THE OCCURRENCE OF *ASPERGILLUS* SECTION *FLAVI* IN SOIL AND MAIZE FROM MAKUENI, COUNTY, KENYA

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This is my original work and has not been submitted for a degree in this or any other University.

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DEDICATION

Dedicated to my lovely family (Daniel, Ladislaus, Faustina and Adrian) for their love and patience and to my dear mother for her immeasurable support. God bless you all.

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LIST OF ABBREVIATIONS

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M1
AGPP	Grain Postharvest Programs
ANOVA	Analysis of Variance
ASF	*Aspergillus* section *Flavi*
ASL	Above sea level
aw	Water Activity
AZL	Agro Ecological Zones
CAST	Council for Agricultural Science and Technology
CDC	Centers for Disease Control and Prevention
CFR	Case fatality rate
CFU	Colony Forming Units
CIMMYT	International Maize and Wheat Improvement Center
CYA	Czapek Dox Agar
CZA	Czapek Yeast Extract Agar
EC	European Commission
ELISA	Enzyme-Linked Imunosorbent Assay
EU	European Union

FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GAP	Good Agricultural Practices
GC	Gas Chromatography
GOK	Government of Kenya
GPS	Global Positioning System
HBV	Hepatitis B Virus
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IFPRI	International Food Policy Institute

KARI	Kenya Agricultural Research Institute
KBC	Kenya Broadcasting Cooperation
KEPHIS	Kenya Plant Health Inspectorate Services
KNCPB	Kenya National Cereals and Produce Board
LC	Liquid Chromatography
LD 50	Median lethal dose
LCC	Liver Cell Cancer
MDRB	Modified Dichloran Rose Bengal Agar
MoA	Ministry of Agriculture
MoH	Ministry of Health
MS	Mass Spectrometry
NCPB	National Cereals and Produce Board
nm	nanometer
PPB	Parts per Billion
PPM	Parts per Million
RH	Relative Humidity
SGR	Strategic Grain Reserve
TLC	Thin Layer Chromatography
UoN	University of Nairobi
WFP	World Food Program
WHO	World Health Organization
YES	Yeast Extract Sucrose broth

ABSTRACT

Maize (*Zea mays* L.) is the staple food for most households in both urban and rural areas in Kenya and has an estimated annual per capita consumption of about 125 kg per person, which is among the highest in the world. Ear rot fungi belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium* are the commonly encountered mycotoxigenic agents on maize in tropical regions of Kenya. Amongst the three, *Aspergillus* genus is the most important aflatoxigenic fungal species that infect maize along the value chain. Contamination of maize by these toxigenic fungi has become a challenging problem especially in many Counties in Kenya where repeated cases of acute aflatoxicosis have been reported yearly for over three decades now (2013).

The study focused on the maize value chain to determine the occurrence of *Aspergillus flavus* (S and L strain), *Aspergillus parasiticus* and *Aspergillus alliaceus* in soil and maize. The samples were collected from Kaiti Location in Makueni County for three seasons from 2009-2011. A stratified sampling approach was used to collect 521 samples consisting of 80 soil samples, 96, 190 and 155 maize samples for pre-harvest, farmer stores and market stores respectively. Isolation of ASF was done on Modified Dichloran Rose Bengal (MDRB) agar at 30 ^{0} C. Thin Layer Chromatography (TLC) was used to screen isolates of *A. flavus* and *A. parasiticus* for aflatoxin production and type using aflatoxin standards (AFB_1, AFB_2, AFG_1 and AFG_2).

Total colony forming units (CFU) and prevalence data was taken. *Aspergillus* and Penicillium species were recovered in every stage of the maize value chain. Ninety percent of the sampled farms had *Aspergillus* section *Flavi* (ASF) fungi, A. *flavus* (S and

L strains), and *A. parasiticus.* The mean population (CFU $g^{-1}$of maize) of ASF was significantly different ($P<0.05$) in the three seasons (mean = 1090 in 2009, 5552 in 2010 and 25664, in 2011) and their population in maize was higher in 2011 than in soil. *A. flavus* S strain had the highest population in maize (mean = 24256 CFU g^{-1} of maize), followed by *A. flavus* L strain (mean =13421 CFU g^{-1} of maize) and *A. parasiticus* (mean=1643 CFU g^{-1} of maize). An association was noted between soil type and distribution pattern of fungal species in maize growing farms in Kaiti. Loam (mean= 213 CFU g^{-1} of soil) and red loam (mean = 222 CFU g^{-1} of soil) soils had the highest fungal population while black loam (mean= 31 CFU g^{-1} of soil) and sandy (mean= 17 CFU g^{-1} of soil) soils had the least. There was a significant difference ($P\leq0.05$) in the population of ASF in local (mean = 6347 CFU g^{-1} of maize) and hybrid (mean = 30933 CFU g^{-1} of maize) maize varieties. Local maize was more tolerant to fungal infection than hybrid maize varieties at pre-harvest stage. Fungal populations in the two maize types were not statistically different at post harvest stage.

The population of *A. flavus* S strain was higher in all sample types but significantly higher ($P<0.001$) in market than in farmer stored samples. Maize hanged on roof and drying on mats had a significantly ($P<0.001$) lower ASF incidence, with means of 65 and 1200 CFU g^{-1} of maize respectively. Samples in storage for 15-25 days had a significantly lower ($P<0.001$) mean fungal population of 1100 CFU g^{-1} of maize which increased with time to 7200 colonies at 105 days in market and 11000 CFU g^{-1} of maize at 330 days in farmer stored maize. Four aflatoxin types (AFB_1, AFB_2, AFG_1 and AFG_2) were detected from colonies isolated from maize and soil but the incidence differed in soil (AFB_1 = 50

and AFB_2 = 47) and maize samples (AFB_1 = 63 and AFB_2 = 59). *Aspergillus flavus* S and L strains were found to be present in every stage of the maize value chain but severity of contamination at pre and post-harvest stages depended on the moisture content, source of maize, duration and type of storage (P = 0.042). Data gathered showed a potential risk of contamination of maize in the study area hence farmers need to be sensitized on good agricultural practices to minimize maize contamination. The results are useful as a guide towards the management of aflatoxin occurence in Kenya.

Key words: Aflatoxin, *Aspergillus* section *Flavi*, Maize, Value chain.

CHAPTER ONE:

GENERAL INTRODUCTION

1.1 Background Information

Maize *(Zea mays* L.) is one of the world's widely cultivated cereal and ranks third in the world production after wheat and rice (FAO, 2002). It is a staple food for most Kenyan households in both urban and rural areas. The estimated annual per capita consumption is about 125 kg (Pingali, 2001; Kimanya *et al*., 2008) and this is among the highest in the world. In Kenya, maize is grown in the tropics, either in large or small scale farms (Odendo *et al*., 2002). Its low productivity has been associated with various constraints espcially drought. Drought is a, common phenomenon in tropical environments contributing to annual maize yield losses of between 17- 60 % in severe drought conditions (Zaidi *et al*., 2004). Some of Kenya's large portions of Eastern, Central and Coastal Provincies are characterized by drought, semi-arid to arid conditions, high temperatures, occasionally heavy rainfall at harvest time and sometimes warm humid conditions (Craufurd *et al*., 2006).

Ear rot fungi belonging to the genera *Fusarium, Aspergillus* and *Penicillium* are commonly encountered on maize in tropical regions. These genera have species capable of producing mycotoxins shown to be toxic to man and animals (Orsi *et al*., 2000). The most common seed-borne maize mycoflora include *Fusarium* spp., *Aspergillus flavus*, *Aspergillus parasiticus, Cladosporium* spp. and *Penicillium* spp. Other constrains are

storage pests and fungal infections that attack maize in storage (Gichuki *et al*., 2000). Research by Kossou and Aho (1993) revealed that fungal infections are responsible for about 50-80 % of damage on farmer's maize during storage especially if conditions are favorable for proliferation of *A, flavus* and *A. parasiticus* growth, resulting to subsequent aflatoxins production in food and feeds.

This shows that majority of Kenyans are exposed to low and medium level doses of a wide spectrum of fungal poisoning through regular consumption of maize products. Low levels of exposure are asymptomatic but are cumulatively likely to be of severe health risk. It is confirmed that ingestion of mycotoxin contaminated products by humans and animals can result in acute aflatoxicosis and deaths (Lewis *et al*., 2005) if the contaminated products are consumed. Besides human consumption, maize is a major component in livestock and poultry feeds hence exposure to humans through consumption of milk and poultry products from animals, fed with contaminated feeds (Boudra *et al*., 2007).

Aflatoxin a metabolite of *A. flavus* is the most popular, widespread and of great economic importance in the agricultural sector. Aflatoxin-producing strains in the *Aspergillus* section *Flavi* group are *A. flavus, A. parasiticus, A. nomius, A. pseudotamarii* and *Aspergillus bombycis* (Peterson *et al*., 2001). The saprophytic nature of these fungi allows them to be present in soil allowing them to infect other plants to complete their life cycle even in absence of maize in the field (Scheidegger and Payne, 2003). Their conidia are assumed to be the primary inoculum and can survive for long periods in soils

because they are well adapted for survival. The most common aflatoxin types produced by these fungi are Aflatoxin B_1 (AFB_1), AFB_2, AFG_1, and AFG_2. According to Alabi (1989), AFB_1 is the most common and toxic of the four aflatoxins, with a lethal dose (LD50) of less than 0.5 mg/kg body weight in susceptible ducklings. Aflatoxin B_1 is a natural contaminant in human and animal food crops before, during and after harvest (Shanahan *et al.,* 2003).

To date (2013), Kenya is the only nation with a population that has repeatedly experienced epidemics of acute aflatoxicosis especially in Machakos and Kitui Counties in 2001, 2004, 2005 and 2006 where many people and especially children have died (Bennett and Klich., 2003; Azziz-Baumgartner *et al*., 2005; Lewis *et al*., 2005; WHO, 2006, KEPHIS, 2006). It is also in Kenya where the largest outbreak reported in the world during the last 20 years was reported in 2004. According to Lewis *et al*. (2005), the 2004 outbreak resulted in 125 deaths out of the 317 reported cases. Studies conducted in various parts of Kenya established a positive relationship between prevalence of hepatitis infections and aflatoxin exposure which is thought to increases the risk of primary hepatocellular carcinoma (Williams, 2004).

Information on prevalence of aflatoxin in the maize value chain in Eastern Kenya is fragmented creating a need for generation of consistent database of aflatoxin prevalence and occurrence of *Aspergillus* section *Flavi* contamination along the maize value chain. This information will be crucial in quantifying the level of chronic exposure in the maize

consuming population within Makueni County as a first step towards management of future aflatoxicosis outbreaks and long-term exposure to aflatoxins

1.2 Problem statement

Aflatoxin contamination is a global problem and close to 55 billion people worldwide suffers from uncontrolled exposure to aflatoxin. The impact of aflatoxin contamination is felt more in the developing countries, affecting the health of the nation's population. It also affects trade hence aflatoxin content of food and feed is strictly regulated in most of the world. However, the standards set have little relevance to poor, small-scale farmers in Africa, who often rely on maize for daily nutrition and income. This is partly because many people in these countries are not knowledgeable of the potential dangers posed by these toxins in food. Two decades after the largest aflatoxicosis outbreak was reported in Kenya in 2004, there are no measures nor are monitoring systems to determine aflatoxin exposure hence chronic incidences are usually attributed to other causes. The available information is very fragmented leaving the society wondering whether aflatoxin contamination is a field or storage problem. Likewise, the producers are not aware of the outcomes of their pre and post-harvest practices which sometimes result to severe food contaminations. Research on the prevalence of *Aspergillus* section *Flavi* in the maize value chain have not extensively been conducted in East Africa. Few studies have been done in Kenya but not many researchers have followed maize from production to consumption as a way of establishing where contamination occurs.

1.3 Justification

There have been sporadic aflatoxicos outbreaks in Makueni for over three decades. The largest outbreak reported in the world during the last 20 years was in Kenya in 2004. This outbreak was associated with consumption of aflatoxin contaminated maize that contained as 4,400 ng/g aflatoxin B1, a quantity that is 440 times greater than the 10 ng/g tolerance level set by the Kenya Bureau of Standards. Since it is difficult to eliminate aflatoxin once it is established in foods, it is important to carry out a systematic follow-up of maize, from the field to the table to identify the critical points where contamination is high, and where, if intervention strategies are adopted, significant reductions in aflatoxin contaminations may be achieved. This is the first study conducted in Makueni with an aim of investigating the entire maize value chain to determine the occurrence of aflatoxigenic fungi in maize and soil in different seasons.

1.4 Objectives

To evaluate the population, occurrence and distribution of *Aspergillus* section *Flavi* in the maize value chain in Makueni County Kenya

The specific objectives were:

1. To determine the occurrence and diversity of aflatoxigenic fungi (*Aspergillus* section *Flavi*) in soils of maize growing farms in Kaiti Location of Makueni County.

2. To determine changes in the occurrence and distribution of *Aspergillus* section *Flavi* group in soil and pre-harvest maize in Kaiti Location of Makueni County
3. To determine the seasonal changes in the population of *Aspergillus* section *Flavi,* in post-harvest maize collected in 2009-2011 in Kaiti Location of Makueni County

CHAPTER TWO

LITERATURE REVIEW

2.1 Maize production and consumption in Kenya

The average maize yield in Kenya is about 1.5 tons ha^{-1} to 2 tons ha^{-1} while the potential is over 6 tons ha^{-1} (Pingali, 2001; Makokha *et al*., 2001). The area under maize cultivation has stabilized at around 1.5 million hectares, producing about 26 million bags of maize per annum. This falls short of the annual domestic maize consumption estimated at 34 million bags (Kamau, 2002; FAO, 2003). To bridge the gap between maize production and demand, Kenya has been importing maize formally and informally across the border from Uganda and Tanzania in addition to large offshore imports from as far as South Africa, Malawi, United States of America and other Southern America countries like Brazil and Argentina (Nyoro *et al*., 1999). Locally, maize production can be improved through increased use of improved seeds, fertilizers and good crop husbandry (GOK, 1997).

Maize has many uses in Kenya. It is utilized as a food; feed for livestock and as a raw material for industry (FAO, 2002). As food, maize plays an important role in the diet of millions of people due to its high yields per hectare, its ease of cultivation, adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Asiedu, 1989). A report by Kumar *et al*. (2007) indicates that about 50 to 55 % of the total maize produced is consumed as food in most developing countries. It also accounts for 28 % of

gross farm output from the small-scale farming sector (Jayne *et al*., 2001) in Kenya's crop production patterns. As a feedstuff, it has a more efficient conversion to animal products such as meat, milk and eggs compared to other grains (Gatch and Munkvold, 1999). According to Lynch *et al*. (1999), maize is also used worldwide as a basic raw material for the industrial production of starch, corn syrup, dextrose, oil, protein, alcohol, biodegradable chemicals, paper, textiles, ready-to-eat snack foods, breakfast cereals, cornmeal and more recently fuel.

2.2 Maize production practices and constraints in Makueni County

The average maize yield in lower parts of Counties in Eastern Province is 0.7 tons ha^{-1}. This is 1.3 tons lower than the average maize yield in Kenya. Low maize productivity is associated with both biotic and abiotic factors. The most common abiotic factors are drought, extreme temperatures, land degradation, high soil aluminum (soil acidity), flooding and salinity (Tuberosa *et al*., 2005). The soils that have low organic matter contents and are predominantly sandy to loamy sand texture, hence they are susceptible to erosion and are limited in their capacity to retain water and plant nutrients. The average annual rainfall in these regions varies between 250 mm and 500 mm. July is usually the driest month, and November the wettest. These seasonal fluctuations result to recurrent famine (Keating *et al*., 1992).

To increase maize production for the marginal areas, the government through public and private institutions such as International Maize and Wheat Improvement Center (CIMMYT) and Kenya Agricultural Research Institute (KARI) is currently investing

considerable resources in developing and promoting maize varieties that are suitable for the marginal areas.

Given that production of aflatoxin by *A. flavus* is influenced by weather (temperature, and humidity), crops grown in warm regions like Makueni County have greater likelihood of infection (Sanders *et al*., 1984; Schmitt and Harburgh, 1989). Despite the efforts by the government to increase maize production in the semi-arid areas, maize is affected before storage.Pests and diseases are the main biotic factors and insects are most often considered as the principal cause of grain losses in storage (Kacaniova, 2003). In Kenya, majority of post-harvest losses are attributed to storage pests and some are known to attack maize both in the field and while in storage (Kacaniova, 2003). Storage pest like *Sitophilus zeamais* (Motsch) (Maize weevil), causes between 10-20 % losses while *Prostephanus truncatus* (Horn) (larger grain borer - LGB) causes between 30-90 % of the harvested maize (Likhayo *et al*., 2004). Research by Schulten (1988) indicates that post-harvest losses are generally around 4±5 % per annum of maize stored on-farm in Africa (McFarlane, 1988). In Kenya, losses due to these pests have been estimated at 1.8 million bags of maize estimated at 8.1 billion Kenya shillings annually.

Existing pest management practices such as chemical control (Kibata *et al*., 2003) and biological control (Giles *et al*., 1996) have had little impact in eradicating storage losses. Farmer practices have also been associated with pre and post-harvest loses. Traditionally, most farmers leave their maize after it has matured in the field with the hope that it will

dry naturally to a moisture content is reduced to about 18 % by the time it is harvested (Rombo, 1986). During this long pre-harvest drying period in the field, this maize is exposed to negative effects of wind and rain and the attacks of insects, birds and aflatoxigenic molds.

2.3 Mycotoxins in crops

Turner *et al*. (2009) described mycotoxins as toxic secondary metabolites produced by organisms of the fungus kingdom, commonly known as molds. Toxigenic molds occur worldwide in air and soil. Example of these mycotoxigenic molds are *Aspergillus*, *Penicillium* and *Fusarium* species. Cole, (1981) indicated that there are over 200 known mycotoxins but only those occurring naturally in foods are of significance in terms of food safety. Turner (2009), described 'mycotoxin' as toxic chemical products produced by fungi that readily occur in agricultural crops including maize (Scudamore *et al*., 1998) and other cereals, legumes, cotton seeds and tree nuts. The toxins can also occur during growth, after harvest, during handling in storage and in processing (Silva *et al*., 2000; Tancino *et al*., 2001; Cardwell and Cotty, 2002).

The Food and Agriculture Organization (FAO) estimates that, mycotoxins contaminate between 25 and 50 % of agricultural crops worldwide (Fandohan *et al*., 2003; Lewis *et al*., 2005; Wagacha and Muthomi, 2008). The fungi find many commodities being good substrate for growth because of the large number of enzymes which they can use for their development (Hell, 1997). This revelation confirm that mycotoxin problems affect the

agricultural economies of many countries in the world because of the significant economic losses associated with their impact on human health, animal productivity and trade (Wagacha and Muthomi, 2008). In this respect, aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxin A, Patulin, T-2 Toxin and zearalenone are considered as the most important mycotoxins on a worldwide scale (FAO, 2001; Miller, 2008). Zearalenone for example, is an estrogenic compound that causes infertility and other reproductive problems in animals (Ngoko *et al*., 2001). Fumonisins are reported to have oesophageal cancer-promoting activity, in addition to causing several diseases in animals (Rheeder *et al*., 2002). These toxins are not destroyed by cooking and freezing or upon digestion by either human or animal and they remain in the food chain in meat and dairy products.

2.3.1Contamination of maize with members of *Aspergillus* section *Flavi* at production stage

Maize may be at a risk of fungal contamination if produced in soils that are highly contaminated with aflatoxigenic fungi. At the beginning of the growing season, sclerotia are exposed to the soil surface and they quickly germinate sporogenically to produce large quantities of conidia on successive crops (Widdow and Donahue 1984). Crops grown under conditions favoring aflatoxin contamination become covered with large quantities of *A. flavus* propagules. Germination of the fungal conidia in maize fields occurs just before silking. During this growing season, infected plant tissues serve as a source of secondary conidial inoculum, especially when environmental conditions are conducive for disease development (Scheidegger and Payne, 2003) Maize canopy

provide shade that may help to retain moisture at the soil surface and thus promote their germination (Wicklow and Wilson, 1986). Infection is most severe when the maize crop is caught by rain just prior to or during harvest (Cotty, 2001; Jaime-Garcia and Cotty, 2003).

The second stage of contamination continues in field especially when maize is stored in piles, wind-rows, and units, during drying and even in the hands of the end-user (Waliyar *et al*., 2003). Contamination could also occur when mature crop is exposed to warm, moist conditions either in the field, during transportation, storage, or use (Cotty, 1991). It can therefore be speculated that aflatoxin problem starts in the field at production stage and may extend in the maize value chain due to poor handling, storage and packaging. Boken *et al*. (2008) indicated that an understanding of the interactions between host plant and environment during the growing season can enable quantification of pre-harvest aflatoxin risk and its potential management.

2.3.2 Aflatoxins and aflatoxigenic fungi

Aflatoxins are naturally occurring, secondary metabolites that were first discovered in England in the early 1960s when more than 100,000 young turkeys on poultry farms died within a few months (Blout, 1961) after consuming an *Aspergillus flavus* contaminated peanut meal that had originated from Brazil. The disease contracted by these animals was called Turkey X disease (Klich *et al*., 2000; Papp *et al*., 2002).

Aflatoxin derives its name from the fact that it was originally found to be produced by *Aspergillus flavus* (Agrios, 1978), but is now known to be produced by other *Aspergillus*

species in the section *Flavi* group most notably *A. flavus* (S and L strains) and *A. parasiticus* (Bennett and Klich, 2003) on variety of food products. *Aspergillus flavus* and *A. parasiticus* are the two most important species in agriculture as they are found through-out the world, being present in both soil and the air (Klich, 2002; Abbas *et al*., 2005). When conidia (spores) encounter a suitable nutrient source and favorable environmental conditions (hot and dry conditions) the fungus rapidly colonize, establish and produce aflatoxin (Payne, 1992) on susceptible crops.

Presence of aflatoxin in human foods causes acute and chronic health effects (aflatoxicosis) depending on the duration and level of exposure. Naturally occurring mixtures of aflatoxins have been classified as a class 1 human carcinogen (IARC, 1993) and, the toxins have carcinogenic mutagenic, immunosuppressive, hepatotoxic and hepatocarcinogenic (Massey *et al.,* 1995) actions. They are also known to cause growth retardation and even death (Wild and Turner, 2002; Gong *et al*., 2004; Williams *et al*., 2004; Azziz-Baumgartner *et al*., 2005). Contamination also occurs in animal products when animals that are intended for dairy production consume aflatoxin-contaminated animal feed (Reed and Kasali, 1987).

2.4 The genus *Aspergillus*

The genus *Aspergillus* is a Deuteromycetes (Fungi Imperfecti; Hyphomycetes), a large genus composed of more than 180 accepted anamorphic species with teleomorphs described in nine different genera (Pitt *et al.,* 2000). Their teleomorphs can be found in the Ascomycetes (Pelczar *et al.,* 1998) structures that produce sexual spores (ascospores),

endogenously in a well-differentiated ascocarp Generally, Deuteromycetes reproduce vegetatively by conidia. According to Klich (2002), the genus is subdivided in seven subgenera, which in turn are further divided into sections.

