergillus niger* was the predominant *Aspergillus* species in maize and soil from the eastern region of Kenya in both seasons followed by *A. flavus*. The presence of other putatively toxigenic *Aspergillus* species in the region indicated the possibility of other mycotoxins apart from aflatoxins.

Seasonal variations in climatic conditions influence the aflatoxin content of maize. It was concluded that total aflatoxin in the short-rains period was higher than in the long-rain season. However, all the maize samples were contaminated with aflatoxins even at low levels. These findings implied that the risk of chronic exposure to aflatoxins remains through all planting seasons.

The short-rains harvest had more aflatoxin variations (B1, B2 and G1 types) than the long-rains harvest, which had only the B1 aflatoxins. Therefore, these findings implied that the conditions of the short-rains favoured the production of more toxin types.

6.2 Recommendations

Quality control bodies such as Government Chemist and Kenya Bureau of Standards focus their efforts in screening maize and other food products for the presence of the four aflatoxins. However, taking into account the predominance of *Aspergillus niger* and other *Aspergillus* species and their documented secondary metabolite profiles suggests that maize from the Eastern region of Kenya is likely to be contaminated by other mycotoxins apart from aflatoxins. Therefore, measures should be put in place to screen for aflatoxins as well as other mycotoxins such as ochratoxins and fumonisins. The quantities of these other toxins can be monitored in relation to aflatoxin levels to see if aflatoxins can be a good indicator of possibility of contamination.

The government should subsidize the cost of testing for mycotoxins by purchasing standards from taxpayers' money because it is very expensive to the ordinary farmer. Such a move will also act as an incentive to the unregulated market, which is often a source of contaminated maize.

It has also been reported that the aflatoxin-producing potential of fungal populations vary with geography with some regions having higher potentials than others do. In Kenya, the Eastern region has been shown to have high-potential aflatoxin producing fungi. Therefore, a cocktail of approaches of precluding future cases of aflatoxicosis are necessary in this region.

In addition, the farmers in the region should be educated on ways of preventing aflatoxin contamination of maize in the farms, at harvest and during storage.

It is suggested that a combination of factors such as the rate of decomposition of organic matter in the soil as well as cultivation practices influence aflatoxin contamination of crops. Insufficient moisture levels in the soils lead to slow decomposition of organic matter, which provides favourable environment for the growth of aflatoxin-producing fungi and the subsequent contamination of crops. It is recommended that measures to promote the rapid decomposition of organic matter in soil be explored as a way of reducing the populations of toxigenic fungi in the soil and the subsequent aflatoxin contamination of maize crops.

Numerous biotic and abiotic factors in the environment of the fungi affect the formation of aflatoxins in maize. Not all strains of aflatoxigenic species respond in the same manner to the various conditions. It is recommended that further studies be performed to understand the biology of these fungi and their variability in order to develop control strategies that will be effective on all strains of the fungi. In addition, more work should be done to understand the mechanism of the influence of various factors on aflatoxin production. These include the molecular facet of aflatoxin biosynthesis, physiology of the fungi, and physiology of the plants susceptible to aflatoxin contamination. Information from these studies will enable the development of novel ways of controlling aflatoxin contamination.

There is the need to do phylogenetic sequencing for the fungi to confirm their identities since morphological features may overlap between species.

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APPENDIX

List of Publications and Manuscripts

1. Abundance and composition of putatively toxigenic *Aspergillus* species in maize and soil in Kenya
2. Seasonal variation of aflatoxins in maize in the Eastern region of Kenya (In preparation).

ANOVA Output

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	177.8863636	3	59.29545	0.723276	0.544054	2.838745
Within Groups	3279.272727	40	81.98182			
Total	3457.159091	43				