2.4.1 *Aspergillus* section *Flavi* group

Aspergillus section *Flavi* comprises of a closely related group of fungi that are found throughout the world and are present in soil and the air (Klich, 2002; Abbas *et al*., 2005), decaying vegetation, hay, and grains undergoing microbiological deterioration. Members of *Aspergillus* section *Flavi* appears to spend most of their life growing as saprophytes in the soil (Accinelli *et al*., 2008a). A*spergillus flavus* and *A. parasiticus* are the two fungal species in the *Aspergillus* section *Flavi* group that are most important in agriculture. The species have received major considerations due to their ability to produce potent carcinogenic aflatoxins (Cary *et al*., 2005; Klich 2007; Cotty *et al*., 2008; Pildain *et al*., 2008) on important food crops prior to harvest, during harvest, storage and processing (Yu *et al*., 2004). Their aflatoxin producing ability varies widely (Cotty, 1997; Horn and Domer 1998). Other members of this section *Flavi* known to produce aflatoxin are *A. pseudotamarii* and *A. bombycis* (Peterson *et al*., 2001).

In culture, members of the section *Flavi* group grows as yellow-green molds and like other *Aspergillus* species produces a distinctive conidiophore composed of a long stalk supporting an inflated vesicle. Colonies on potato dextrose agar at 25° C are olive to lime green with a cream reverse. Conidiogenous cells on the vesicle produce the conidia that

are more variable in shape and have a relatively thin, usually with finely roughened walls. *Aspergillus flavus* grow better with a water activity (aw) of between 0.86 and 0.96 (Vujanovic *et al*., 2001) and can be divided into S and L strains on the basis of sclerotia morphology (Mellon and Cotty, 2004). The typical or L strain isolates vary widely in aflatoxin producing ability and a significant percentage are non-toxin producers. The S-strain isolates have a tendency to produce greater quantities of smaller sclerotia and also produce more aflatoxin than L strain isolates.

Populations of *A.parasiticus* are characterized by considerable diversity in morphology and aflatoxin production (Wicklow *et al*., 1998). The fungus appears to be adapted to soil environment and is prominent in peanuts. It has a woolly/ cottony texture to granular with a rapid growth at both 25 and 37^0 C. The fungus produces darker green conidial heads and more pronounced conidium ornamentation (Klich and Pitt 1988). This characteristic is used to separate *A. flavus* from *A. parasiticus*. When present, sclerotia are dark brown. Conidia and sclerotia serve as infective propagules of the *Aspergillus* species and these propagules can contaminate various crops through direct contact (Bayman and Cotty, 1993). Phenotypically, *A. parasiticus* produces AFB_1, AFG_1, AFB_2, and AFG_2 (Bennett and Klich, 2003). These abbreviations are indicative of the colours they exhibit/fluorescence under the ultraviolet light (385 nm) on thin layer chromatography plates (Sweeney and Dobson, 1998; Bennett and Klich, 2003). Thus B is for blue and G is for green -yellow.

A third species, *A. nomius* has a mycotoxin profile similar to *A. parasiticus* but morphologically resembles *A. flavus* (Kurtzman *et al*., 1987; Peterson *et al*., 2001). The species was considered rare, but recent studies indicate that *A. nomius* is widely distributed and might be of economic importance (Ehrlich *et al*., 2007). Other fungi known to produce aflatoxins but encountered less frequently in nature are *A.bombycis, A. ochraceoroseus* and *A. pseudotamarii* (Goto *et al*., 1996; Klich *et al*., 2000; Peterson *et al*., 2001).

2.4.2 Maize post harvest loses

In Kenya, post-harvest losses have previously been estimated at 30 % of all stored produce but with advent of the larger grain borer (LGB), *Prostephanus truncatus*-Horn (Coleoptera: Bostrichidae) and aflatoxin, the loss can be 100 % depending on the severity of the outbreak (Songa and Irungu, 2010). In their study, Songa and Irungu, (2010), indicated that less than 50 % of the farmers in Counties in Eastern, Central and Coast regions control insect pests in their stored grains. Delayed insect control and use of ineffective insecticide increase insect activities leading to loss of maize quality through damaged grains and fungal infection.

Grains stored in polypropylene material and with relatively high moisture content for longer than one month are likely to develop fungal infection. A recent survey by the Ministry of Agriculture (2007) indicated that farmers store their maize in the living houses due to insecurity. Due to poor aeration in the houses, the relative humidity is high predisposing the produce to both storage pests and fungal attack. Other post-harvest loses

occur while the harvest is held for short-term storage or while the farmer awaits a selling opportunity or a rise in prices. The rest of the grains may be sold either in one go or over a period of time, through a variety of different marketing channels. Additionally, post-harvest operations such as shelling and mechanical drying also account for wounding of kernels in the field and in store (Zuber *et al*., 1986) hence aflatoxin contamination.

2.5 Implications of aflatoxins on human and animal health

2.5.1 Aflatoxins and human health

A positive correlation has been established between consumption of aflatoxin-contaminated foods and the increased incidence of liver cancer worldwide (Aly, 2002). Humans are exposed to the toxins by consuming foods contaminated with products of fungal growth. In Africa for example, maize consumption is a primary avenue through which humans become exposed to aflatoxins (Shephard, 2008). Intake of low, daily doses of the toxins over long periods may result in chronic aflatoxicosis expressed as impaired food conversion, stunting in children (Gong *et al*., 2004; Abbas, 2005), immune suppression, cancer and reduced life expectancy (Cardwell and Henry 2004; Williams *et al*., 2004; Farombi, 2006).

Chronic dietary exposure to aflatoxins is a major risk factor for hepatocellular carcinoma, particularly in areas where hepatitis B virus infection is endemic. However, chronic, sub clinical exposure does not lead to symptoms as dramatic as acute aflatoxicosis. Ingestion of high concentrations of aflatoxin results in rapid development of acute aflatoxicosis

which manifests as hepatotoxicity or, in severe cases, fulminant liver failure (Fung and Clark, 2004). After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide intermediate or hydroxylated and become the less harmful aflatoxin M_1. No animal species is immune to the acute toxic effects of aflatoxins.

Aflatoxicosis is characterized by severe liver damage leading to jaundice, hepatitis, mutagenicity and nervous disorders (KEPHIS, 2006) and when most severe, death (Williams *et al*., 2004). Other health conditions in which aflatoxin has been implicated are Kwashiorkor and Reye's syndrome, sicknesses that are more prevalent in Africa than in other parts of the world (Wild *et al*., 1991). From liver biopsy of 27 Sudanese children suffering from Kwashiokor, Coulter *et al*. (1986) reported the detection of aflatoxin B_1, B_2 and aflatoxicol in the organs of the children. In a different study, Stora *et al*. (1983) found aflatoxin B_1 levels of between 120 and 180 μg^{-1} in the livers of five infants suffering from Reye's syndrome.

2.5.2 Aflatoxin and animal health

Toxic and especially carcinogenic effects of aflatoxins have been reported in several different animals, but susceptibility to these toxins varies greatly with sex, age, species and strain within a species (CAST, 2003). Aflatoxins cause liver damage, decreased milk and egg production, recurrent infection as a result of immunity suppression, in addition to embryo toxicity in animals consuming low dietary concentrations (Saad, 2007). Aflatoxin B_1 is the most potent mycotoxin to affect cattle. When significant quantities of B_1 are consumed, the metabolite M_1 appears in milk within 12 hours. Young animals of all

species are more susceptible than mature animals to the effects of aflatoxin. Pregnant and growing animals are less susceptible than young animals, but more susceptible than mature animals (Saad, 2007).

2.6 Factors affecting occurrence and production of aflatoxin in maize

2.6.1 Environmental factors

The occurrence of aflatoxins is influenced by various climatic factors (Widstrom 1992). The *Aspergillus* fungi are native to warm arid, semi-arid, and tropical regions with changes in climate resulting in large fluctuations in the quantity of aflatoxin producers (Bock *et al*., 2004). These fungi compete poorly under cool conditions and the quantity of *A. flavus* in cool areas with temperatures below 20° C is low compared to warmer regions with temperatures above 25°C.

Crops grown in warm climates have greater likelihood of infection by the fungi and in some regions, infection only occurs when temperatures rise in association with prolonged drought (Sanders *et al*.,1984; Schmitt and Harburgh, 1989) and high insect activity. Climate influences not only the quantity but also the types of aflatoxigenic fungi present in the soil. It also influences the extent to which crops become wounded by mammals, birds and insects. For insects, survival between seasons, dispersal across regions, and rates of population increases are all influenced by climate. Mold growth and aflatoxin contamination are not formed by the climatic factors in isolation but depend also on the moisture content of the maize. Kawasugi *et al*. (1988) showed that maize harvested early at moisture content of about 30 % had the highest aflatoxins levels.

Aflatoxin-producing fungi differ geographically (Cotty, 1997; Horn and Dorner, 1998). As a result, contamination varies with geographic location, agricultural and agronomic practices, and the susceptibility of the crop to fungus before they are harvested, and during storage, and/or processing.

2.6.2 Agronomic factors

Widstrom (1992) and Cotty (1994) identified plant stress, irrigation, cropping pattern, variety, date of planting, date of harvesting and storage conditions as some of agronomic factors that may influence aflatoxin development. Physical and chemical characteristics of husk and grain also render maize susceptible to mold infection (Barry *et al*., 1986; Cardwell *et al*., 2000). The variety of maize planted is an important factor in fungal infection and toxin development. Nwogu *et al*. (1979) found that yellow maize was more susceptible to microbial and fungal attack than white maize.

Planting and harvesting dates may be managed to reduce aflatoxin contamination (Jones and Duncan, 1981). Many authors have reported that drought, especially during the terminal growth stages, lead to high aflatoxin contamination (Jacques, 1988; Kumar *et al*., 2000). If maize is grown with a crop that is also susceptible to aflatoxin development, there will be an increased risk of toxin metabolism (Cotty, 1994). Cole *et al*. (1982) investigated the effect of peanut, maize, soyabean crop rotation on aflatoxin development. They found more aflatoxins in maize that was planted after groundnut.

2.6.3 Biotic factors

Relationship between insect damage and aflatoxin development has been studied and reported (Fortnum, 1986). Insect attacks in the field and in farmer store facilitate easy fungal infection. Zuber *et al*. (1986) reported that insects that feed on maize ears in the field predispose grains to *A. flavus* infection through the physical damage caused by their feeding. Sinha and Sinha (1992) observed that the rapid multiplication of insects lead to increased moisture content, dust production, *A. flavus* infection and aflatoxin contamination. Thus varieties with high resistance to ear-infesting insects and tough kernels are less susceptible to *A. flavus* infection and aflatoxin contamination. Insects also act as vectors of fungal spores (McMillian *et al*., 1990) and help to increase the infection process. Competition with other organisms could lead to slowing down of the development of *A. flavus* in the substrate.

2.7. Strategies for aflatoxin management

Although the first reported outbreak of acute aflatoxicosis in Kenya occurred more than three decades ago, (Nagindu *et al*., 1982), annual outbreaks have continued to affect people in Eastern part of the Kenya (Azziz-Baumgartner *et al*., 2005; World Health Organization AFRO Food Safety Newsletter, 2006). Therefore serious efforts must be made to control the aflatoxin production since it is unavoidable and unpredictable (FAO, 1997). Bankole and Adebanjo (2003) have proposed many solutions against aflatoxin production in food, and some of the strategies may be applicable in Kenya.

2.7.1. Regulatory measures

To minimize potential human exposure, the aflatoxin content of food and feed is strictly regulated in most of the world (Shephard, 2008). However, the standards set have little relevance to poor, small-scale farmers in Africa, who often rely on maize for daily nutrition and income. Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains, for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply. Kumar *et al.* (2000) listed (Table 2.1) the general levels permitted and the consequences of ingestion of products with aflatoxin (AFB_1) levels beyond those levels.

Table 2.1: Aflatoxin (AFB_1) limits and consequencies of their ingestion

Aflatoxin level (parts per billion)	Limitation / consequence
20	Highest level allowed for humans
50	Highest level allowed for animals
100	Slowed growth of young ones
200-400	Slowed growth of adults
Beyond 400	Liver damage and cancer

Kumar *et al*. (2000)

2.7.2 Prevention of mold contamination and growth

2.7.2.1 Mechanical grain drying

Most situations leading to mycotoxin contamination relate to non-maintenance of stored products at safe moisture content. Rapid drying of agricultural products to low moisture content is often emphasized among the recommendations for solving the mycotoxin problem. Drying harvested maize to moisture content of 15.5 % or lower within 24 to 48 hours have been shown to reduce the risk of fungus growth and consequent aflatoxin production (Hamilton, 2000). Siriacha *et al*. (1989) found that if shelled grains are immediately sun-dried the chance of contamination was reduced as compared with that of non-dried shelled maize. Most farmers in Africa, sun-dry their harvests which often require longer durations for the product to attain 'safe' moisture level especially in times of cloudy weather. The effectiveness of drying was demonstrated in a report by Awuah and Ellis (2002) in which groundnut kernels with 6.6 % moisture content were found to be free of fungi regardless of storage protectant used for six months.

2.7.2.2 Constraints of traditional drying, storage and improved storage structures

Traditionally, grains are spread out on polyethylene sheets on the floor, and the stirring or turning is done manually till the product is dry. Due to the high rainfall at the time of harvest, farmers opt to stack maize in the field as they prepare their fields for the next season. Other farmers dry their grains over the fire and then mix the moist and dry grains (Begun, 1991).

Traditional storage structures used by farmers for on-farm storage include structures made of plant materials like wood, bamboo, thatch or mud placed on raised platforms and covered with thatch or metal roofing sheet. Essentially the stores are constructed to prevent insect and rodent attack and to prevent moisture from getting into the grains.

Experiments carried out by Eriksson (1986) with the different types of storage structures used in Kenya, established that the main constraint in most traditional storage structures is that they do not allow for optimal free ventilation and thus a long pre-harvest drying is required before maize can be stored safely in such stores.

Eriksson found out that molds were present in maize stored in all types and sizes of traditional structures and it was difficult to establish if mold infection had occurred during storage or in the field. It was also observed that prolonged storage, regardless of the storage structure encouraged mold growth. Insect and mold infestation might not be noticeable at harvest, but the build-up of the infection will accelerate later during long storage periods. Study by Bett, *et al.* (2011) shows that maize stored in metal silos had aflatoxin levels reduced by 60 % on average. However, it has been very difficult to promote metal silo to small-scale farmers due to their high cost.

CHAPTER THREE:

GENERAL MATERIALS AND METHODS

3.1 Site selection

Maize samples analysed in this study were collected for three years from January 2009-July 2011 in Kaiti division in Makueni County (Figure 3.1). Makueni County is in former Eastern Province of Kenya. The area is semi-arid to arid with low and erratic rainfall, hence highly prone to frequent drought, resulting in food insecurity and scarcity of water. The area has been considered a high aflatoxin risk area and contamination of food by aflatoxins contributes to food insecurity in the area.

The selection of the study area was based on availability of maize, high frequency of drought and previous reports of aflatoxin outbreaks. Actual site selection was determined by a Global Positioning System (GPS) in farms where pre-harvest maize samples were collected from 2009-2011. Kaiti was selected because cases of aflatoxicosis had previously been reported and studies by Kaswii, (2009) revealed that *A. flavus* had high incidence (mean = 32.6 %) The selection of farmers was done through the assistance of field extension officers in the Ministry of Agriculture (MoA) and scientists from Kenya Agricultural Research Institute (KARI).

Makueni County rainfall data was acquired from the Kenya Meteolorogical department through KARI agromet department.

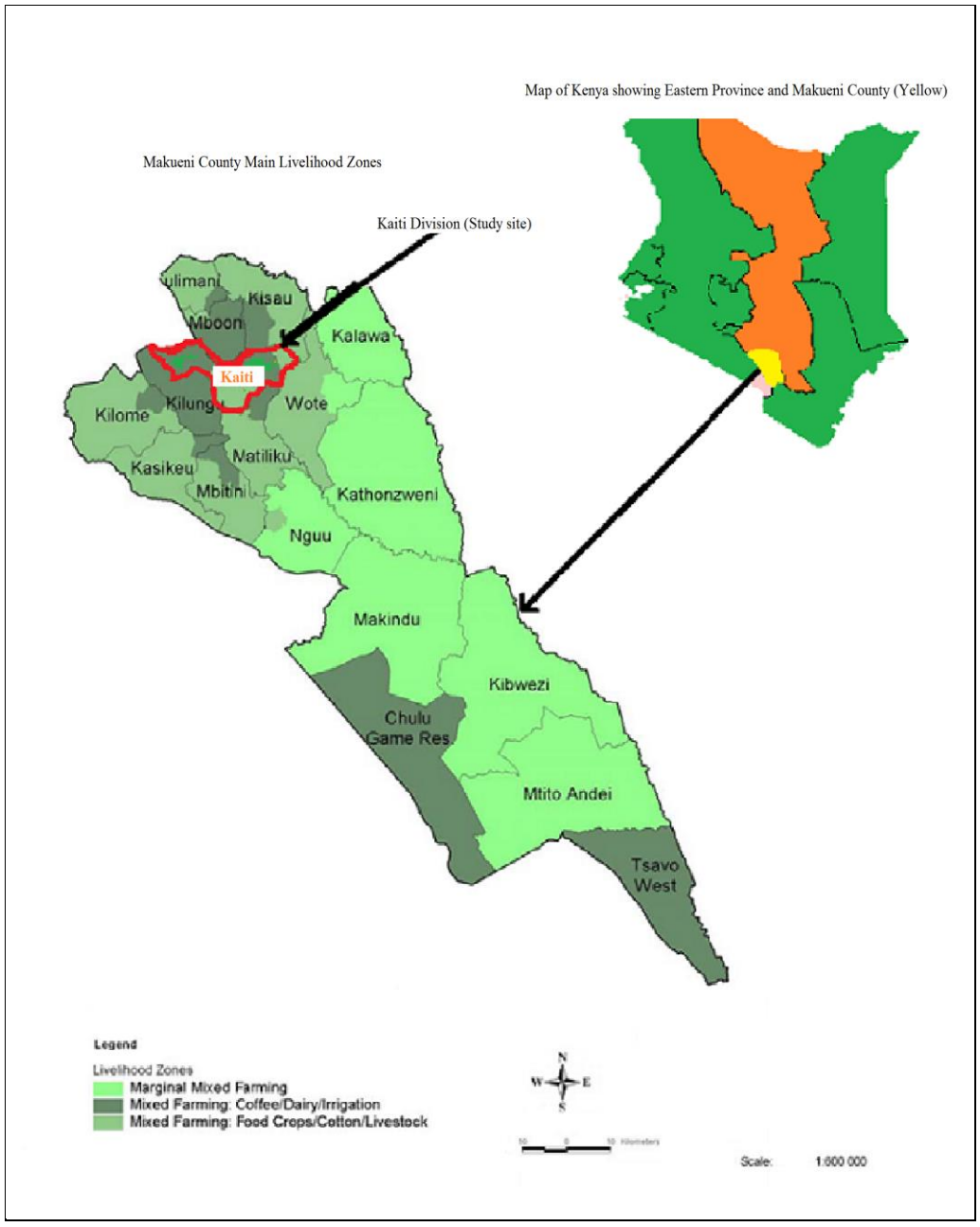

Figure 3.1: Map of Makueni showing administrative boarders, main livelihood zones and the study area (Karanja, L.W)

3.1.1 Farmer and Market vendor selection

Individual farmers were selected based on the distance from one farm to another (not less than 3 km of each other), presence of maize crop in the field and willingness of the farmer to participate in the survey. Farmers from 11 locations were selected (Table 3.1) Individual agricultural markets in each district were selected on the basis of information obtained from interviews with the District Agriculture Officer in each district. Markets that represented potential exposure to aflatoxin among all market maize consumers within the study area were selected. Vendors with maize from a variety of sources were included. Vendors were systematically selected based on location within the market in order to obtain a fair distribution within the market place (Table 3.1). Selected maize vendors were interviewed and requested to provide samples of each of their maize products for aflatoxin analysis.

Table 3.1: Markets and villages where samples were collected from

Farmer stores	Market
Iuani	Itooni
Kilala	Itumbule
Kivani	Kaiti
Makongo	Kaumoni
Mukuyuni	Kilala

Nzuuni	Kyambalasi
Ukia	Kyuasini
Utaati	Makutano
Kaumoni	Mukuyuni
Nziu	Mwea
Kyuasini	Ukia

3.1.2 Maize sample collection

A one kilogram (kg) sample was taken from every maize product offered by the farmers and vendors interviewed. The samples included dried maize grains, maize flour (commercial or locally milled), and *muthokoi* (kernels with the outer hull removed). Where the vendor offered the same product from different sources (purchased from local farmers, Lorries and other distributors) then a one kg sample was taken from each. Most of the samples were collected from 90 kg bags of maize.

Multiple samples were taken from different parts of one bag or several bags belonging to one vendor and combined to produce a one kg sample for analysis. The samples were milled using a commercial mill available in the districts where the samples were collected from. A one kg sub-sample was drawn from the milled samples for laboratory analysis. The samples were then placed in polypropylene bags, labelled and transported to CABI-

laboratories in Nairobi for cold storage at 4^0 C in a cold room, until analysis was done. Microbial analysis was conducted for a period of seven months from October 2011-May 2012.

3.2 Determination of moisture content of maize samples

The ground samples were analyzed to determine initial moisture content (MC) of the maize samples after milling. An aluminum container measuring 6 cm x 5 cm was made by wrapping a 15 cm x 15 cm sheet of aluminium foil around a 6 cm diameter glass. The container was weighed and its weight recorded as "empty container weight". Ten gram (10g) sub sample of ground maize were determined by weighing in the aluminium container and its weight noted as "wet weight of sample" (Wwt) .The samples were replicated three times and incubated at 105^0 C for 24 hours, cooled in a desiccator containing self-indicating silica gel then weighed again to determine the "dry weight of sample"(Wdt). The following formula was used to calculate percentage moisture content of the samples.

$$MC = ((Wwt - Wdt)/ Wwt) \times 100\ \%$$

3.3 Preparation of Modified Dichloran Rose Bengal Medium (MDRB)

A semi-selective medium, Modified Dichloran Rose Bengal medium (10 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 25 mg Rose

Bengal, per litre of distilled water) was used for isolation of *Aspergillus* section *Flavi* (Horn and Dorner, 1998). The pH of the medium was adjusted to 5.6 using 0.01 M HCl before autoclaving for 20 minutes at 121^0 C and 15 Pressure per .Square.Inch (P.S.I). The medium was then cooled to 55^0 C in a water bath. Two antibiotics were added to the the culture medium to enhance sensitivity of each other. Streptomycin sulphate (in 5 ml distilled water) and Chlortetracycline (in 10 ml distilled water) were prepared and passed through a 0.25µm filter to sterilise before adding them to the cooled media at the rate of 40 and 2 mg/l, respectively.

A fungicide, dichloran was dissolved in acetone and 5 ml of the suspension added to the medium at the rate of 4 mg/l. This was aimed at inhibiting other fungal and bacterial growth and ensuring that the medium was semi selective. The culture media was poured into sterile plastic Petri dishes to a depth of 0.5cm (15 ml in a 9 cm diameter Petri dish) under the laminar airflow. This was allowed to cool in a sterile air cabinet at room temperature, packed in polypropylene bags and left for 2-3 days at room temperature before use.

3.3.1 Preparation of 2 % water agar

Two percent water agar was used as a diluent and was prepared by adding 2 g agar in 100 ml distilled water and sterilizing for 20 minutes at 121^0 C and 15 P.S.I

3.4 Population of *Aspergillus* section *Flavi* group in maize and soil samples

Fungal isolates were recovered by dilution plate technique on MDRB (Cotty, 1994). The ground maize samples were vigorously shaken to ensure proper mixing. From the one kg samples, two sub-samples each weighing 2.5 g was drawn and put in a 15 ml graduated dilution tube. Two percent water agar was used to dilute the samples. This diluent was added to the samples to make 10 ml of the stock solution. The solution was vortex mixed thoroughly then serial diluted by transferring 1 ml of the stock to 9 ml of the diluent. The diluted samples were placed in a rack in a water bath at 40^0 C until plated on the MDRB media. The procedure was repeated with soil samples but one gram of soil was diluted in 9 ml of the diluent.

Using a micro applicator, a 200 µl aliquot of the diluted samples (soil and maize) were transferred aseptically onto the semi selective medium in the 90 mm diameter Petri dishes and spread on the surface of the culture medium using a sterile glass rod. A total of 6 replicates were plated for each sample. The inoculated plates (0.2 ml/plate) were incubated at 30^0 C for 3-7 days.

The plates were removed from the incubator and visually observed for microbial growth. Culture plates that had 10-60 colonies were selected for counting the colony forming units (CFUs) Lines were drawn on the under-side of the plates for ease of counting the colonies. *Aspergillus* species were identified as described by Horn and Dorner (1998). A stereomicroscope was used to identify fungi in the section *Flavi* group directly from the dilution plates in plates that were heavily colonized by other filamentous fungi.

Morphological characteristics (colour, texture, size of colony) were used to separate the fungi into groups.

Six *Aspergillus* section *Flavi* reference cultures (Figure 3.1) obtained from University of Georgia, United States of America were used to aid in identification. The reference cultures were plated on MDRB medium each time isolation was done. The plates were incubated for 5-7 days at 30^0 C. Colony characteristics of the reference cultures were compared to those that appeared on the maize and soil sample cultures.

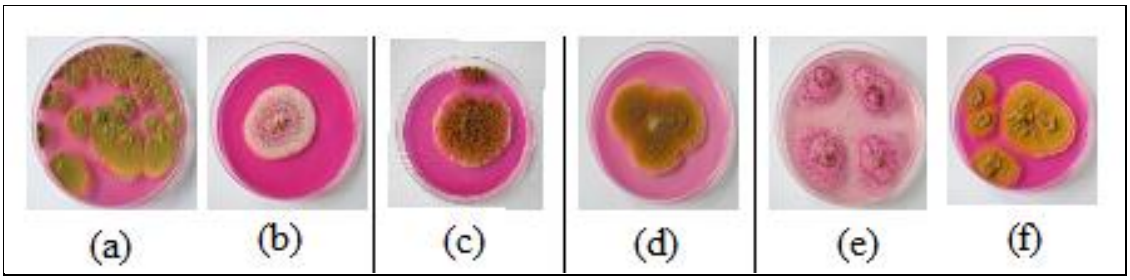

Figure 3.2: Fungal reference strains showing *A. flavus* L strain (a), *A. flavus* S strain (b), *A. caelatus* (c), *A. tamarii* (d), *A. alliaceus* (e) and *A. flavus* (L strain) (f) (Plates by L. Karanja, 2011)

Aspergillus flavus (S and L strains) and *A. parasiticus* colonies were plated in Czapek Dox Agar (CZA), prepared by mixing 35 g of Czapek Dox concentrate with 15 g agar in one litre of distilled water. Czapek Yeast Extract Agar (CYA) was prepared by mixing 1 g of K_2HPO_4, 10 ml Czapek concentrate, 5 g powdered yeast extract, 30 g sucrose and 15 g agar in one litre of distilled water for color differentiation hence further identification.

The pH of the medium was then adjusted to 7.2 before autoclaving for at 121^0 C and 15 P. S. I. for 20 minutes. The molten media was cooled and poured as described in section 3.3.

Aspergillus species and total filamentous fungi were enumerated. The total numbers of colony forming units were counted using a tally counter and the number of colonies of the target fungi (*Aspergillus flavus* L and S strains, *A. parasiticus*, *A. tamarii*, *A. alliaceus* and *A. caelatus*) per plate was recorded. Other fungal isolate such as *A. niger, Fusarium, Penicillium, Rhizopus* spp. and bacterial colonies were recorded too. Target species (*A. flavus* and *A. parasiticus*) colonies were sub-cultured on CZA in slants and preserved in a refrigerator at 4^0 C after sporulation.

3.5 Screening *Aspergillus flavus* isolates for aflatoxin production and types

A liquid broth Sucrose Yeast Extract (YES) was prepared by mixing 150 g of sucrose, 40 g of glucose, 20 g yeast extract and 10 g soy stone in one litre of distilled water. The pH was adjusted to 5.9 using 0.25 M HCl. Aliquots of 2 ml of the broth were dispensed into 6 ml glass vials, loosely screwed and autoclaved for 30 minutes as described in section 3.3.

Fungal spores from pure *A. flavus* L and S strains and *A. parasiticus* culture plates were picked with an inoculating loop and transferred into the liquid medium and the inoculated vials were loosely screwed before incubation at 30^0 C for 7 days. Toxin was extracted from the vials with the growing cultures by adding 2 ml of chloroform to each vial,

mixing vigorously. The vials with a mixture of chloroform and the target fungal colony were left overnight for the aflatoxin to settle at the bottom of the vial.

Thin layer chromatography (TLC) plate (A Silica gel 60; EMD Chemicals Inc., Darmstadt, Germany) was used to determine and confirm presence of different aflatoxins in the maize samples. A line was drawn quarter way from the bottom of the TLC plate. Some marks were made along the drawn line (1 cm apart). A spot (5µl) containing mixture of the analytical grade aflatoxins standards (AFB_1, AFB_2, AFG_1 and AFG_2) was put on the first mark and a similar amount of the chloroform extract was placed from the second spot onwards. This was repeated with different samples until all the vials were completed. The spotted plates were placed in a glass container (5 cm x 3 cm) containing a developer solvent made by mixing distilled water, acetone and chloroform, in a ratio of 1.5: 12: 88 respectively, until the solvent covered 80 % of the TLC plate. The plates were allowed to air dry before viewing under the UV light in a dark room. The bands were compared with the standard aflatoxins and scored based on presence or absence of specific aflatoxin types.

3.6 Statistical analysis

All data was captured in Microsoft excel and subjected to exploration and cleaning prior to analysis. Excel, R and Genstat softwares (VSN International (2011) were used to analyse the data. In Microsoft excel, means and standard errors were used to compare means while pie charts and bar graphs were used to show incidence levels of aflatoxins in

different sites. R was used to display means and confidence intervals (C.I) using the bargraph within the sciplot package. Confidence intervals were set to 1.96 standard errors (translates to 0.05 P value). Further, associations between specific fungal incidences were investigated based on analysis of contingency tables with appropriate chi-squared tests using different variables, including soil type of the areas where the samples were collected from. Analysis of variance was performed on all data with the general linear model (GLM), suitable for unbalanced data. Tukey's honestly significant difference (HSD) test was performed to compare treatment means at the 5 % level, the studentised range to determine a single critical value that all comparisons must exceed for significance. This method was used for experiments that had equal numbers of observations (N) in each group or in cases where N varied significantly between groups.

CHAPTER FOUR

OCCURRENCE AND DIVERSITY OF AFLATOXIGENIC FUNGI (*ASPERGILLUS* SECTION *FLAVI*) IN SOILS OF MAIZE GROWING FARMS IN KAITI LOCATION, MAKUENI COUNTY

Abstract

Makueni, a County in Kenya has been occasioned by sporadic, yearly recurrent aflatoxicosis outbreaks since 1981. *Aspergillus flavus* and *Aspergillus parasiticus* are the two common fungi in the *Aspergillus* section *Flavi* (ASF) group that are associated with aflatoxin contamination of agricultural commodities along the value chains. These fungi reside in soil as conidia, sclerotia and hyphae all of which act as the primary inocula for the two fungi that produce carcinogenic aflatoxins in the commodities. Information on prevalence and occurrence of aflatoxigenic fungal populations in soils of maize growing regions in Makueni is fragmented. The aim of this study was to determine the occurrence and distribution of aflatoxigenic fungi at the production stage of the maize value chain.

Soil samples (80) were collected from 37 maize growing farms in Kaiti Location. In the laboratory, plating and subsequent isolation of ASF population was done on Modified Dichloran Rose Bengal (MDRB) agar. Thin layer chromatography (TLC) technique was used to determine and confirm presence of different aflatoxin types in fungi isolated from the the soil samples. Out of 80 samples analyzed, 105 isolates were recovered 70 of which was *Aspergillus* in the section *Flavi* group. The most common member of ASF was *A. flavus* (S and L strains) and *A. parasiticus* with frequencies of 81, 65 and 43 colonies respectively. Of the cultured isolates, 55 % were aflatoxigenic and exhibited the

four toxin types but with varying percentage incidences of 24, 22, 21, 22 and 12 % for aflatoxins (AF) B_1, AFB_2, AFG_I, AFG_2 and AFB_1/ AFB_2 respectively.

Generally, *A. flavus* S and L strain and *A. parasiticus* were present in 90 % of the sampled farms. Spatial analysis of fungi in the ASF group assumed a patchy distribution in Kaiti Location and the highest incidence was in loam and red loam and the least in black loam and sandy soils. All the sampled farms had soils with high incidences of other *Aspergillus* spp (86 colonies), *A. niger* (78 colonies) and *Penicillium* spp (73 colonies). The study revealed that soil collected from Kaiti contained a mixture of *Aspergillus* and other fungal species. The finding of this study stresses the need of studying the soils further to understand the possible mycotoxin interactions in existence, in addition to recurrent aflatoxicosis outbreaks reported yearly.

Key words: Soil, Global Positioning System, Contamination, Kaiti

4.1 Introduction

Kenya has been experiencing sporadic recurrent aflatoxicosis outbreaks since 1981. Scientists have shown that worldwide contamination of foods with mycotoxins is a significant health and economic problem (Hussein and Brasel, 2001; Bennett and Klich, 2003). The occurrence of toxic secondary metabolites in foods and feeds is natural and unavoidable and is influenced by certain environmental factors (Smith *et al*., 1994).

In maize producing regions of Makueni, the economic impact from yield loss and contamination is not directly associated with contamination of soils with aflatoxigenic fungi. The contamination is associated with other factors such as drought, extreme

rainfall variability (Ogallo, 1994) and soils of low to medium fertility (Republic of Kenya, 2002). The saprophytic nature of these fungi allows them to be present in the soil allowing them to infect other plants (Scheidegger and Payne, 2003) hence completing their life cycle. Their conidia are assumed to be the primary inoculum. These fungi are also thought to be seed-borne, but Cotty, *et al*. (1994) suggested that *A. flavus* was soil-borne and that it survived for long periods in soils because it is well adapted for survival and may exist as conidia, sclerotia or hyphae. Sclerotia are commonly produced by strains of *A. flavus* (S and L) and *A. parasiticus* in culture (Cotty, 1989; Wicklow *et al*., 1998) and likely serve as resistant structures for surviving adverse environmental conditions (Coley-Smith and Cooke, 1971). They contaminate many commodities used as food and animal feed (Doner, 2004). The two fungi are able to survive and out-compete other fungi under hot and dry conditions.

To effectively prevent or minimize future outbreaks of aflatoxicosis and reduce long-term exposure to aflatoxins, data on possible contamination sources along the maize value chain is required in order to establish a long-term intervention that target all the stakeholders. Geographical information systems (GIS) have been used for early and accurate detection, diagnoses and pathogen surveillance on local, regional, and global scales to predict outbreaks and allow time for development and application of mitigation strategies. Emerging, re-emerging and endemic plant pathogens like the genus *Aspergillus* continue to challenge the ability to safeguard plant health in Kenya and specifically in areas where aflatoxin contamination has been earlier reported.

In the current study, GIS was used to map the risk areas in Kaiti Location as a way of helping in understanding the relationship between soil contamination and recurrent aflatoxicosis outbreaks in the region. Data gathered from the study will give guidelines for reducing human exposure to aflatoxin contamination through maize and maize products consumptionas a first step towards developing reliable methods to avoid future exposure of vast human populations to unacceptable aflatoxin levels. This study aims at evaluating the occurrence and distribution of ASF in different soils in Kaiti. The results from this study will show the relationship between aflatoxin contaminations, with soil contamination.

4.2 Materials and methods

4.2.1 Sample collection and preparation

Eighty soil samples were collected from 37 farms in Kaiti Location where pre-harvest maize samples were collected in 2009, 2010 and 2011 seasons. In the farms, five sample points were identified. One kilogram of soil was collected from the top 5 cm layer of soil. The samples from the five sample points in a farm were thoroughly mixed to form a composite sample. Spoons used to scoop the soil and were surface sterilized using 70 % ethanol to avoid cross contamination. The same procedure was repeated for all the randomly selected sample points in the same farm and at least four meters apart. A one kilogram sub-sample was drawn from the composite soil sample and labelled with the name of the farmer, village, GPS co-ordinates, soil type and the date of collection. The labeled samples were put in ziplock bag and placed in a cool box for transportation to

CABI laboratories in Nairobi where they were stored at 5^0 C until plated for microbial analysis.

4.2.2 Determination of the diversity and population of *Aspergillus* section *Flavi* in soil samples

In the laboratory, the soil samples were air dried and then hammered to break it into a powder. It was then passed through a 2 mm aperture laboratory test sieve (Endecotts limited, London, England) to get a fine powder. Two sub-samples weighing one gram each were put into a 15 ml graduated dilution tube. Nine millilitres of two percent water agar was added to make a 10 ml stock solution. The stock solution was serial diluted by transferring 1 ml of the stock to 9 ml of the diluent until a 10^{-3} dilution was attained. The diluted samples were placed in a rack in a water bath at 40^0 C until plated.

A semi-selective medium, MDRB was prepared as described in section 3.4. Fungal isolates were recovered by dilution plate technique (Cotty, 1994). Using a micro applicator, 200 µl aliquot of the diluted samples were transferred aseptically onto the semi selective medium in a 90 mm diameter Petri dishes and spread on the surface of the culture medium using a sterile glass rod. A total of 6 replicates were plated for each sample. The inoculated plates (0.2 ml/plate) were incubated at 30^0 C for 3-7 days without sealing. The plates were removed from the incubator and visually observed for microbial growth. Lines were drawn on the reverse-side of the culture plate for ease of counting the colonies.

Aspergillus species (*Aspergillus flavus* L and S strains, *A. parasiticus*, *A. tamarii, A. alliaceus* and *A. caelatus)* and total filamentous fungi were enumerated. The total numbers of colony forming units (CFU g^{-1}) were counted using a tally counter. Other fungal isolate such as *A.niger, Fusarium* and *Penicillium, Rhizopus* spp. and bacterial colonies were recorded too.

Aspergillus species were identified as described by Horn and Dorner (1998). A stereomicroscope was used to identify fungi in the section *Flavi* group directly from the dilution plates in Petri dishes that were heavily colonized by other filamentous fungi. Other plates were macroscopically observed for microbial growth. Morphological characteristics (colour, texture, size of colony) were used to separate the fungi into groups. Reference cultures (Figure 3.2) were used to aid in identification. Colonies identified as *A. flavus* (S and L strains) and *A. parasiticus* were sub-cultured on Czapek Dox Agar (35 g of Czapek Dox concentrate with 15 g agar in 1 litre of distilled water). The agar slants were incubated for the fungi to grow and preserved in a refrigerator at 4^{0} C after sporulation.

Thin layer chromatography (TLC) plates (A Silica gel 60; EMD Chemicals Inc., Darmstadt, Germany) were used to determine and confirm presence of different aflatoxins in the soil samples. *Aspergillus flavus* (S and L strains) and *A. parasiticus* colonies were grown in a liquid Sucrose Yeast Extract (YES) broth prepared, dispensed and inoculated as described in section 3.5. The spotted plates were placed in a glass container (5 cm x 3 cm) containing a developer solvent made by mixing distilled water,

acetone and chloroform, in a ratio of 1.5: 12: 88 respectively, until the solvent covered 80 % of the TLC plate length. The plates were allowed to air dry before viewing under the UV light in a dark room. The bands were compared with the standard aflatoxins and scored based on presence or absence of specific aflatoxin types.

4.3 Statistical analysis

Altitudes and study site GPS coordinates of the sampled farms were entered into an excel sheet and converted into ArcGIS files using Esri's ArcGIS (ESRI, 2011) software. Soil maps and boundary files were downloaded then opened in ArcMap 9 program. The points were geo-referenced and overlaid on soil maps then exported into various file formats. Microbial data was first inputted into Microsoft excel spread sheets and subsequent analysis was done using GenStat (Version 13 and 14) statistical package (VSN International (2011). Percentage incidence was calculated as a proportion of total number of samples analyzed. In excel, means and standard errors were used to compare means while pie charts and bar graphs were used to show incidence levels of aflatoxins in different sites. Multivariate analysis was used to investigate the relationships/interactions between the incidence of *A. flavus* (S and L strains) and *A. parasiticus* with other fungal species including *A. niger*, *Penicillium* and other *Aspergillus* species.

4.4 Results

There was morphological diversity in fungal species isolated from the different soil samples (Figure 4.0, plate a-f). Fungi in the ASF group, *Rhizopus, Penicillium* and other *Aspergillus* species were all isolated from soil in Kaiti. The most common species from section *Flavi* in this study, *A. flavus* (S and L strains) and *A. parasiticus*, were identified directly from soil dilution plates in MDRB medium. Colonies of *A. flavus* (L strain) were yellow to gray green with no sclerotia and those of *A. parasiticus* (Figure 4.0, plate a) were yellowish to dark green and more compact than *A. flavus* (L strain). Both *A. flavus* (S strain) and *A. alliaceus* produced large whitish brownish black sclerotia with prolonged incubation (over 10 days). A. *alliaceus* produced relatively larger sclerotia than *A. flavus* (S strain) which produced numerous small and dark sclerotia and had sparse conidial heads. *Aspergillus flavus* (L strain) and *A. parasiticus* (Figure 4.0, plate a-f) were found to be morphologically indistinguishable at first. *Aspergillus caeletus* and *A. tamari* were not isolated from the soil samples analyzed. Fungi identified as other *Aspergillus* species were characterized by a bright yellow colour on MDRB (Figure 4.0, plate, c), which gradually turned dark green with age (Figure 4.0, plate, e)

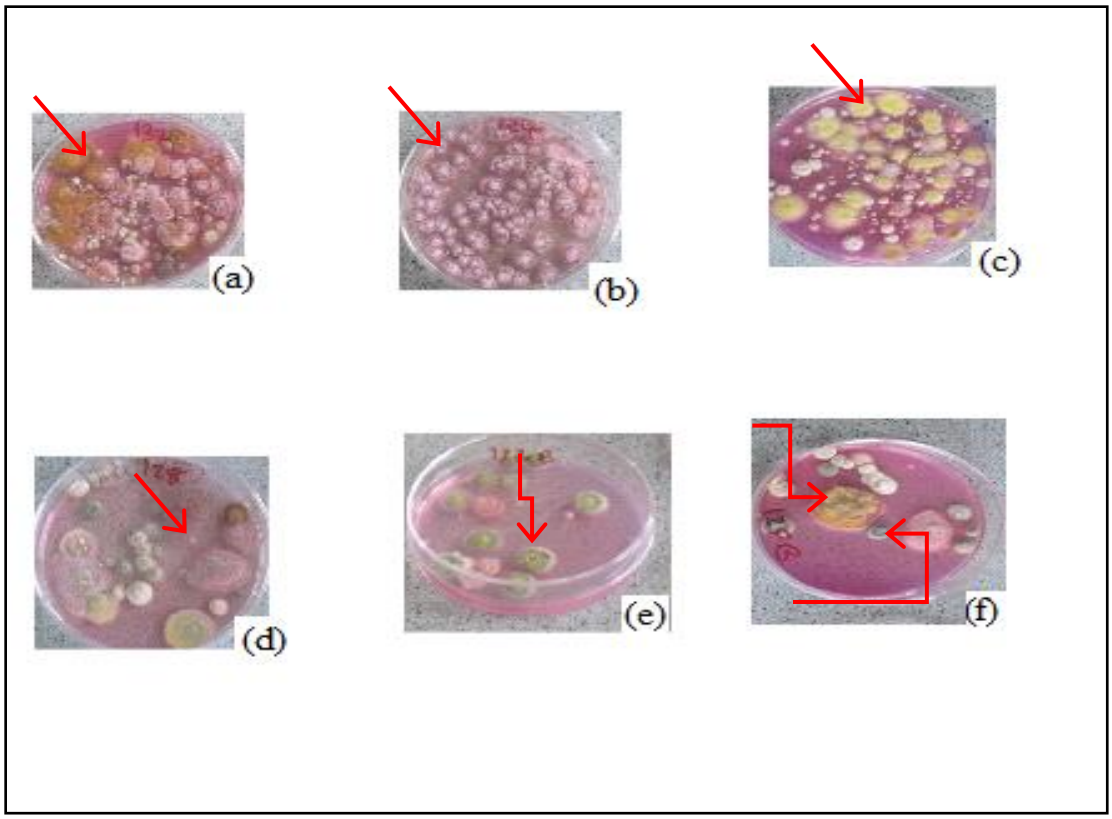

Figure 4.0: Morphological diversity of different fungal isolates in soil samples. *A.parasiticus* (a), *A. flavus* (S strain) (b), Other *Aspergillus* spp (c), *A. niger* (d), Other *Aspergillus* spp (yellow colour darkened with prolonged incubation) (e), *Penicillium* spp (small green colony) and *A. flavus* (L strain) (f)

Different fungal isolates showed different levels of abundance (Figure 4.1.). 86 of the fungal colonies isolated were grouped as other fungi.This group consisted of all other fungal isolates other than species within the *Aspergillus* section *Flavi* group (*Aspergillus alliaceus*, *A. caeletus*, *A. parasiticus*, and *A. flavus* (S and L strains), *A. parasiticus*), *Rhizopus*, *Penicillium* and other *Aspergillus* species. The incidence of fungi categorized as other fungi was generally higher (86 colonies). The prevalence of *A. niger* and

Penicillium species was higher, 78 and 73, respectively than that of *A. flavus* (L strain) and *A. parasiticus*, 65 and 43 colonies respectively.

Fungi categorized as other *Aspergillus* species had the highest number of colonies (86) while *A. alliaceus* had the lowest number of colonies. *Aspergillus tamarii* and *A. caeletus* were not isolated from any of the soil samples analyzed.

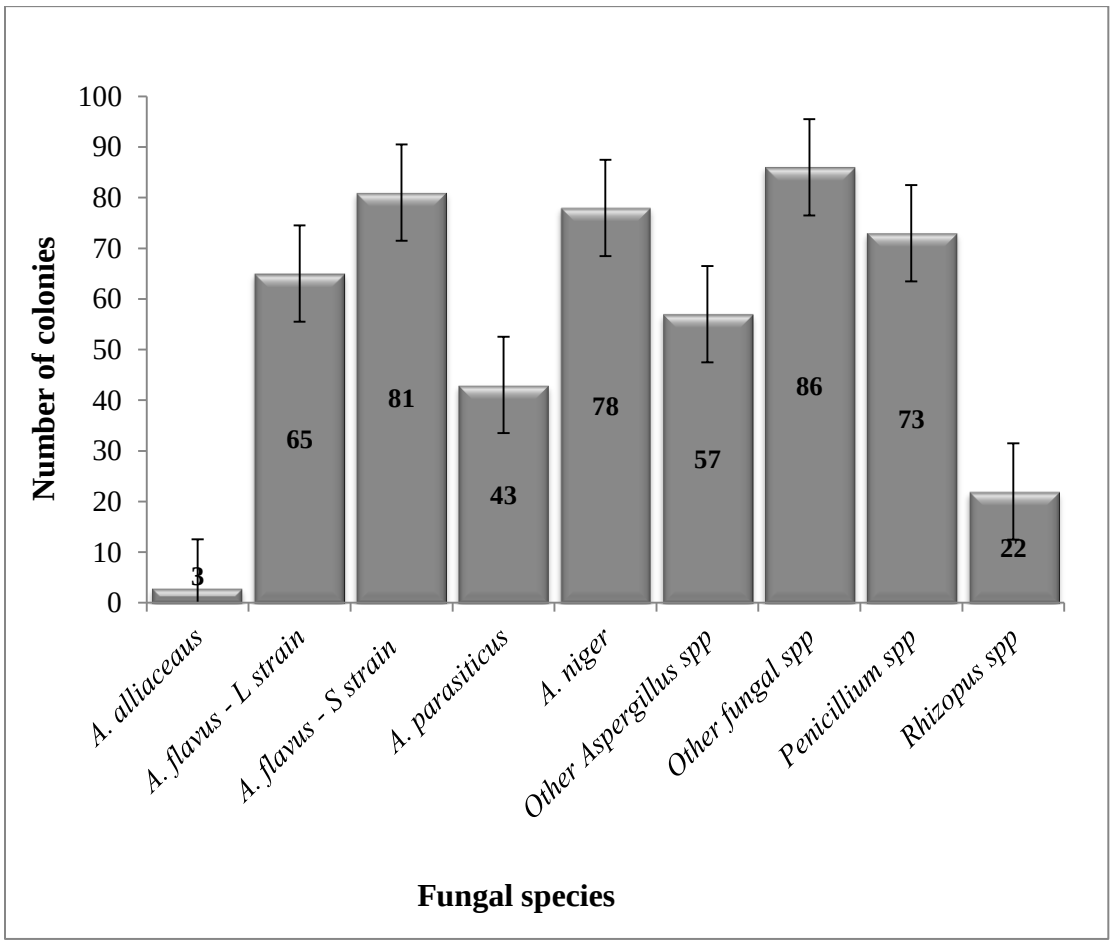

Figure 4.1: Occurrence and diversity of fungal species in soil samples collected from Kaiti in Makueni County - (Error bars represent the standard error of the mean).

The prevalence of *A. flavus* (S strain) was generally higher followed by that of (*A. flavus* L strain) and *A. parasiticus* respectively (Figure 4. 2) with percentage incidence of 43, 34 and 23 % respectively.

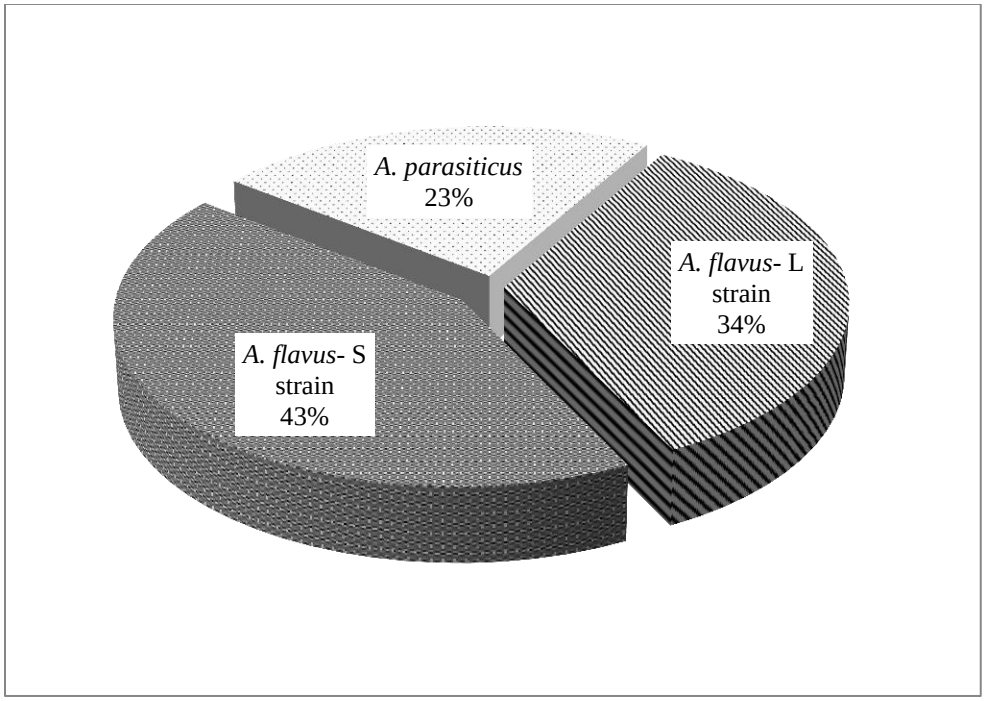

Figure 4.2: Prevalence of *A. flavus* (S and L strains) *and A. parasiticus in* soil collected from Kaiti in Makueni County.

A correlation was noted between soil type and fungal species distribution pattern in the different soil types in maize growing regions of Kaiti Location. The fungi showed diversity in the different sub-location due to soil types (Figure 4.3) and altitudes (Figure 4.4). There was a significant difference ($P=0.028$) and ($P<0.01$) in fungal species

population found in the different soil types and and altitudes respectively in Kaiti. Highest numbers of species in the *Aspergillus* section *Flavi* were recovered from clay sandy, loam, red loam and sandy loam in that order.

The general distribution of fungi in soil irrespective of the fungal species is shown in Figure 4.4. Black loam (mean= 31 CFU g^{-1} of soil) and sandy (mean= 17 CFU g^{-1} of soil) soils had the least number of fungal colonies with a total of below 50 species. The highest fungal population was in loam (mean= 213 CFU g^{-1} of soil) and red loam (mean= 222 CFU g^{-1} of soil) soils (Figure 4.3).

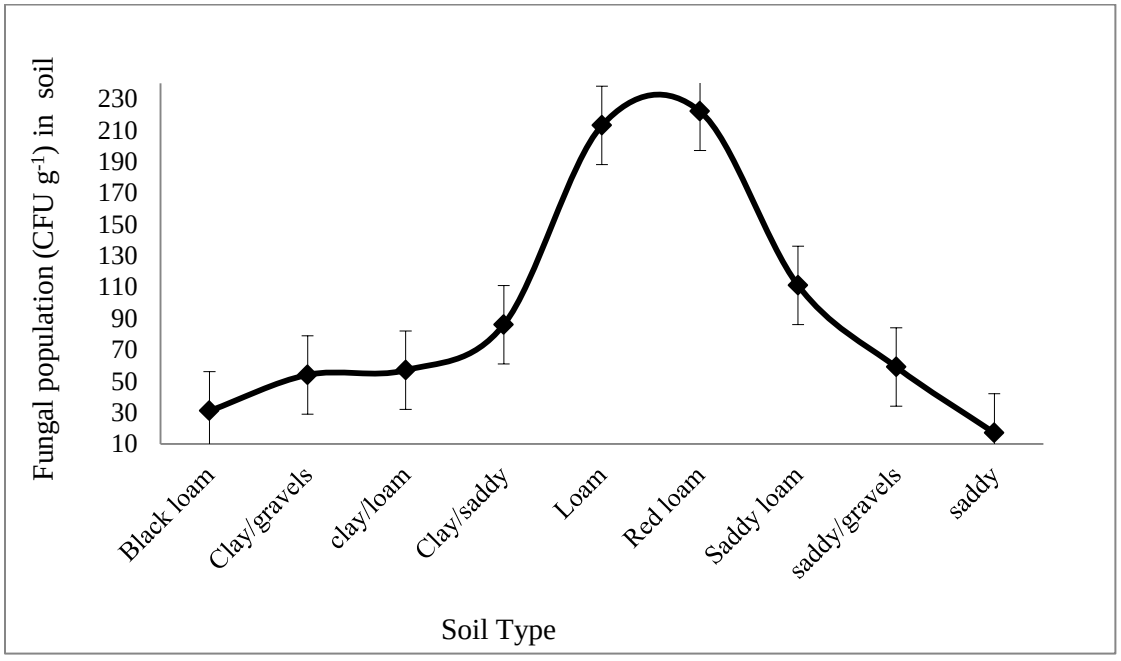

Figure 4.3: Distribution of *Aspergillus* section *Flavi* in different soil types. Error bars represent the standard error of the mean.

There was a significant difference ($P<0.01$) in populations of all fungal species in soil collected from different altitudes. Highest population (CFU g^{-1} soil) of *Penicillium* species and other *Aspergillus* species was in the altitude range of 1289-1438 m Above Sea Level (ASL). *Aspergillus niger* had the highest population in lower altitude range of 1138-1288 m asl whereas the population of *A. flavus* (S and L strains) and *A. parasiticus* did not seem to be affected by the altitude (Figure 4.4). A difference in the distribution of all fungal species in soil was not significant in altitudes beyond 1481 m asl. Other *Aspergillus* species and *Penicillium* species had their highest CFUs in altitudes range of 1291-1480 m asl.

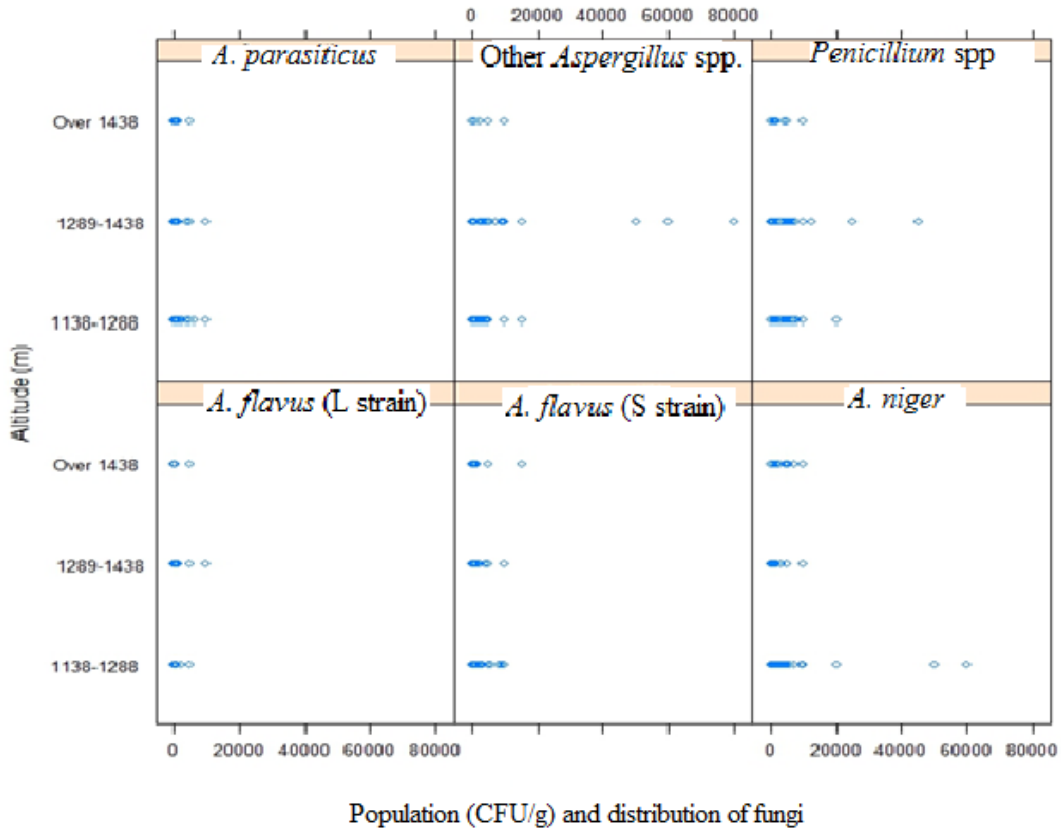

Figure 4.4: Fungal species population (CFU g^{-1} of soil) and distribution in soil in three altitude range, in Kaiti Location.

Ninety percent of the sampled farms and locations had either of the *Aspergillus* section *Flavi* species. Figure 4.5 shows the distribution of *A. flavus* (S and L) and *A. parasiticus* fungi in maize growing farms in Kaiti Location. The spots show distribution of ASF but do not quantify the population of the fungi present.

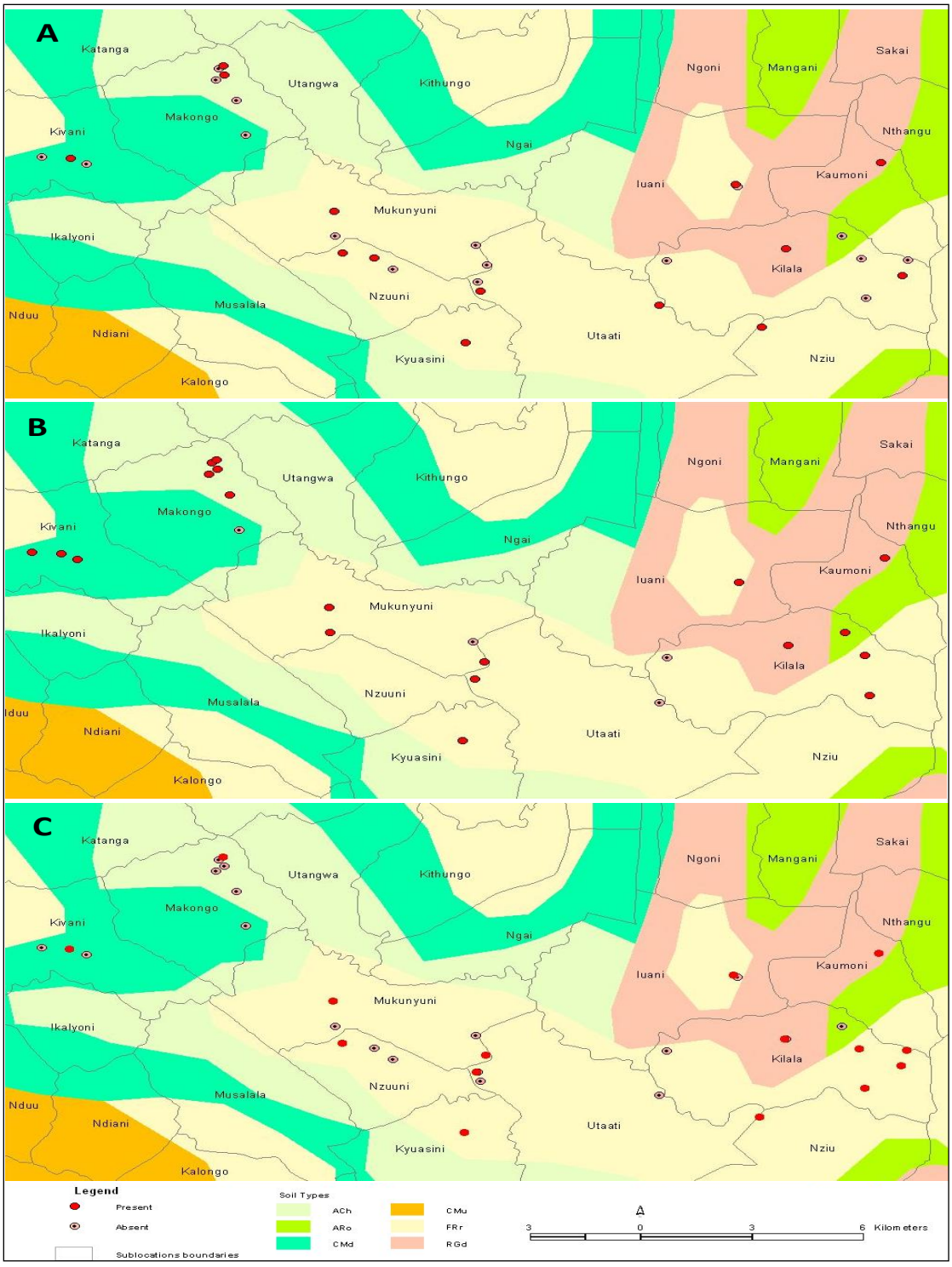

Figure 4.5: Distribution of *A. flavus* L strain (A), *A. flavus* S strain (B) and *A. parasiticus* (C) in different sub-locations in Kaiti, Kenya

The assayed soil samples contained fungal colonies with different toxin types. Fifty (50) percent of the soil samples had colonies suspected to be in the *Aspergillus* section *Flavi* group. Out of the 80 soil samples analyzed, 38 colonies were cultured on YES medium for production of aflatoxins. Only55 % of the cultured colonies was aflatoxigenic. Generally, the most common toxin type was AFB_1 with 50 % prevalence followed by AFB_2 (47 %). Both AfG_1 and AFG_2 had 45 % prevalence (Figure4.6). However, there was no significant relationship between soil types and Aflatoxin types. Fungal isolates from 29 % of the samples had all four toxin types (AFB_1, AFB_2, AFG_1 and AFG_2). Twenty six (26) % of the samples had both (AFB_1, and AFB_2).

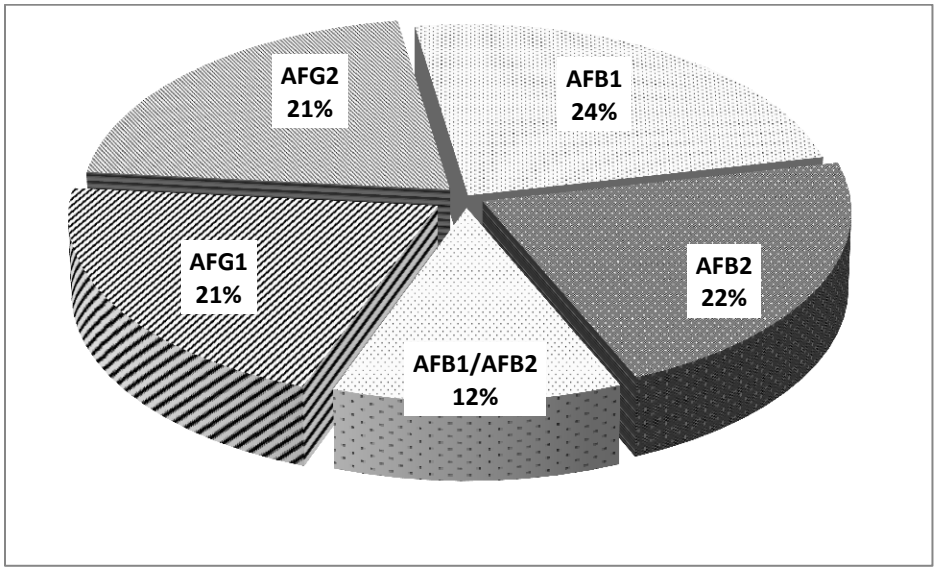

Figure 4.6: Percentage incidence of different aflatoxin types of *A. flavus* and *A. parasiticus* isolated from Kaiti soils

4.5 Discussion

Makueni County, one of the aflatoxin hot spots in Kenya is located in the south-eastern rangelands and has thirteen agro-ecological zones (Jaetzold *et al*., 1983). The area's mean temperature ranges from 18-24° C in the cold seasons and 24-33° C in the hot days. Temperatures as high as 32° C have been recorded during drought periods resulting to high evaporation (Jaetzold and Schmidt, 1982). Such shifts in weather patterns favor the proliferation of *A. flavus* and *A. parasiticus* fungi and subsequent production of aflatoxins. Aflatoxin contamination in particular is favored by environmental conditions of a region and has been found to be more in years with above average temperature and below average rainfall (Wilson and Abramson, 1992).

Presence of members of *Aspergillus* section *Flavi* in 90 % of the sampled farms in 11 sub-locations and high incidences of A. *flavus* (S strain) in the study area could explain causes of aflatoxicosis outbreaks in Eastern Kenya from as early as 1981(Moturi, 2008; Nangindu *et al*., 1982). Muthomi *et al*. (2012) related the higher levels of *A. flavus* in the region with the warmer temperature conditions (25 - 35^0 C) prevalent in Eastern Kenya. The results are also in agreement with Bock *et al*. (2004) who indicated that aflatoxigenic fungi are native to warm arid, semi-arid, and tropical regions and changes in climate result in large fluctuations in the quantity of the fungi. The presence of *A. flavus* in these soils is an indication that inoculum of this fungus is widespread in maize growing areas and in the environment of the semi-arid Eastern region of Kenya.

Different fungal species were present in the soil samples. It is likely that food contamination in Makueni could be due to a combination of more than one toxin type especially when the different fungi coexist in food (Speijers and Speijers, 2004). The study revealed that other fungal species such as *Penicillium*, *A. niger*, *Rhizopus* species and other *Aspergillus* spp were present in the analysed soil samples in higher populations than *A. parasiticus*. Some of these fungi are known to produce other types of toxins. *Aspergillus niger* for example has been shown by Wilson *et al*. (2002) to produce ochratoxins. Some *Penicillium* species are known to produce ochratoxins (Sweeny and Dobson, 1998), patulin (Spadaro *et al*., 2009; Welke *et al*., 2009) and citrinin (Singh *et al*., 2008). *A. alliaceus* is a rare fungus and had an incidence of about 20 % in the analysed samples. Similarly, Horn (2005) documented low incidences of *A. alliaceus*, and *A. tamarii* in the United States of America.

Aspergillus flavus is classified as storage fungi but this results show that *A. flavus* is present in soils of maize growing farms in Kaiti in Makueni. This confirms that crop infections usually start in the field before harvest as indicated by Wilson and Abraham (1992) and Morenoa *et al*. (2009). Infection further increases during harvest, drying, and storage (Wilson and Abramson, 1992). The results further indicated that *A. flavus* (S and L strains) and *A. parasiticus* had percentage incidence above 70 CFU g^{-1} of soil. . This result agrees with findings by Abbas *et al*. (2004a) who indicated that in any environment, the frequency of aflatoxigenic fungal isolates can range from 50 – 80 %.

Other research findings by Abbas *et al*. (2004b) indicated that factors including plant species present in an area, soil composition, cropping history, crop management, and environment conditions, including rainfall and temperature affect the relative distribution of aflatoxigenic versus non aflatoxigenic isolates in an environment. Aflatoxigenic strains of *A. flavus* and *A. parasiticus* have different toxigenic profiles. In the present study, $AFB_{1,}$ AFB_2, AFG_1, AFG_2 toxins profiles were detected in 55 % of the isolated colonies. These results agree with Abbas *et al*. (2004b) who reported that not all *A. flavus* and *A. parasiticus* isolates produce aflatoxins. Pitt, (1993) also indicated that *A. parasiticus* and *A. nomius* produce the four toxin types.

Spatial analysis of fungi in the ASF group (A. *flavus* S and L strains and *A. parasiticus*) assumed a patchy distribution in Kaiti soils and that highest incidence was in loam, red loam and sandy loam while the least were in black loam and sandy soils. These results are in agreement with Horn, (2003) that *A. flavus* has an aggregate or patchy spatial distribution pattern in the soil in a cultivated field. From spatial distribution maps, Jaime-Garcia and Cotty (2006) reported that on average; areas with high aflatoxin contain high percent clay and low percent sand. Simillarly in the current study, high incidences of *A. flavus* (S and L) strains and *A. parasiticus* were also found in some soil types that contained sand. The major soil property associated with maintaining soil populations of *A. flavus* is soil organic matter. Soils high in organic matter are considered suitable for agricultural production. These results indicate that the highest numbers of *Aspergillus* species were found in soils considered good for crop production. The result contradicts

with findings by Zablotowicz *et al*. (2007) who observed that higher populations of *A. flavus* are maintained in the soil surface of no-till compared to conventional-till soils.

A suitable and sustainable way of controlling mycotoxins would be by prevention of the initial contamination in the field and can be accomplished by reducing fungal infection in growing crops through adoption of suitable agricultural practices. These practices consist of planting transgenic plants resistant to fungal infection as well as crops capable of delaying toxin production. According to Bhatnagar (2010), host resistance is the widely explored and probably the best strategy for control of mycotoxins. Others strategies include proper fertilization, weed control, and necessary irrigation as well as crop rotation, cropping pattern, and use of bio pesticides as protective actions that reduce mycotoxin contamination of field crops.

4.6 Conclusion

The results of this study revealed high incidences of *A. flavus* (S and L) strains and *A. parasiticus* in addition to other fungal species like *A. niger* and *Penicillium* spp. This finding stresses the need of studying Kaiti soils to further understand the possible mycotoxin interactions in existence, in addition to recurrent aflatoxicosis outbreaks reported yearly. This information would be useful in understanding toxin interactions as a first step towards development of sustainable mycotoxin control strategies and prevention of future aflatoxicosis outbreaks in Eastern Kenya.

4.7 Recommendations

There is a need to carry out more studies in other locations in Makueni County to get a true picture of the distribution of members of *Aspergillus* section *Flavi* in the County Farmers need to be informed on the occurrence of *A. flavus* in soils of maize growing farms and the relationship of the fungi to maize contamination. They should also be trained on good agricultural practices to prevent crop contamination at production stage.

CHAPTER FIVE

OCCURRENCE AND DISTRIBUTION OF *ASPERGILLUS* SECTION *FLAVI* IN SOIL AND PRE-HARVEST MAIZE IN KAITI LOCATION OF MAKUENI COUNTY

Abstract

Maize consumption is one of the primary avenues through which human become exposed to aflatoxin contamination especially in developing countries. Makueni County is one of the aflatoxin hot spot**s** and there has been a dilemma on whether aflatoxin contamination of maize is storage or a soil problem. This study was therefore conducted to determine the occurrence and distribution of aflatoxigenic fungi (*Aspergillus* section *Flavi* ASF) in soil and pre-harvest maize samples collected from Kaiti Location in Makueni County and the relationship between soil contamination and pre-harvest maize grown in ASF infected soils.

Maize growing farms were identified and 80 soil and 96 pre-harvest maize samples collected. Isolation of ASF and other soil fungi and identification of aflatoxin types was done on Modified Dichloran Rose Bengal (MDRB) and Thin Layer Chromatography (TLC) technique. Data gathered showed a significant difference ($P<0.001$) in the population (colony forming units per gram of substrate-CFU g^{-1} of maize) of all fungi in maize in different years. There was a significant difference ($P<0.001$) in fungal species in the section *Flavi* group in different years (2009, mean= 1090 in 2010, mean= 5552 and in 2011, mean= 25664 of maize). *Aspergillus flavus* (S strain, mean=13421 $CFUg^{-1}$ of

maize) had the highest population and *A. flavus* (L strain- mean= 24256 CFU g^{-1} of maize) had the lowest. Generally, there was high rainfall variability in all the years (2009-2011) and mean annual rainfall was below the normal range of what is expected in the region per annum (between 250 mm and 500 mm). Fungal population in hybrid and local maize varieties was highly significant ($P<0.001$) in the three years but the population was lower in local (2010, mean= 3167 CFU g^{-1} of maize, and 2011= mean= 19775 CFU g^{-1} of maize) than in hybrid maize varieties (2010, mean = 6347 and 2011, mean = 30933 CFU g^{-1} of maize)

Conclusion drawn from the current study was that maize at post harvest stage of the maize value chain contained high population of fungi in the *Aspergillus* section *Flavi* group. The incidence of *A. flavus* (S and L strains) was high in both market and farmer stored maize and in the three seasons (2009-2011). This is a concern especially where incidences of ASF fungi is directly associated with aflatoxin contamination.This information is useful in determining appropriate measures in controlling ASF to prevent or minimize future aflatoxicosis outbreaks in Kenya. Farmers should be educated on good agricultural practices to enhance the communities' interventions to improve maize harvesting, drying and storage practices to reduce the likelihood of contamination with aflatoxigenic fungi and aflatoxicosis outbreaks in Kenya.

Key words: ***Aspergillus*** section *Flavi*, Inoculum, Soil, Maize value chain

5.1 Introduction

Maize is one of the world's widely cultivated cereal and ranks third in the world's production of cereals after wheat and rice (FAO, 2002). Majority of Kenyans are exposed to low and medium level doses of a wide spectrum of fungal poisoning through regular consumption of maize and its products (Lewis *et al.,* 2005). With climate change, weather patterns have changed resulting in extreme weather conditions such as prolonged drought or flooding in areas that were initially suitable for agriculture. Due to these changes, pest and diseases distribution have also changed. Climatic conditions such as dry weather followed by wet weather during harvesting have also been associated with fungal contamination of cereals before and after harvesting. For example, fungal analyses carried out after the 2004 aflatoxicosis outbreak in Kenya indicated that the outbreak was caused by *A. flavus* (S strain) (Probst *et al.*, 2007). This fungal strain produces more aflatoxin and was previously not found in Africa (Bennett and Klich, 2003). The Ministry of Agriculture (2007) indicated that samples collected from maize depots in at least 14 districts in Eastern and Coast Provinces contained aflotoxin levels beyond 10 ppb,, raising fears of a serious health risk.

In 2010, Kenya experienced wet weather during the harvesting period of the short rains crop planted in October/November 2009 mainly in Eastern, Central and Coast Provinces. As a result, about 2.3 million bags of maize were rendered not marketable hence unfit for human consumption. The government offered to buy the contaminated maize from the farmers at a cheaper price for disposal and to cushion the farmers from further loses.

Farmer practices such as dropping of dehusked cobs on the bare ground during harvesting increases the chances of aflatoxin contamination. In a survey conducted by the Ministry of Agriculture in 2007, over 90 % of small scale farmers in Eastern Province were reported to drop maize cobs on the ground during harvesting while 75 % of farmers dried maize cobs on bare ground thus increasing incidences of aflatoxin contamination. The study aimed at determining the occurrence of *Aspergillus* section *Flavi* at the production (soil) and pre-harvest stages of the maize value chain in Kaiti Location in Makueni County. Population of ASF in soil, pre- harvest maize and different seasons was compared to determine the effect of soil contamination and season on maize contamination at pre- harvest stage.

5.2 Materials and Methods

5.2.1 Site selection, soil sample collection and analysis

The study site was Kaiti Location in Makueni County as described in chapter three sections 3.1.

5.2.2 Collection and preparation of soil and pre-harvest maize samples.

In June 2011, 96 pre-harvest maize samples were collected from Kaiti for three consecutive years from 2009-2011. In 2011, farms were revisited for soil collection. Using a GPS and a list of farmers, farms where pre-harvest maize samples were collected were located. In the farms, five sample points were identified and 80 soil samples

collected as described in section 4.2.1. Each sample was a composite of approximately one kg of top soil. Pre-harvest (about a week before harvest) maize samples were drawn from five sample stations, each measuring 5 rows by 5 m long, identified in each plot. Five cobs were collected from five randomly selected maize plants at each sampling station, four at the corners and one in the middle of the plot as described by Mahuku *et al.*, (2010).

To compare seasons (years), all the 96 pre-harvest maize samples were selected for analysis. For comparison of fungal populations in soil and pre-harvest maize samples, soil and pre- harvest maize samples from 33 farms were compared.

Twenty five maize cobs were collected from each farmer field, hand-shelled, and thoroughly mixed to form a composite sample. This was labeled and considered as one sample. Pre-harvest maize samples from the sampled farms were sun-dried to 13 % moisture content then milled separately (one composite sample per farmer) using a commercial mill avaialbe in the villages. A one kg sub-sample was drawn from the milled sample and analyzed for moisture content as described in section 3.2. The samples were then placed in polypropylene bags, labelled and transported to CABI-laboratories in Nairobi for cold storage at 4^0 C in a cold room, until analysis was done. In the laboratory, soil was air dried and hammered into a fine powder before weighing one gram for microbial analysis.

5.3 Determination of the population of *Aspergillus* section *Flavi* group in soil and pre-harvest maize samples

A semi-selective medium, Modified Dichloran Rose Bengal medium was prepared as described in section 3.4. From the pre-harvest maize samples, a one kg composite sample was drawn from the milled mixture and shaken vigorously to ensure proper mixing. Two sub-samples each weighing 2.5 g were drawn from the composite sample and put in a 15 ml graduated tube then diluted with two percent water agar (prepared by adding 2 g agar in 100 ml distilled water and sterilizing for 20 minutes at 15 P.S.I) to make a 10 ml stock solution. The procedure was repeated with soil samples but 1 g of soil was diluted in 9 ml of the diluent. The stock solution was vortex mixed thoroughly then serial diluted by transferring 1 ml of the stock to 9 ml of the diluent. The diluted samples were placed in a rack in a water bath at 40^0 C until plated.

Fungal isolates were recovered by dilution plate technique on MDRB (Cotty, 1994). Using a micro applicator, a 200 µl aliquot of the diluted samples (soil and maize) were transferred aseptically onto the semi selective medium in the 90 mm diameter Petri dishes and spread on the surface of the culture medium using a sterile glass rod. Six replicates were plated for each sample.

The inoculated plates (0.2 ml/plate) were incubated at 30^0 C for 3-7 days without sealing. The plates were removed from the incubator and visually observed for microbial growth. Lines were drawn on the under-side of the culture plates for ease of counting the colonies. *Aspergillus* species were identified as described by Horn and Dorner (1998). A

stereomicroscope was used to identify fungi in the section *Flavi* group directly from the dilution plates in plates that were heavily colonized by other filamentous fungi. Other plates were macroscopically observed for microbial growth.

Morphological characteristics (colour, texture, size of colony) were used to separate the fungi into groups. Reference cultures (Figure 3.2) were used to aid in identification. *Aspergillus* species and total filamentous fungi were enumerated using a tally counter as described in section 4.2.2.

5.4. Screening *A. flavus* and *A. parasiticus* isolates for aflatoxin production and types

Thin layer chromatography (TLC) plates (A Silica gel 60; EMD Chemicals Inc., Darmstadt, Germany) were used to determine and confirm presence of different aflatoxins in the cultures isolated from pre-harvest and soil samples. *Aspergillus flavus* and *A. parasiticus* colonies were grown in a liquid Sucrose Yeast Extract (YES) broth prepared, dispensed and inoculated as described in section 3.5. The spotted plates were placed in a glass container (5 cm x 3 cm) containing a developer solvent made by mixing distilled water, acetone and chloroform, in a ratio of 1.5: 12: 88 respectively, until the solvent covered 80 % of the TLC plate length. The plates were allowed to air dry before viewing under the UV light in a dark room. The bands were compared with the standard aflatoxins and scored based on presence or absence of specific aflatoxin types.

5.5 Statistical analysis

Data collected was first keyed into Microsoft excel spreadsheets and subsequently analysed using GenStat 13 and 14 th edition (SP2) statistical packages (VSN International (2011). The data were subjected to analysis of variance (ANOVA) to test if the resulting frequency distributions were similar for the two samples (soil and pre-harvest maize samples). R Statistical Package (2012): was also used for statistical analysis, simulation modeling and advanced data analysis. R was used to display means and confidence intervals (C.I) procedure using the bargraph within the sciplot package. Confidence intervals were set to 1.96 standard errors (translates to $P \leq 005$). Further, associations between specific fungal incidences were investigated based on analysis of contingency tables with appropriate chi-squared tests using different variables, including season, maize variety and soil type and altitudes of the areas where the samples were collected from. The data were subjected to Kolmogorov-Smirnoff and the Mann-Whitney U two samples tests to assess whether soil population was larger than that of maize. Tukey's honestly significant difference (HSD) test was performed to compare treatment means at the 5 % level.

5.6 Results

5.6.1 *Aspergillus* section *Flavi* in pre-harvest maize and soil samples

Data gathered from this study showed that, *Aspergillus flavus* S strain had a higher incidence in pre-harvest maize collected in July and February 2010 than in 2011. There were varying incidences of different ASF fungi in the different seasons but generally

A.flavus S strain was higher in 2010 and 2011 maize samples. *Aspergillus* section *Flavi* was also present and the population (CFU g^{-1}) in soil ranged from 2,000 to $\leq$ 5,000 colonies per gram of soil. The population of ASF (CFU g^{-1} of maize) in the different years was significantly variable ($P<0.001$) irrespective of the fungal species (Figure 5.1). The lowest population of ASF in pre-harvest maize was in 2009 (mean= 1090 CFU g^{-1} of maize) followed by 2010 (mean= 5552 CFU g^{-1} of maize) then 2011 (mean= 25664 CFU g^{-1} of maize).

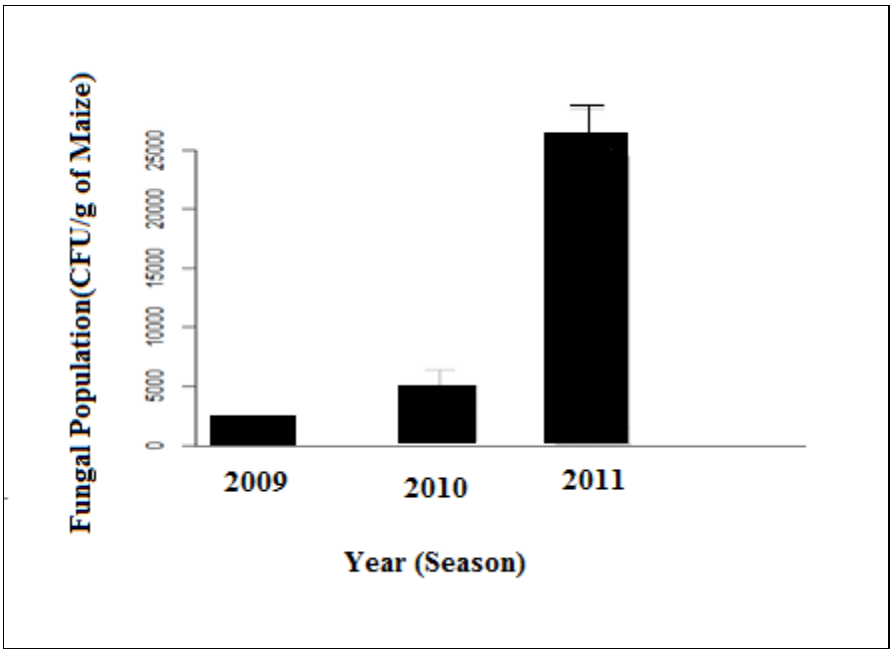

Figure 5.1a: Fungal population (CFU g^{-1} of maize) in pre-harvest maize in different seasons (2009, 2010 and 2011) Error bars are confidence intervals set to 1.96 Standard Errors.

There was a significant difference (P<0.001) in the population of *A. flavus* S and Lstrains and *A. parasiticus* in 2009, 2010 and 2011 (Table 5.1). *Aspergillus flavus* S strain had the highest population, followed by *A. flavus* L strain and *A. parasiticus* as shown below in Table 5.1. Generally, there was a highly significant (P<0.001) interaction between season and fungal species in the pre-harvest maize samples and the population of fungi in *Aspergillus* section *Flavi* was not detectable in 2009. The population of other *Aspergillus* species was significantly higher (P<0.01) in maize samples than that of *A. flavus* L strain and *A. parasiticus* in year 2010.

Table 5.1: Population of *Aspergillus spp* in pre- harvest maize samples in three different seasons

Fungal species	**Year**		
	2009	**2010**	**2011**
A. flavus S train	0[a]	7134 [c]	55885 [c]
A. flavus L strain	0[a]	880[b]	32570 [b]
A. parasiticus	0[a]	167 [a]	3422 [a]
Other *Aspergillus* species	958[b]	10771 [c]	5781 [a]

Means that do not share subscripts (within the years) differ by p <0 .05 according to Tukey's Honestly Significant Difference.

In comparison of fungal population in soil and pre- harvest maize samples, fungal population in maize samples collected in 2011 season was higher than that of soil in the same year (Figure 5.1b). Kolmogorov-Smirnoff (K-S) and the Mann-Whitney U two samples tests showed that distribution in maize and soil were significantly different (K-S $P = 0.2344$; Man-Whitney U-test: $P = 0.001$).

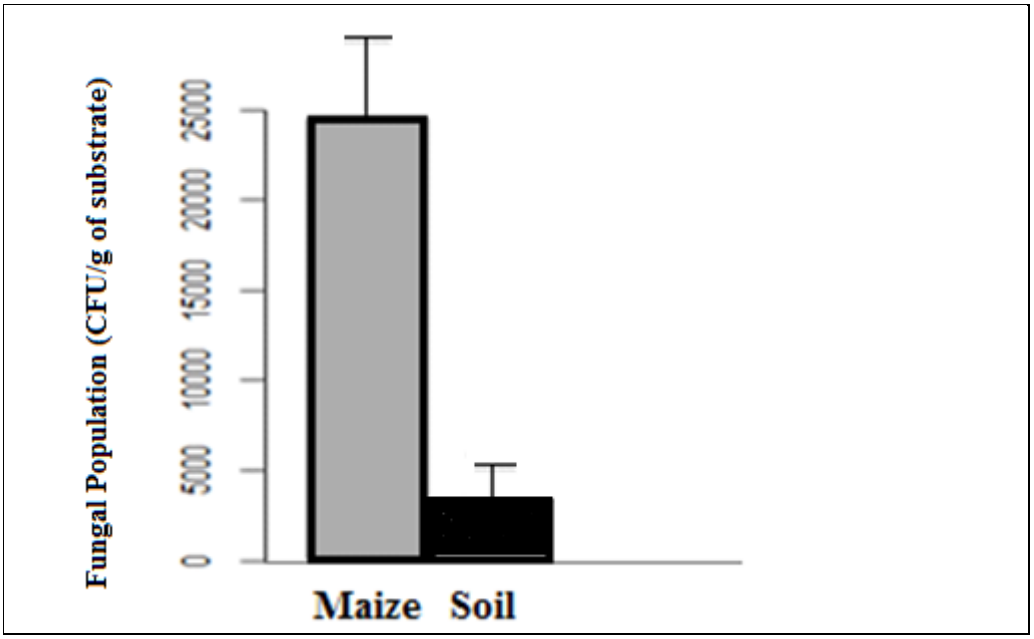

Figure 5.1b: Fungal population (CFU g^{-1}) in soil and pre-harvest maize collected. Error bars are confidence intervals set to 1.96 Standard Errors.

Rainfall data gathered from KARI agro- meteological department revealed that the mean annual rainfall in Makueni County was highly variable and was below the normal range

of what was expected in the region per annum (between 250 mm and 500 mm) in normal seasons. The rainfall data showed high variability in the different years in terms of amount and distribution pattern (Table 5.2). Year 2007/2008 had an annual mean rainfall of 32.44 mm followed 41.33 mm in 2009, 59.75 mm in 2010 and 502 mm in 2011. The highest amount of rainfall was experienced in year 2011 and in the months of March and November/December in all years except in March 2009 which had the least amount (1.1 mm) and December 2007/2008 which had 58 mm. The first eight months of 2012 had a mean annual rainfall of 19.8 mm.

Table: 5. 2: Rainfall distribution in Makueni (2008-2011)

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	(mm)
2008	57	7.3	222	13	1	0	0	0	0	1.2	82	5.2	32.44
2009	29.3	5.5	1.1	38.2	13.1	0	0.5	1	0.2	57	189	161.1	41.33
2010	78	83	93	14	25	0	0	3	2	11	154	254	59.77
2011	9	22	124	3	0	0.2	0.4	0	0	75	149	119	501.6

5.6.2 Population of *Aspergillus* section *Flavi* in different maize varieties at pre-harvest stage

There was a significant ($P<0.001$) difference in fungal population in local and hybrid maize varieties and an even greater significant ($P=0$) differences in fungal population in maize varieties in the different years with means of 6347 in 2010 and 30933 CFU g^{-1} of maize in 2011 for hybrid maize varieties. Local maize varieties had the lowest fungal population (mean = 3167 in 2010 and 19775 CFU $g^{-1}$of maize in 2011) (Figure 5.2a). However, in year 2009 hybrid varieties had a significantly lower ($P<0.001$) fungal population than local varieties in the same year while the highest fungal population in both local and hybrid varieties was in 2011. Fungal population in 2009 in both hybrid and local varieties was below 5000 colonies per gram of maize (Figure 5.2a).

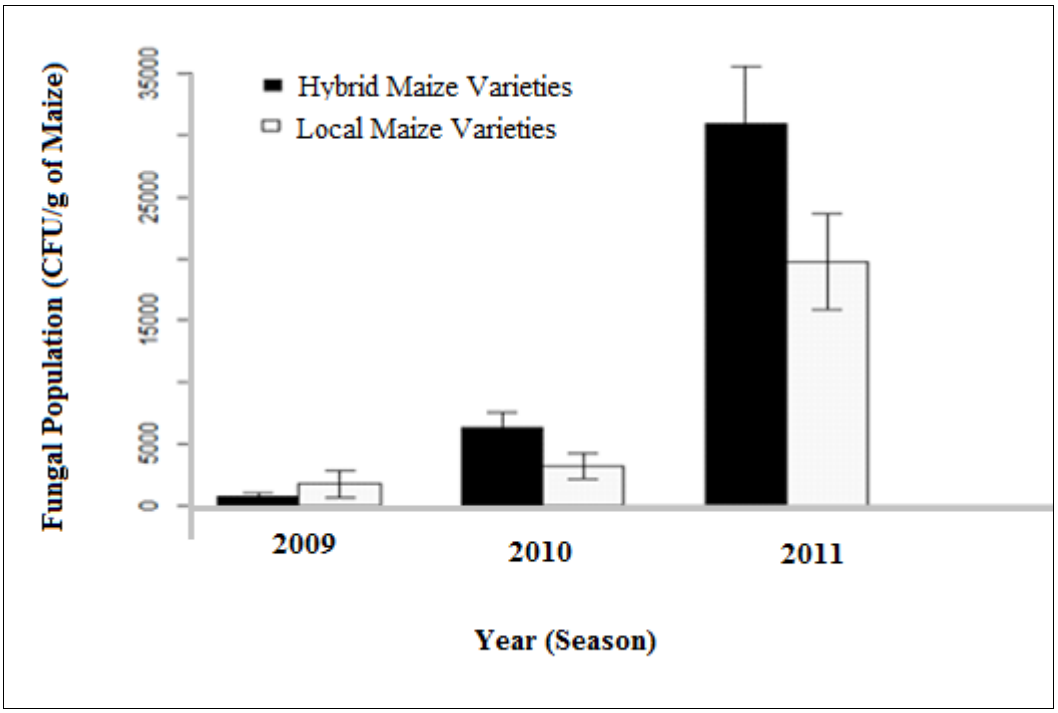

Figure 5.2a: Fungal Population (CFU g^{-1} of maize) in hybrid and local maize varieties in three seasons (years). Error bars are confidence intervals set to 1.96 Standard Errors

Further analysis showed that hybrid maize varieties grown in the study area had higher population of ASF species compared to local varieties grown in the region (Figure 5.2b). *Aspergillus flavus* S strain had a higher population (CFU g^{-1}) than *A. flavus* L strain and *A. parasiticus* in both hybrid and local maize varieties. The population of S strain was significantly higher ($P<0.001$) in hybrid than in local maize varieties. The population of *A. flavus* L strain and *A. parasiticus* were not statistically different in both local and hybrid maize varieties (Figure 5.2b).

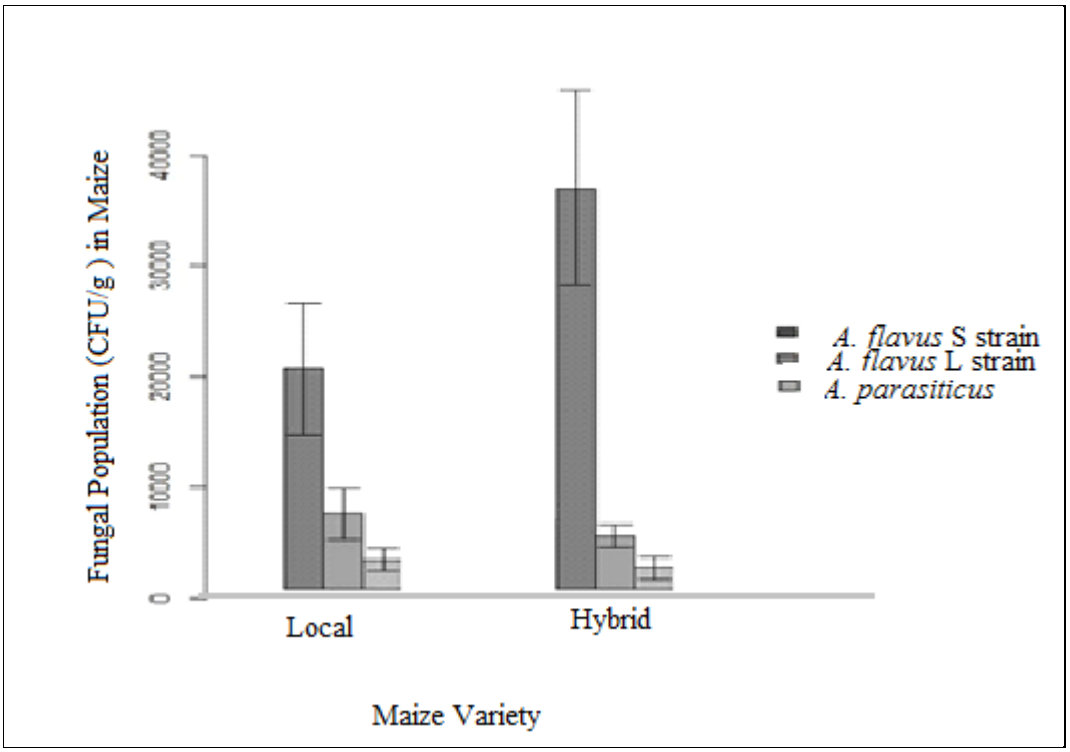

Figure 5.2b: Population (CFU g^{-1}) of *A. flavus* (S and Lstrains) and *A. parasiticus* in local and hybrid maize varieties. Error bars are confidence intervals set to 1.96 Standard Errors.

Further analysis revealed a statistical difference ($P<0.001$) in populations of *Aspergillus* section *Flavi* fungi in soil and maize in different altitudes (Figure 5.2c). Generally, altitude had an effect on maize variety with the highest fungal population recorded in hybrid maize grown in altitude above 1500 m asl (Figure 5.2c). There were no statistical differences between local and hybrid maize varieties in altitudes below 1500 m asl but the differences were very significant ($P<0.001$) in maize grown in altitudes above 1550 m

asl. The population of all fungal species in soil was not statisticall different in altitudes beyond 1400 m asl (Figure 4.3).

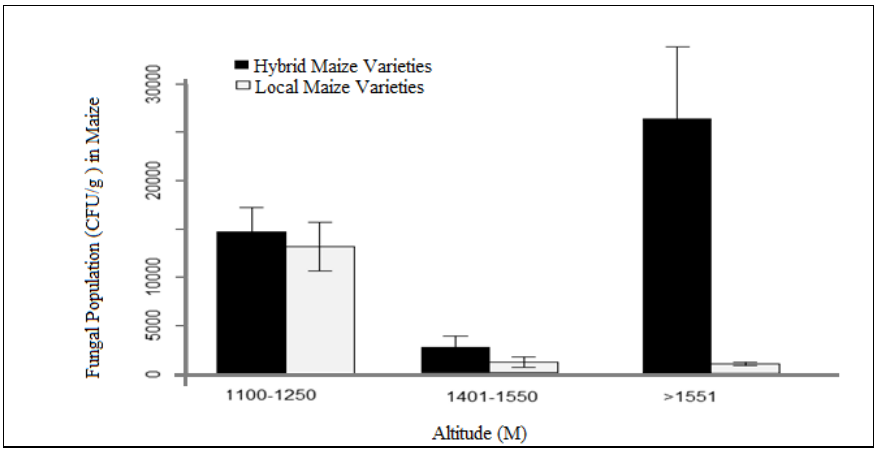

Figure 5.2c: Fungal population in hybrid and local maize varieties in different altitudes. Error bars are confidence intervals set to 1.96 Standard Errors

5.6.3 Incidence of aflatoxin types in soil and pre-harvest maize samples

Fungal isolates isolated from maize and soil samples analysed showed that the samples had all the four aflatoxin types, AFB_1, AFB_2, AFG_1 and AFG_2 but the percentage incidences differed between maize and soil samples fungal isolates (Figure 5.3). Generally, AFB_1 and AFB_2 had higher incidences of 63 and 59 respectively in fungal isolates from pre-harvest maize compared to 50 (AFB_1) and 47 (AFB_1) in soil samples. The incidences of the four toxin types were not statistically different in fungal isolates

from soil samples (Figure 5.3). Generally, incidences of AFB_1, AFB_2 were significantly different ($P<0.001$) from those of AFG_1 and AFG_2 in pre-harvest maize samples. Fungal isolates had aflatoxin types AFG_1 and AFG_2 but the incidence was lower in both maize and soil samples but were lower in soil isolates than in maize ones.

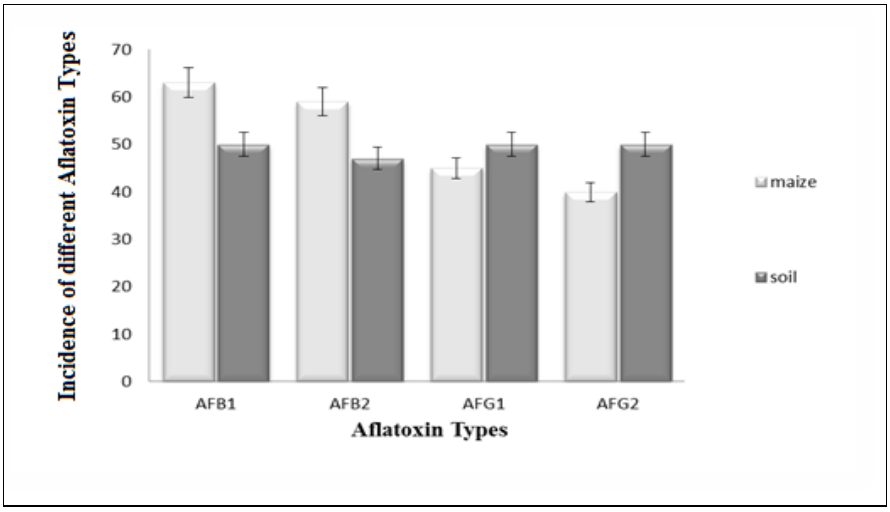

Figure 5.3: Incidence of different aflatoxin types in isolates of *A. flavus* and *A. parasiticus* isolated from soil and pre-harvest maize. Error bars set at 5 % confidence interval.

5.7 Discussion

Results from this study indicated that *A. flavus* is present in both soil and pre-harvest maize samples hence risk of maize contamination when conditons are suitable for fungal growth. This in conformity with findings by Abbas *et al.* (2004a) and Zablotowicz *et al.* (2007) who indicated that soil populations of *A. flavus* in soils under maize cultivation can range from 200 to >300,000 colony forming units. They also agree with findings of Saito *et al.* (1989) and Okazaki *et al*. (1992) who suggested that *A. flavus* persists as a saprophyte in the soil.

Presence of *A. flavus* (S and L strains) and *A. parasiticus* in soil and maize samples in is an indication that initial soil contamination is present in the area. This is a challenge and

may probably explain yearly aflatoxicosis outbreaks in Makueni County. Presence of the fungi may also act as the source of primary inoculum but the extent of contamination in the maize value chain may depend on pre and post-harvest practices. Probst *et al.* (2007) reported that the cause of aflatoxicosis in Makueni in 2004 was due to presence of *A. flavus* S strain in home grown maize. This fungal species that produces more aflatoxin was previously not found in Africa (Cotty and Cardwell, 1999; Bennett and Klich, 2003).

The study also showed that Makueni rainfall data for 2007/2008-2011 had significant rain variability probably due to climate change. Annual rainfall received in the region in the four years was below what was expected in the region (250 – 500 mm) per annum (Ogallo, 1994). Year 2009 experienced unusual variations with dry spells interspersed with off-season rains in the months of January and February. The poorly distributed rains in October – December 2008 caused by delayed onset and low amounts resulted to crop failure in 2009 season resulting in famine. Research findings have revealed that drought stress, a feature that commonly occurs in most maize growing areas in Kenya and most of sub-Saharan Africa, favors *A. flavus* growth, enabling it to have modest advantage over other grain infesting fungi (Nyangito and Ndirangu, 1997; Udoh, 1997). Similarly, high mean rainfall in 2010 following a dry year in 2009 provided suitable condition for growth and survival of soil micro-organisms hence high fungal population in pre-harvest maize in 2010 and 2011 seasons.

However, our study did not establish why year 2009 had the lowest incidence of the fungi despite having a higher rainfall in 2007/2008 season. This shows that climatic conditions

are not the only determinants of aflatoxin contamination in maize at pre-harvest level. Other *Aspergillus* species were present in the three seasons. This could be because they are native in the soil, have adapted to the regions climatic conditions and are not necessarily affected by drastic weather changes. Year 2009 was also food insecure and resulted in provision of relief food by the government.

To increase maize production, the government, public and private institutions such as CIMMYT, KARI and IITA are currently working together and investing considerable resources in developing and promoting maize varieties that are suitable for the marginal areas in Kenya. Some of the varieties are used as an alternative to the landraces and hybrid varieties (Pixley and Bänziger, 2004; Pixley, 2006), that have been perceived as costly by the small scale maize farmers in the rural areas. Hybrid varieties may offer a solution to food insecurity as they are early maturing, drought and disease resistance to an extent but results obtained from this study shows that they are not resistant to *Aspergillus* contamination.

Munkvold (2003) reported that cultivated hybrids frequently have serious fungal infection. This conforms with the results of this study that showed that local varieties had lower ASF population (CFU g^{-1} maize) at pre- harvest stage, compared to hybrids varieties grown in the area. This shows that unless suitable hybrid varieties with resistance to infection by aflatoxigenic fungi are developed, the ones promoted in the study area only improves food security through increased yield but not food safety.

The current study showed that species density and diversity had a certain distribution patterns in Kaiti region, but the current study did not look at all the other influencing factors. The study revealed that the highest population of members of ASF was in lower attidudes.this result is in agreement with findings by Klich *et al.* (1992) that majority of *Aspergillus* species from section *Flavi* group appear to be most abundant in lower altitudes and their density decrease with increase in latitude. This is probably due to differences in soil types. Generally fungi in the section *Flavi* group in the sampled farms shows an aggregated form of distribution for *A. flavus* and *A. parasiticus* rather than random distribution in soil.

All the four toxin types (AFB_1, AFB_2, AFG_I and AFG_2) were detected in cultures isolated from soil and pre-harvest maize samples. Based on the profiled toxins, it can be assumed that *A. parasiticus* and/or *A. flavus* were present in both maize and soil samples but the prevalence of *A. flavus* was significantly higher than that of *A. parasiticus*.

5.8 Conclusions

Aflatoxigenic fungi were isolated from soil and pre-harvest maize samples collected from Kaiti Location. This is an indication that the fungi are present in the production and pre-harvest stages of the maize value chain but probably the magnitude of aflatoxin contamination may depend on the farmer's knowledge on aflatoxin management practices. Maize variety, season and altitude were found to have an effect on the incidence and diversity of members of *Aspergillus* section *Flavi* in Makueni County. This

information is necessary to farmers for advice on suitable agronomical and cultural practices that would reduce aflatoxin contamination at the production stage of the maize value chain.

5.9 Recommendations

More studies on development of resistant varieties should be done. There is a need to repeat this kind of study for more than three seasons to come up with the true picture of the relationship between soil health and contamination of products grown in infected soils.

Farmers should be educated on good agricultural practices to enhance the communities' interventions to improve maize harvesting, drying and storage practices to reduce the likelihood of contamination with aflatoxigenic fungi.

CHAPTER SIX

SEASONAL CHANGES IN POPULATION OF *ASPERGILLUS* SECTION *FLAVI* IN POST-HARVEST MAIZE COLLECTED IN 2009-2011 IN KAITI LOCATION OF MAKUENI COUNTY

Abstract

Contamination of maize by mycotoxigenic fungi is a serious problem in Kenya and particularly in Makueni County where yearly aflatoxicosis outbreaks have been reported for the last three decades. Aflatoxins are potent carcinogenic secondary metabolites produced by some fungi in the *Aspergillus* section *Flavi* (ASF) group on important food crops, including cereals and oil seeds. *Aspergillus flavus* and *Aspergillus parasiticus* have been associated with the risk of aflatoxin contamination at post-harvest stage of the maize value chain. The current study involved 190 farmers and 155 traders in kaiti Location for three seasons (2009-2011). Duration of storage, source and maize variety were some of the criterion used to select 345 maize samples. In the laboratory, plating and subsequent isolation of ASF was done on Modified Dichloran Rose Bengal agar. Thin Layer Chromatography was used for determination of toxin types. Data gathered showed that the highest fungal population of ASF in maize was in 2011 (mean= 16045 CFU g^{-1} of maize) and 2010 (mean =11431 CFU g^{-1} of maize) and the lowest was in 2009 (mean 1312 CFU g^{-1}). The population of *A. flavus* S strain was significantly higher ($P<0.001$) in market samples and six out of 10 ten markets had samples with a mean incidence above 5000 colony forming units per gram of maize. Highest population of ASF was in

Itumbule (mean= 12900 CFU g^{-1} of maize) market and a farmer store in Ukia (mean= 8180 CFU g^{-1} of maize). The lowest was in Itooni market (mean=128 CFU g^{-1} of maize) and a farmer store in Nzuuni (mean= 299 CFU g^{-1} of maize). 100 % of the farmers used traditional maize storage methods.

Samples drawn from maize hanged on roof (mean= 65 CFU g^{-1} of maize) and drying on mats (mean = 1200 CFU g^{-1} of maize) and those in storage for 15-25 days (mean= 1100 CFU g^{-1} of maize) had a significantly ($P<0.001$) lower incidence of all fungal species. The fungal populations in farmer stores increased to a mean of 11000 CFU g^{-1} of maize at 330 days in storage. Overall, results from farmer and market samples showed that moisture content, storage type and duration of storage were the most important factors contributing to occurence of aflatoxigenic fungi at post-harvest stage. This study showed that *.A. flavus* was present in thee seasons, in farmer and market stores. The population of the fungi was not statistically different in local and hybrid maize varieties in the three seasons. The incidence of members of ASF was dependent on post-harvest handling practices and the type of storage method employed by farmers.

Key words: Aflatoxin, Post-harvest, Aflatoxigenic, Colony forming units

6.1 Introduction

Aspergillus, *Fusarium* and *Penicillium* species are some of the major fungal genera associated with mycotoxin grain contamination. Of all mycotoxins, aflatoxins probably cause the most concern (CAST, 2003). The toxins have carcinogenic, immunesuppressive (Cardwell and Henry 2004; Gong *et al.,* 2004; Williams *et al.,* 2004; Farombi, 2006),

stunted growth (Gong *et al.,* 2004; Abbas, 2005) effects in both humans and domestic animals (Turner *et al.,* 2003). Aflatoxin contamination is caused by fungi that belong to *Aspergillus* section *Flavi* (Cotty *et al.,* 1994) and the species most frequently implicated with food contamination along the value chain are *A. flavus* and *A. parasiticus* (Cotty *et al*., 1994).

Contamination of food with the toxin is a widespread problem across Africa (Bankole and Adebanjo, 2003; Bankole *et al*., 2006; Wagacha and Muthomi, 2008) where maize consumption is suspected to be the primary avenue through which humans become exposed to aflatoxins (Egal *et al*., 2005; Shephard, 2008). Intake of low, daily doses of the aflatoxins over long periods may result in chronic aflatoxicosis expressed as impaired food conversion and reduced life expectancy (Gong *et al.,* 2004; Williams *et al.,* 2004). Aflatoxins also cause economic losses and serious food safety problems worldwide (Strosnider *et al.,* 2006; Williams *et al.,* 2004) hence regulatory limits on the quantity of aflatoxins permitted in food and feed exist in several countries (FAO, 2006).

Food and Agriculture Organization (FAO) estimates that 25 % of the world food crops are affected by mycotoxins each year and constitute huge annual losses at post-harvest stage (FAO 1997; Cardwell *et al*., 2004). Aflatoxin contamination can occur at any stage, in the field or during harvest, drying, and storage and even in food and animal feeds (Salunkhe *et al*., 1992). However, contamination is more likely to occur at the post-harvest stage if the produce is not handled properly to ensure fungal growth is minimised (Yadgiri *et al.,* 1970; Wilson and Abramson, 1992). Another important post-harvest

factor affecting aflatoxin contamination is storage type. Fungi on grains previously attacked in the field can develop rapidly during storage when conditions are suitable for its establishment and mycotoxins production (Turner *et al*., 2005). Jolly *et al*. (2009) also indicated that post-harvest losses of crops are greater than the improvements made in primary production. Lack of appropriate storage facilities and poor storage conditions in Kenya‘s aflatoxin prone areas have contributed to great food losses. The average volume of storage losses reported by small scale farmers is less than 1% (Stephens and Barrett, 2011) but according to a report by Bett and Nguyo (2007) storage loses can amount to 5-10 % of the total loss.

Maize trade is another stage in the maize value chain where aflatoxin contamination occurs. In Kenya, maize is typically sold by small farmers immediately after harvest, saving few bags for their own consumption. Lewis *et al.* (2005) assessed maize in markets in Eastern Kenya and found out that it represented a significant source of exposure to aflatoxin, long after contaminated household stores were consumed or discarded. Commercially marketed maize in Kenya have been found to exhibit contamination levels up to twice the current regulatory limit of 10 parts per billion (ppb) (Muriuki and Siboe, 1995). Much higher levels of contamination have been detected in maize sold in informal markets. In 2004, a year in which 317 hospitalizations and 125 deaths due to acute aflatoxin poisoning were recorded in Kenya, 55 % of samples purchased from informal markets in Eastern Province contained aflatoxin levels greater than 20 ppb aflatoxin, 35 % contained more than 100 ppb, and 7 % had levels exceeding

1000 ppb (Lewis *et al.,* 2005). Maize from the affected area contained aflatoxin levels above 4,400 ng/g aflatoxin B1, which is 440 times greater than the 10 ng/g tolerance level set by the Kenya Bureau of Standards (CDC, 2004). Given that maize is the primary staple grain for Kenyans (Muriuki and Siboe, 1995), even relatively low levels of exposure may have significant health effects (Shephard, 2008). The current study therefore sought to assess occurrence and distributions of species and strains within *Aspergillus* section *Flavi* in market and farmer stores maize samples collected for three seasons across different agroecological zones where most maize is produced in Kaiti region of Makueni County.

6.2 Materials and methods

6.2.1 Site selection and sample collection

The study was conducted from January to May 2012. Farms were selected as described in chapter three sections 3.1. Locating the site was done using a Global Positioning System (GPS) for ease of identifying the sites in subsequent visits.

6.2.2 Post-harvest sampling for stored maize grain

6.2.2.1 Sampling from farmer stores

Samples were drawn from maize samples collected in 2009 to 2011 seasons. Samples were initially collected between 15 and 25 days post-harvest, and then monthly for that in storage for 45-330 days or until the grains were depleted. The samples were collected

from 90 kg bags of maize. Multiple probing was done and five sub-samples were taken from different parts of one bag or several bags belonging to one vendor or farmer and combined to produce a 1 kg sample for analysis.The maize samples were collected using the farmer or vendor's sampling tools such as spikes and scoops. The sub- samples were mixed to form a composite sample that was milled using a commercial mill and 1 kg sample drawn from the mixture. Moisture content of the milled samples was determined before the samples were transported to CABI laboratories in Nairobi for storage at 4^0 C in a cold room awaiting aflatoxin analysis. A total of 190 samples representing the three seasons (year 2009-2011), local and hybrid maize varieties, different storage methods, sources of grain and different days in storage were picked for analysis. Maize samples were grouped either into hybrid or local varieties.

The procedure was repeated for samples taken from market stores and 155 samples were collected. Sampling interval in the market stores was 0-20, 30-50, 60-70 and 90-100 days due to the nature of market business. When picking the samples for analysis, different storage methods, sources of grain, maize variety and moisture content at time of sampling were considered.

6.2.3 Determination of diversity and population of *Aspergillus* section *Flavi* group in post harvest maize

A semi-selective medium, MDRB was prepared and inoculated as as described in section 3.4. Fungal isolates were recovered by dilution plate technique (Cotty, 1994). The

inoculated plates, replicated six times (0.2 ml/plate) were incubated at 30^0 C for 3-7 days without sealing. The plates were then removed from the incubator and visually observed for microbial growth. *Aspergillus* species were identified as described by Horn and Dorner (1998). *Aspergillus* species (*Aspergillus flavus* S and L strains, *A. parasiticus, A. tamarii, A. alliaceus* and *A. caelatus)* and total filamentous fungi were enumerated as described in chapter three sections 3.4.

Other fungal isolate such as *A. niger, Fusarium Penicillium, Rhizopus* spp. and bacterial colonies were also recorded. A stereomicroscope was used to identify fungi in the section *Flavi* group directly from the dilution plates in Petri dishes that were heavily colonized by other filamentous fungi. Morphological characteristics (colour, texture, size of colony) were used to separate the fungi into groups and reference cultures were used to aid in identification. Colonies identified as *A. flavus* and *A. parasiticus* were sub-cultured on Czapek Dox Agar (35 g of Czapek Dox concentrate with 15 g agar in one litre of distilled water) agar slants with sporulating fungi werepreserved in a refrigerator at 4^0 C

Thin layer chromatography (TLC) plates (A Silica gel 60; EMD Chemicals Inc., Darmstadt, Germany) were used to determine and confirm presence of different aflatoxins in *A. flavus* and *A. parasiticus* fungal colonies isolated from post- harvest maize samples. The fungi were were grown in a liquid Sucrose Yeast Extract (YES) broth, prepared, dispensed and inoculated as described in section 3.5. The spotted plates were placed in a glass container (5 cm x 3 cm) containing a developer solvent made by

mixing distilled water, acetone and chloroform, in a ratio of 1.5: 12: 88 respectively, until the solvent covered 80 % of the TLC plate length. The plates were allowed to air dry before viewing under the UV light in a dark room. The bands were compared with the standard aflatoxins and scored based on presence or absence of specific aflatoxin types.

6.3 Statistical analysis

Data was analysed using GenStat 13 and 14 th edition (SP2) statistical packages (VSN International (2011). The data were subjected to analysis of variance (ANOVA) to test if the resulting frequency distributions were similar for market and farmer store samples. R Statistical Package (2012): was also used for statistical analysis, simulation modeling and advanced data analysis. R was used to display means and confidence intervals (CI) procedure using the bargraph within the sciplot package. Confidence intervals were set to 1.96 standard errors (translates to $P \leq 005$). For graphs made in Excel, error bars represented the standard error of the mean. Further, associations between specific fungal incidences were investigated based on analysis of contingency tables with appropriate chi-squared tests using different variables, including season, maize variety storage type and duration of storage in market and farmer store samples.

6.4 Results

6.4.1 Fungal population in different seasons

The highest fungal population (CFU g^{-1} of maize) was in 2010 and 2011 and the least in 2009. In 2009, fungal contamination in both storage and market samples was

significantly lower ($p<0.001$) compared to 2010 and 2011. Further analysis using Turkeys 95 % confidence interval showed a significant difference ($P<0.001$) in CFU g^{-1} of maize in year 2009 (mean= 1312^{a}), 2010 (mean= 11431^{b}) and year 2011 (mean= 16045^{c}) (Figure 6.1).

Aspergillus flavus S strain had a higher incidence than *A. flavus* L strain and *A. parasiticus* but the three had similar incidence in 2010 and 2011 in market samples but a totally different incidence in storage samples in the same years. The differences in fungal population in different years were contributed by higher population of *Penicillium* spp and *A. flavus* S strain, irrespective of the sample type (Table 6.1).

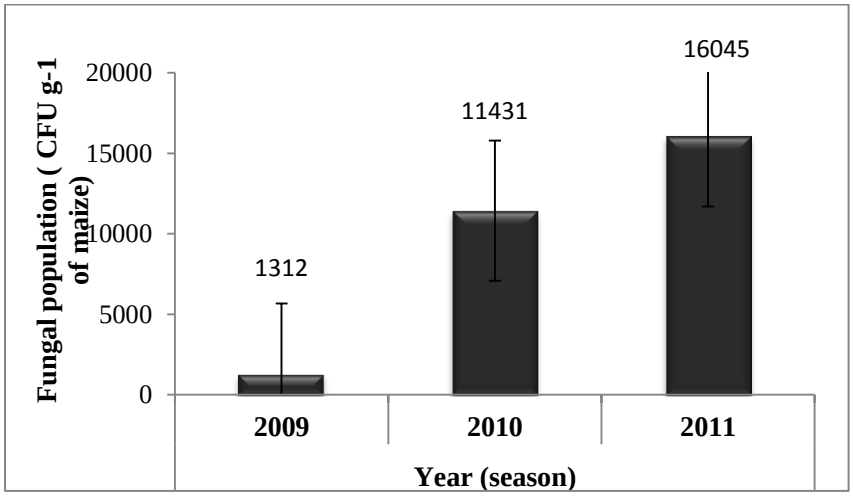

Figure 6.1: Fungal population (Mean CFU g^{-1} maize) in different seasons.
Error bars represents the standard error of the mean.

The population of ASF in maize from market and farmer stores was significantly different ($P<0.001$). Data gathered in this study further showed that differencies in CFU

g^{-1} of maize were contributed by the distribution of individual fungal species in the analysed maize samples (Table 6.1). Generally, fungi in the ASF group had their highest population in market samples with mean CFU g^{-1} of maize of 16812 for *A. flavus* S strain, 3181 for *A. flavus* L strain and 1520 for *A. parasiticus.*

On the other hand, mean CFU g^{-1} of maize for ASF in farmer stores were significantly lower ($P<0.01$) than those of the market samples. For example *A. flavus* S strain had a mean of 9671 CFUg^{-1} of maize. *Aspergillus parasiticus* had the least incidence in both farmer and market store samples (mean = 195 and 1520 CFU g^{-1} of maize respectively) but significantly higher in market samples than in storage samples. The population of *A. flavus* L strain was slightly higher in market than in farmer store samples (Table 6.1)

Penicillium and other fungal species had their highest population in farmer store samples than in market samples. *Penicillium* spp showed a higher incidence in storage samples than in market ones with a mean CFU g^{-1} of maize of 18522 (farmer store) and 14462 (market store) but the difference in farmer and market samples was not scientifically significant.

Table 6.1: Fungal population (Mean CFU g^{-1} maize) in market and farmer store maize samples

Fungal Species	Storage samples (Mean fungal population)	Market samples (Mean fungal population)
A. flavus S strain	9671^{bc}	16812^{ab}
A. flavus L strain	1358^{d}	3181^{bc}
A. parasiticus	195^{c}	1520^{a}
Penicillium spp	18522^{a}	14462^{a}
A. niger	1655^{ab}	3299^{bc}
Other *Aspergillus* spp	8304^{b}	5999^{b}

Means that do not share same subscripts differ by $p < 0.05$ according to Tukey's Honestly Significant Difference.

6.4.2 Fungal population in market, farmer store and in different storage methods

All the farmers and market vendors interviewed in this study revealed that they used traditional methods of storage, with some putting their maize in traditional granaries and others in their own houses and/or market stalls (Figure 6.2a). All the 155 market vendors stored their maize in both polypropylene and sisal bags. 80 % of the vendors placed the maize bags on wooden pallets and 20 % on cement floor.

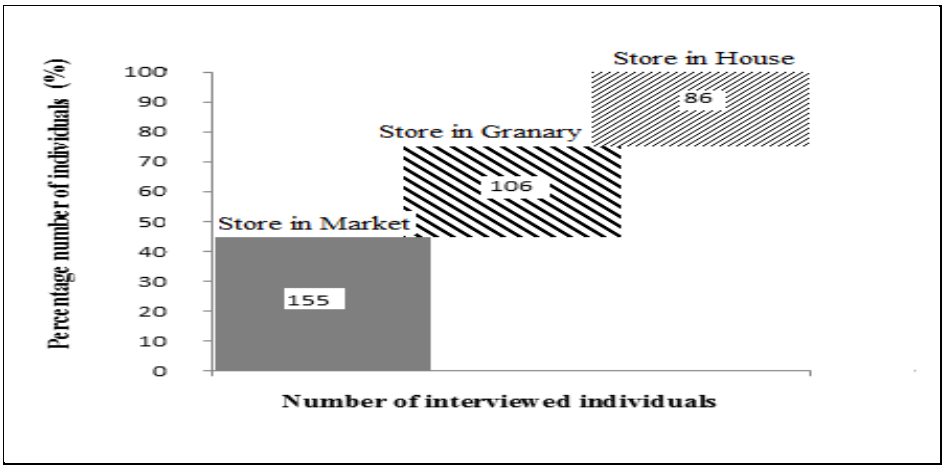

Figure 6.2a: Percentage number of individuals interviewed and the storage methods used

Farmers and traders in the study area were found to store their maize in either polypropylene or sisal bags (Figure 6.2b). Maize stored in sisal bags in market stores had a slightly higher fungal population (mean= 8691 CFU g^{-1} of maize) than that stored in sisal bags in farmer stores (mean= 7339 CFU g^{-1} of maize). The incident was different in farmer stores as maize stored in polypropylene bags had a slightly higher population than that in the market stores. Fungal population in sisal and polypropylene bags was significantly different ($P<0.01$) in farmer stores (mean=7340 and 9109, CFU g^{-1} of maize respectively). Mean fungal population in polypropylene (mean=9109, CFU g^{-1}) and sisal (mean=8691, CFU g^{-1} of maize) bags in market and farmer stores revealed no statistical difference (Figure 6.2b), irrespective of the fungal species.

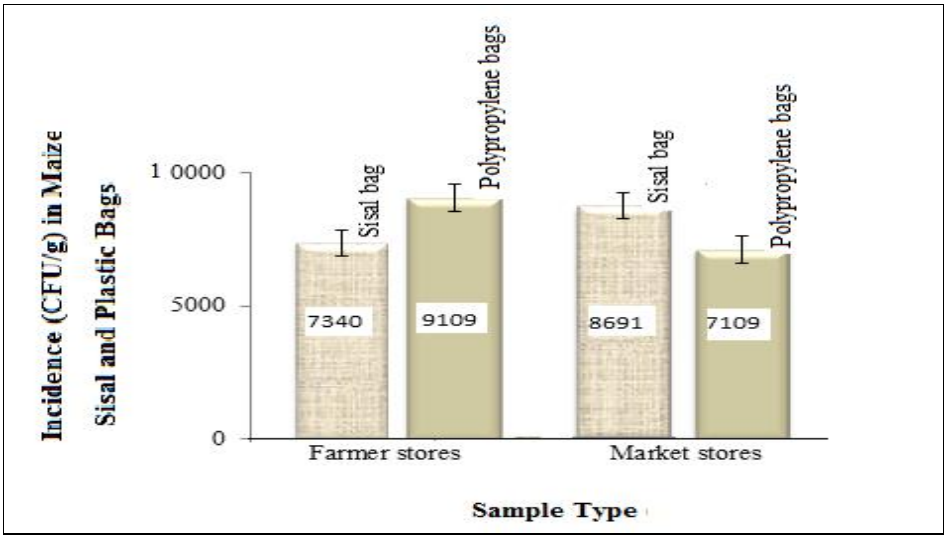

Figure 6.2b: Fungal population (CFU g^{-1} of maize) in maize stored in sisal and polypropylene bags in market and farmer store. Error bars represent the standard error of the mean.

The results further revealed that maize hanged on roof (mean = 65 CFU g^{-1} of maize) and the one dryed on mats (mean = 1214 CFU g^{-1} of maize) had a significantly lower ($P<0.001$) fungal incidence compared to other storage methods/containers. Maize cobs dried on wooden pallets and cement floor and maize kept in house stores had the highest incidence of fungal contamination with a means of 15548, 11703 and 12517 CFU g^{-1} of maize respectively, irrespective of the season (Table 6.2).

Table 6.2: Mean fungal population (CFU g^{-1} of maize) in farmer and market stores

Storage Method	Store type	Mean CFU g^{-1} of maize
Hanged on roof	farmer store	65
Sisal bags	market store	7340
Polypropylene bags	market store	9110
Bags on wooden pallet	market store	7830
Dried on Mats	farmer store	1214
Sacks in Granary	farmer store	6280
Hanging above fire place	farmer store	5799
House store	farmer store	12517
Cobs drying on cement floor	farmer store	11703
Cobs drying on wooden pallet	farmer store	15548

6.4.3 Fungal population (CFU g^{-1} of maize) in market and farmer store maize of different varieties and collected from different sources.

There was a significant difference ($P<0.001$) in fungal population between storage and market samples. Maize samples collected from markets had a significantly higher ($P<0.001$) fungal population (Mean CFU g-1 of Maize) compared to the farmer store ones (Table 6.3). Hybrid and local maize varieties showed no significance difference ($P=0$) when compared within the market or the farmer store. Generally maize variety

alone did not have an effect on changes in fungal population in market or the farmer store.

Table 6.3: Fungal population (Mean CFU g^{-1} of Maize) in different maize types and sources in market and farmer stores

	Market Store	Farmer Store	
Maize Type	**Fungal population (Mean CFUg-1 of Maize)**		**Sig diff**
Hybrid Varieties	12042*	6578	(P<0.001)
Local Varieties	12675*	6678	(P<0.001)
Maize Sources			
Own Store	784	6197	(P<0.001)
Purchased	11320	9396	(P<0.05)
Own Farm	396	4703	(P<0.001)

**Comparison within (market or farmer stores) shows no significant difference (P=0) in the fungal population*

- *Own Store- Maize in a farmer/vendor store but not originating from own farm*
- *Purchased –Maize bought from lorries by market vendors and/or purchased by farmers from other farmers within Kaiti Location.*
- *Own farm-Maize in a farmer and /or market vendor store and originating from their own farms*

The results also showed that fungal incidence was high in maize purchased from other local farmers in both market and farmer stores (mean=11320 and 9396 CFUg $^{-1}$ of maize respectively). Maize in market and farmer store and originating from either farmer or vendor's farm had the least fungal incidence. However maize in farmer store and originating from own farm had a significantly (P<0.001) higher fungal population than own farm sourced maize in market the stores (Table 6.3). Different interaction showed some statistical differences. For example, interactions between variety and source of maize (P=0.025), variety, storage and fungal species (P=0.066). Likewise interaction between variety, moisture and fungal species also showed some significant difference (P=0.048).

Data further revealed that population of *A. flavus* S strain had a significantly higher population in both hybrid and local maize varieties but the difference was not statistically significant in both hybrid and local maize varieties. Population of *A. flavus* L strain and *A. parasiticus* was lower in both maize types and their population was significantly lower than that of *A. flavus* S strain and other *Aspergillus* species. Generally, maize variety alone did not have an effect on changes in CFU g^{-1} of maize.

6.4.4 Fungal population in market and maize samples with different storage time

Fungal population in maize samples stored between 15-330 days in market and farmer stores showed a significant difference (P<0.001). The results also revealed that fungal populations in both farmer and market stored maize kept for different durations assumed a normal growth curve. Maize was stored in in market for a shorter duration compared to

farmer stores because of market dynamics. Fungal populations in market stored maize samples fluctuated with storage duration. Data gathered in this study showed that fungal populations increased rapidly in market stored maize for up to three months to reach a maximum fungal population of 10437 CFU g^{-1} of maize, before dropping drastically to a mean CFU of less than 600 colonies in the fourth month (Figure 6.4a).

Further analysis showed that high populations of individual fungal species contributed to significant differences ($P<0.001$) in fungal populations at different storage times.

In general higher population at 60-70 days was due to high population of all fungal species (*A. flavus* S strain, *A. flavus* L strain, *Penicillium* species, *A. parasiticus,* and *A. niger*). Incidence of *A. flavus* S strain and *Penicillium* spp was higher than that of the rest of the fungi but that of *A. flavus* S strain was significantly ($P<0.001$) higher than the rest in all the samples. Population of other fungi and *A. niger* was not statistically different. Fungi in the section *Flavi* group had extremely low populations in both storage types.

Fungal populations in farmer stored maize for over one year increased gradually to a mean population of greater than 10000 CFU g^{-1} of maize in one year. It was noted that a similar fungal population (mean 10437 CFU g^{-1} of maize) was recorded in maize stored in the market for slightly longer than than a month. Farmer maize stored for less than two month (15-40 days) had a significantly lower ($P<0.001$) fungal populations (mean =1107 CFU g^{-1}) and their means increased to 10342 CFU g^{-1} of maize in one year (Figure 6.4b).

Population of *A. flavus* S strain was significantly high among the section *Flavi* group followed by *A. flavus* L strain. *Aspergillus parasiticus* had the lowest incidence in all storage durations. In general *A. flavus* S strain was present in all samples analysed and was present from the beginning to the end of storage duration. However, the populations differed over time, with fungal growth assuming an almost normal fungal growth pattern with occasional drops and rapid increases in fungal population over time.

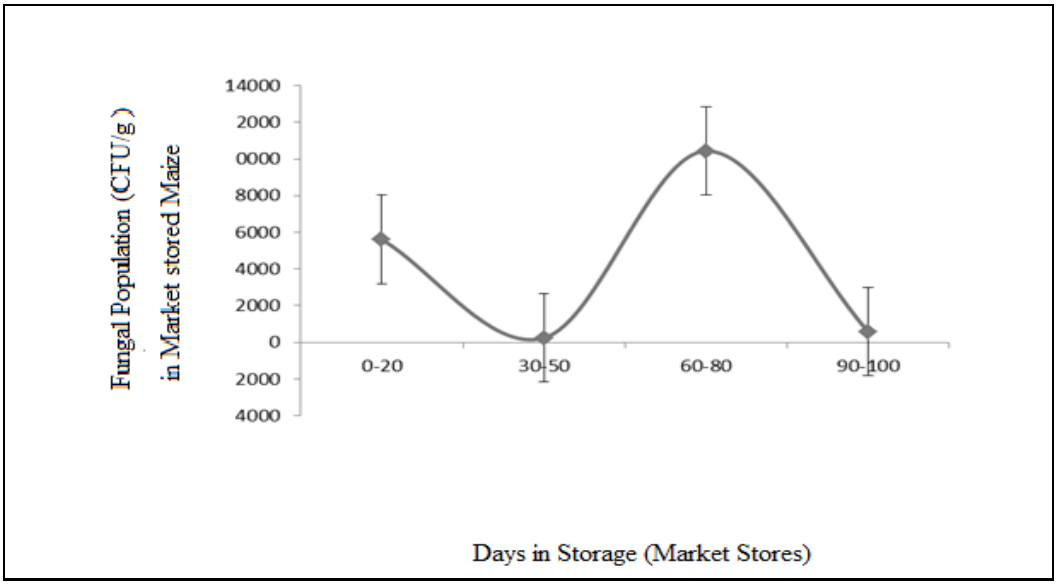

Figure 6.3a: Fungal population (Mean CFU g^{-1} of maize) in market stored maize (100 days). Error bars represent the standard error of the mean.

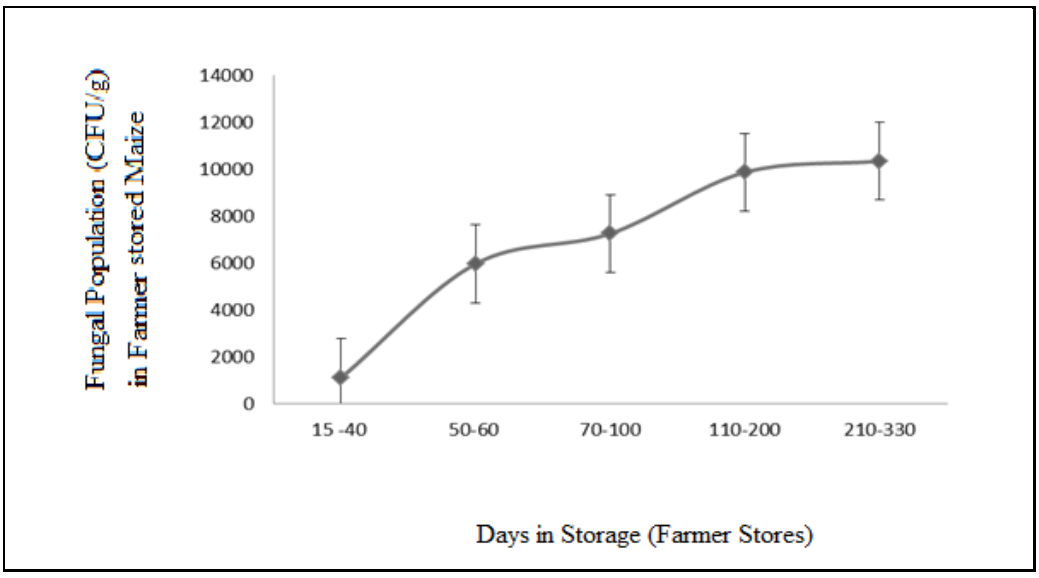

Figure 6.3b: Fungal population (Mean CFU g^{-1} of maize) in farmer stored maize (330 days). Error bars represent the standard error of the mean.

6.4.5 Incidences of *Aspergillus* section *Flavi* in different moisture content and maize sources

Maize samples analyzed in this experiment had a moisture content range of 9-15 %. Data gathered in this study revealed that fungal population was lower in maize samples that had lower moisture content (Figure 6.5a). The results also reveal that moisture content had a significant (P=0.023) effect on type of fungal species (P=0.003), days in storage (P=0.041) and type of storage (P=0.042). In general the effect of moisture on fungal population in storage samples was not statistically different There was no significant

difference (P=0.264) between moisture content and grain sources in storage experiment but fungal species were significantly different (P=0.001) as shown in Figure 6.5b. *Aspergillus flavus* S strain had a significantly higher (p<0.001) population than L strain and *A. parasiticus* in the three moisture content ranges. A. flavus had the highest population in Maize dried to a moisture content of 13-14.9 % .its lowest population was in 9-10.9 % moisture content (Figure 6.5b). *Aspergillus* spp population was not different in all the three MC ranges. Other interactions did not show a statistical difference. In market samples, moisture and source had a significant difference (P<0.001).

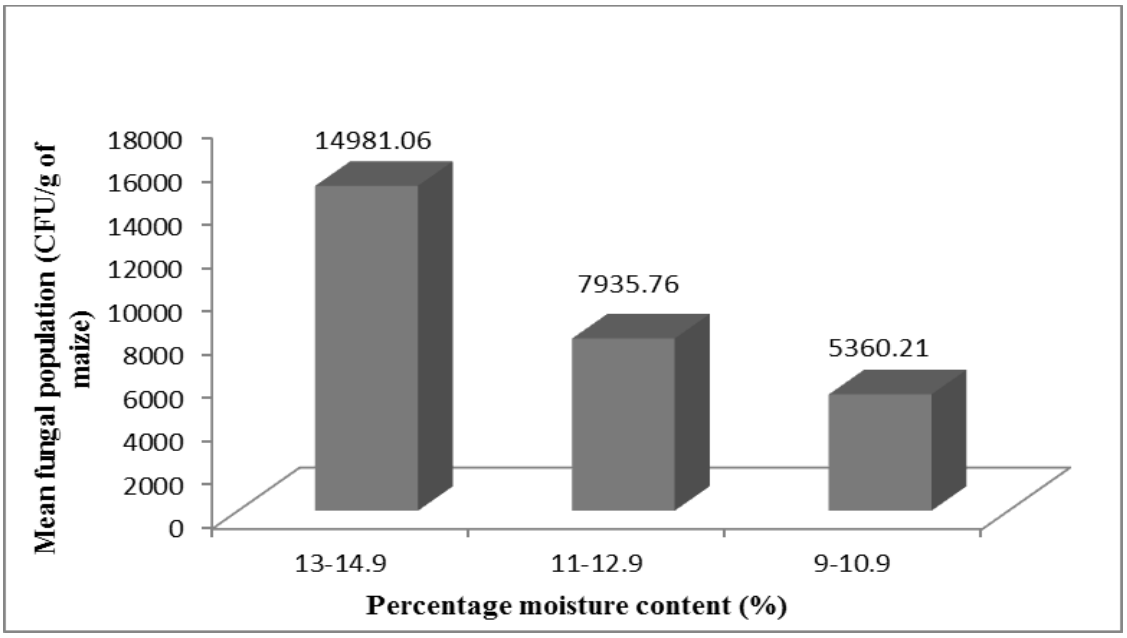

Figure 6.4a: Effect of moisture content (MC) on fungal population (Mean CFU g-1 of maize) in market and farmer stored maize

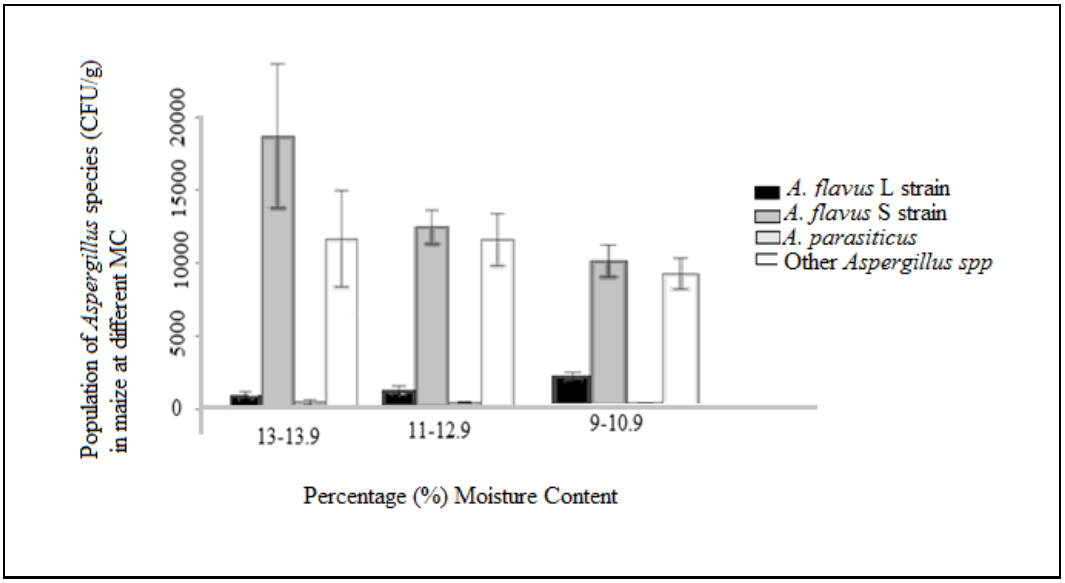

Figure 6.4b: Mean poulation of *Aspergillus* species in maize with different moisture content. Error bars are confidence intervals set to 1.96 Standard Errors.

6.4.6 Fungal population in maize samples from different markets and locations in Kaiti

Market samples had a significantly (P<0.001) higher population of *Aspergillus* section *Flavi* compared to those collected from the farmer stores (Table 6.4). There was no significant difference (P=0.25) in fungal population between the different markets and locations. Six out of ten markets had samples with a mean CFU g^{-1} of maize above 5000. The highest fungal populations were in Itumbule market samples (mean= 12900). Lowest population was in Itooni and Kaiti markets with mean CFU g^{-1} of maize of 128 and 556 respectively (Table 6.3). The highest fungal population among the farmer stores was in a farm in Ukia (mean= 8180 CFU g^{-1} of maize). The lowest population among the farmer storage samples was in Nzuuni (mean= 299) (Ttable 6.4). Different years (seasons)

showed a consistent trend and a significant difference (P<0.001) where 2009 had the lowest CFUs compared to 2010 and 2011 in both market and farmer maize samples.

Table 6.4: Fungal population (Mean CFU g^{-1} maize) in different markets and farms in Kaiti

Location/market place	Market sample	Farmer store sample
Iuani	*	8550
Kilala	12400	6970
Kivani	*	6490
Makongo	*	1220
Mukuyuni	6640	1330
Nzuuni	*	299
Ukia	7200	8180
Utaati	*	1210
Itooni	128	*
Itumbule	12900	*
Kaiti	556	*
Kaumoni	12000	*
Kyambalasi	5890	*
Makutano	1700	*
Mwea	3670	*

**Sample not available; market samples (n=155) farmer store samples (n=190).*

6.4.7 Incidence of different aflatoxin types in *A flavus* and *A. parasiticus* colonies isolated from market and farmer store maize samples

Only 73 % of the analysed maize samples had colonies in the *Aspergillus* section *Flavi* group. The assayed maize samples contained both *A flavus* and *A. parasiticus* coloniesand data gathered showed that all the assayed colonies were aflatoxigenic. The most common aflatoxin type was AFB_1 with a percentage incidence of 60 followed by AFB_2 (53), AFG_1 (38) and AFG_2 (30).

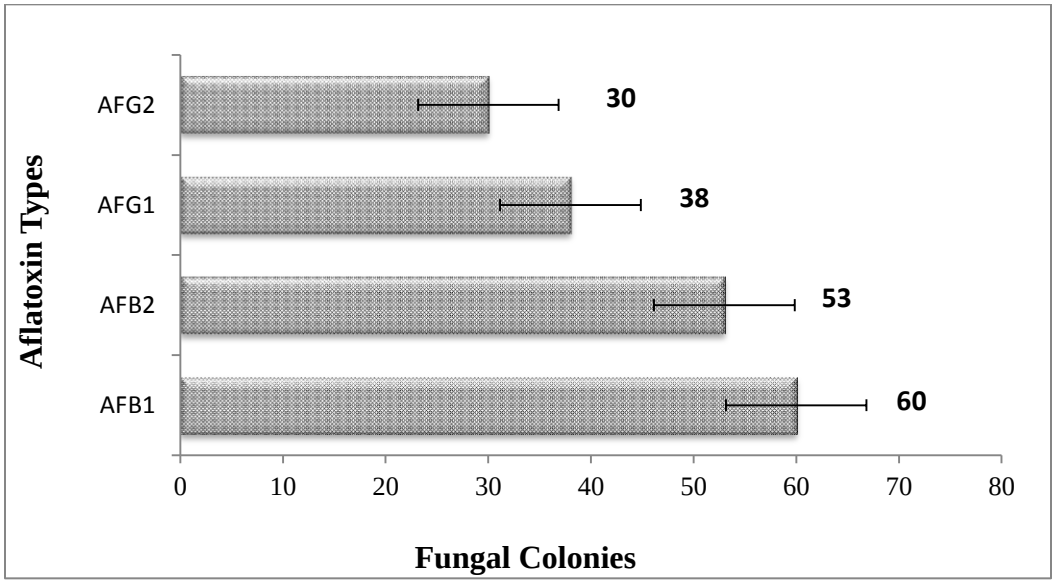

Figure 6.7: Fungal colonies and the incidence of different aflatoxin types in post-harvest maize samples. Error bars represent the standard error of the mean.

6.5 Discussion

In the current study, the population, prevalence and incidence of *Aspergillus* section *Flavi* in post-harvest maize in three consequent seasons (2009-2011) was determined in

samples collected from Kaiti Location in Makueni County. Other *Aspergillus* spp, *A. flavus* S strain and *Penicillium* species were the fungal species encountered in this study in both market and farmer stores maize samples.e samples. The results partly agrees with those of Orsi *et al*. (2000) who found out that *Fusarium*, *Aspergillus* and *Penicillium* are the major genera commonly encountered on maize in tropical regions. *Aspergillus flavus* and *A. parasiticus* both isolated and reported in this study are the major producers of aflatoxins (Yin *et al*., 2008). *Penicillium* and *Fusarium* species produces ochratoxins, zearalenone and fumosins respectively (Wagacha and Muthomi, 2008).

Aflatoxins have powerful teratogenic, mutagenic and hepato-carcinogenic effects (Wang *et al*., 2001). Fumonisins on the other hand are reported to have cancer-promoting activities, in addition to causing several diseases in animals (Rheeder *et al*., 2002). Ueno (2000) have shown the importance of co-occurrence of fumonisin with AFB_1 in the promotion of carcinogenesis. The fact that other mycotoxigenic fungi were encountered in the study is an indication that maize contamination in Makueni County may be caused by a combination of by aflatoxins and other mycotoxins if cross contamination occurs (Bankole and Mabekoje, 2003).

Aspergillus flavus S and L strains is the fungal species that was commonly encountered in the maize samples. The fact that market samples had a higher population of all fungi should be considered as an important public health concern. It is also an indication that contamination of maize with mycotoxigenic fungi and other molds is a complex issue

that involves farmer practices, biotic and abiotic factors both in field and in farmer stores. It is likely that vendors buy maize from individual farmers and mix them. Once the product enters the market distribution system, other factors during transportation and storage (Bhat and Vasanthi, 2003; Wagacha and Muthomi, 2008) set in resulting to cross contamination of products in the market.

Markets have a tendency of having high population of rodents which move from one stall to the other resulting to cross contamination of products in the market. The other practice that could justify higher population of *A. flavus* in the market place is the fact that, farmers sell off old maize or other commodities when they are anticipating a new harvest. In a previous study by Lewis *et al*. (2005) showed that homegrown maize may not remain within the household of a farmer after harvest. Farmers tend to sell maize to market vendors and purchase it back as needed.

The data gathered also showed that farmers in the study area employ different storage conditions some of which promote growth of aflatoxigenic fungi and subsequent production of aflatoxins. Interventions to minimize aflatoxin contamination in the maize value chain must include regional market distribution system as a way of reducing possible continuous exposure to aflatoxin contamination and future aflatoxicosis outbreaks in Kenya.

The results of this study further showed that fungal populations of members of *Aspergillus* section *Flavi* increased with storage duration in both farmer and market store

samples. This is probably because the samples were properly dried by the time they were taken to the market. This result agrees with findings of other researchers. For example in a study carried out in Benin, Hell *et al*. (2000) concluded that aflatoxin levels at the beginning of storage were less than the ones found in maize stored for 6 months. Increase in aflatoxin contamination with storage time especially in Africa could be associated with climatic conditions in the tropics such as excess heat, high humidity and drought.

Likewise, traditional storage practices in developing countries cannot guarantee protection against major storage pests of staple food crops like maize, leading to 20-30 % grain losses at post harvest stage. As a result, smallholder farmers end up selling their grain soon after harvest, only to buy it back at an expensive price just a few months after harvest, falling in a poverty trap. Inadequate drying and storage under damp conditions are primary factors that lead to aflatoxin production and grain contamination (Wilson and Payne 1994) in traditional storage methods. A 100 % of the methods used by farmers who participated in this study used various traditional methods to dry their maize. Wild and Hall (2000) revealed that much aflatoxin contamination of food takes place during post-harvest storage, as opposed to pre-harvest conditions. Controlling maize contamination by aflatoxigenic fungi in post-harvest settings is crucial. The current study showed relationships between storage conditions and *Aspergillus* section *Flavi* populations in both market and farmer stored maize. Maize dried and stored at low moisture content has lower chances of fungal contamination. The Kenya National Cereals and Produce Board-KNCPB (Heather *at al.,* 2006) recommends sun drying maize for

several days until it attains a moisture content of 12-15 %. According to Payne (1992) sun drying maize by spreading it directly on open ground increases the likelihood of contact with soil and contamination by *Aspergillus* spores. It is therefore important to educate farmers on proper post-harvest handling of their produce to reduce contamination by aflatoxigenic fungi.

Storage of maize in polypropylene bags was also found to be a common practice by farmers in interviewed in this study. This is probably because plastic is cheaper and more readily available than the preferred sisal bags (Turner, *et al.,* 2005). Plastic is poorly aerated hence encourage growth of fungi in maize stored especially if it was not dried properly before storage (Hell *et al*., 2000; Udoh *et al*., 2000). In the current study, maize stored in polypropylene bags had a significantly higher fungal population than that stored in sisal bags in farmer stores but this was not the case in market samples.

The results of this study further revealed that moisture content had an effect on the incidence of members of *Aspergillus* section *Flavi*. Lowest fungal population was found in samples with lower moisture content. According to Garuba *et al*. (2011), reduced moisture content is of great significance since high moisture content and rich nutritional components of maize has been reported to encourage mold growth on maize during storage. Studies have shown that physical parameters such as temperature (optimum of 28 to 30 ^{0} C) light, relative humidity (moisture content above 14 %), pH and water activity (0.83 to 0.97) (Dantigny *et al.,* 2005) are some of the factors that can influence

the growth of aflatoxigenic fungi and production of aflatoxin during storage (Viquez, 1994). However, the effect of moisture content is higher than that of temperature.

Lewis *at al*. (2005) assessed and compared the extent of aflatoxin contamination in maize from three sources in Eastern Kenya. He found out that most of the homegrown maize samples collected in 2005 and 2006 had aflatoxin levels above the 10-ppb limit compared with purchased maize samples. similarly, data gathered from this study indicated that maize in markets stores originating from local farmers had a significantly higher fungal population than maize purchased from lorries and that coming from the vendors own farms. This data is consistent with data collected by Lewis *et al.* (2005) that showed a statistically significant association between the locations of aflatoxin-contaminated market maize and cases of aflatoxicosis outbreak. Contamination of homegrown maize and market maize originating from the region should be taken seriously because local trade and distribution of contaminated homegrown maize may facilitate and sustain the cycle of aflatoxin exposure to the communities in Makueni County. This partly explains causes of recurrent aflatoxicosis outbreaks for the last four decades, probably because maize consumed by local farmers is not monitored for aflatoxin or other contaminants through traditional regulatory authorities. As a way of reducing exposure in the rural setting, Shephard (2003); Wild and Gong (2010), Williams *et al*.(2004) and Kimanya *et al*. (2008) indicated that it is the people living in the affected regions where locally grown crops are consumed who should insist for enforced regulatory standards and other protective measures. Such measures could include monitoring food at the subsistence farm level. This may reduce the risk of aflatoxin exposure farmers and consumers. The

government has since reduced aflatoxin regulatory limits to 10 ppb, a level that is still high considering that Kenyans rely of maize for their daily caloric intake and have an annual per capita consumption of about 125 kg (Pingali, 2001), the highest in the world.

Over 60% of the maize samples analysed had colonies that produced different aflatoxin types. This result conforms to those of Abbas *et al*. (2004b) that the frequency of aflatoxigenic isolates can range from 50- 80 % in any environment. *Aspergillus flavus* produces aflatoxin (AF) B_1 (M1), AFB_2 whereas *A. parasiticus* produce AFG_1 and AFG_2 in addition to AFB_1 and AFB_2. Based on the profiled toxins, we can say that both A. *parasiticus* and or *A. flavus* were present in post- harvest maize collected from Kaiti Location. Other colonies were not aflatoxigenic hence could be screened further and used as biological control agents of the aflatoxigenic *A. flavus*.

Seeds planted in Kenya vary from local landraces to composites and hybrids. Local landraces are poor yielding but have a greater advantage of being suited to the local conditions. It is thought that they have a possibility of being more resistant to fungal attack than the improved composites and hybrids. Identification of aflatoxin resistance and incorporation of pertinent traits into maize has been an enormous challenge for breeders. The characteristics that are important in mitigating aflatoxin susceptibility are pest, disease and drought tolerant, husk cover and flintiness of the grains. They are the attributes that the seed developing and bulking companies ought to consider. Unfortunately, these qualities may not be positively related to high productivity per hectare which is currently the single most important standard for release in Kenya.

6.5 Conclusion

Conclusion drawn from the current study is that maize at post harvest stage of the maize value chain contained high population of fungi in the *Aspergillus* section *Flavi* group. The incidence of *A. flavus* (S and L strains) was high in both market and farmer stored maize and in the three seasons (2009-2011). This is is a concern especiallybecause presence of *A. flavus* (S and L strains) and *A. parasiticus* in food can be directly associated with aflatoxin contamination. *A. flavus* produces AFB_1 and AFB_2. These toxins were detected in fungal isolates in over 60 % of the maize samples at post-harvest stage. Aflatoxin BI has been associated wlith liver cirrhosis.

6.5 Recomendations

Farmers should be educated on good agricultural practices to enhance the communities' interventions to improve maize harvesting, drying and storage practices to reduce the likelihood of contamination with aflatoxigenic fungi and aflatoxicosis outbreaks in Kenya.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1General discussion

The local communities in Makueni County like in other aflatoxin hot spots in Kenya are not aware of what poisons their maize. However, they are aware that bumper harvest of maize is followed by molding of grains; a development branded "aflatoxin", that renders their grains unfit for consumption (author's personal communication with a village elder) hence they experience famine in the midst of a good harvest. The communities' argument is in line with a statement by Hassan (1998) that contamination of maize by fungi renders grains unfit for human consumption by discoloration, sometimes leading to serious food insecurity.The current study was meant to evaluate the population, occurrence and distribution of *Aspergillus* section *Flavi* in the maize value chain in Kaiti Location of Makueni County in Kenya. According to CAST (2003), contamination of maize with aflatoxigenic fungi is an additive process starting in the field and multiplying along the value chain depending on the suitability of conditions for fungal growth.

Maize sample collected for three seasons were followed from the field to the market. Soil was collected in maize growing fields with the aim of determining the occurrence of *A. flavus* (S and L stains) and *A.parasiticus* at production stage. Maize was collected to determine the incidence of the fungi at pre and post harvest stages. Generally the differences in fungal population (CFU g^{-1} of substrate) in the different years were contributed by higher populations of *A. flavus* (S and Lstrains) and *Penicillium,*

irrespective of the sample type. In their study, Hussein and Brasel (2001) and Abarca *et al*. (2001) showed that aflatoxigenic and non aflatoxigenic strains of *A. flavus* are capable of growing on maize in the field, in storage and during processing.

Data gathered in this study (chapter 4-6) showed that fungi belonging to the genera *Aspergillus* and *Penicillium* were present in soil. Soil is the primary inoculum, so crops grown in infected fields are prone to contamination but the extent of contamination again depends on farmer practices. The highest incidence of the target fungi was in loam, red loam and sandy loam while the least were in black loam and sandy soils. These results are in agreement with Jaime-Garcia and Cotty (2006) who reported that on average, areas with high aflatoxin contamination have soils with a higher percent of clay than sand. Penicillium and *Aspergillus flavus* (S and L strains) were also present in pre and post harvest maize. Contamination at pre-havest stage may result from leaving maize to dry in the field for an extended period of time, planting maize varieties that open up upon maturity allowing rain water to penetrate into the kennel and cause rotting of the maize while still in the field. Other practices are dropping maize cobs on bare soil when harvesting. This result agrees with those of Samson (1991) and Orsi *et al*. (2000) that ear rot fungi belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium* are commonly encountered on maize in tropical regions. With reccurent aflatoxicosis outbreaks in Makueni since 1981, then the results are in agreement with those of Orsi *et al*. (2000) that these genera have species capable of producing a wide spectrum of compounds shown to be toxic to man and animals.

The results further showed that year 2009 experienced unusual rainfall variations with dry spells interspersed with off-season rains in the months of January and February. Based on these findings, we would have expected year 2009 to have the highest incidence of *Aspergillus* section *Flavi* but this was not the case in this study. This shows that climatic conditions are not the only determinants of aflatoxin contamination of maize with aflatoxigenic fungi. In chapter five and six, the study revealed that maize variety and post-harvest practices that include, storage methods and duration and presence of insect and rodent in the granaries. All these practices expose maize to contamination with ASF fungi and subsequent aflatoxin contamination even before maize is processed for storage. Aflatoxin is not localised in Kenya only. All the toxigenic fungi isolated in this study have been previously associated with contamination of stored maize in other African countries like Nigeria as reported by Udo *et al*. (2000), Bankole and Mabekoje (2003), Atehnkeng *et al*. (2008) and Garuba *et al*. (2011).

7.2 Conclusion

Aspergillus flavus (S and L strains) and *Aspergillus parasiticus* are the two members of *Aspergillus* section *Flavi* that were isolated in maize and soil in Kaiti Location. They were present at pre-harvest stage, in farmer and market stored maize and in three seasons. The highest incidence of *Aspergillus flavus* (S and L strains) the target fungi was in loam, red loam and sandy loam while the least were in black loam and sandy soils. The fungi occurred in 90 % of the sampled farms, an indication that the fungi are widely distributed

in Kaiti Location. It was present in the three seasons, 2010 and 2011 had the highest populations. The population of *Aspergillus flavus* (S and L strains) was lowest in soil followed by at pre-harvest stage. The fungal incidence was higher in market than in farmer stored maize. It was also higher in maize stored at higher moisture content, prolonged storage duration and was present in homegrown maize at significantly higher levels than purchased maize. Hybrid maize varieties were more susceptible to fungal attack at pre- harvest stage but the fungal population was not different at post-harvest stage. This is an indication that maize contamination with aflatoxigenic fungi has more to do with post-harvest practices employed by farmers. Recent studies have linked aflatoxin production in foods to environmental conditions, poor processing and lack of proper storage facilities in developing countries (Farombi, 2006). Aflatoxin B_1 and AFB_2 were detected in over 60 % of the analysed samples. AFB_1 produced by *A. flavus* is a potent carcinogen. In conclusion, it should be noted that farmers have no control of climatic conditions, so despite the effort to apply good agricultural practices, aflatoxin contamination may still occur. The magnitude of contamination however, depends on farmer's knowledge of aflatoxin management practices.

7.3 Recommendations

1. Aflatoxigenic fungi in the *Aspergillus* section *Flavi* occurred in all the stages of the maize value chain in the study area. To ensure food security in Makueni County, there is a need to create awareness on the occurrence of this fungi and possible contamination ways. Farmer education and adoption of suitable

agricultural practices would be crucial in reducing aflatoxin contamination in the maize value chain in Makueni County and all aflatoxin prone regions in Kenya. This can be done through regular programs on radio, television, local daily newspaper and magazines. Farmers should be facilitated through government agencies and NGOs to participate in such programs so that they can reach their fellow farmers.

2. Weather experts should come up with weather models that can predict an outbreak before it occurs. They should also map high risk areas in Eastern Province and Kenya at large showing aflatoxin hot spots that are not suitable for aflatoxin contamination susceptible crops to aflatoxin.
3. The government should also provide modern grain preservation methods like Silos and super bags probably at County level. During good seasons, farmers should be encouraged to reserve excess grains for proper storage
4. During harvesting, farmers should be provided with rapid tests and moisture meters. This is to ensure grains are dried and stored at the required moisture content to avoid loses by molds in storage.

7.4 Recommendations for further research

1. More studies need to be carried out in other locations in Makueni County to get a true picture of how aflatoxigenic strains are distributed in the County. Data including farmer practices, crop history soil temperatures, soil types and soil pH

need to be gathered since these parameters are important in colonization and survival of the fungi in soil.

2. There is a need for further studies to show the levels of other mycotoxins and their effect on interaction with aflatoxins.

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7.6 APPENDICES

Appendix 1.0: TLC plates showing different types of aflatoxins from cultures isolated from Kaiti soils