# POST-HARVEST FUNGI DIVERSITY AND LEVEL OF AFLATOXIN CONTAMINATION IN STORED MAIZE: CASES OF KITUI, NAKURU AND TRANS-NZOIA COUNTIES IN KENYA.

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY.**

**APRIL, 2015**

## DECLARATION

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

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## DEDICATION

I dedicate this thesis to my loving mum and dad who have taught me the value of discipline and hard work. Thank you for teaching me that even the largest task can be accomplished if it is carried out step by step. May the Lord bless you always.

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## ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| **AMOVA** | Analysis of Molecular Variance |
| **AFPA** | *Aspergillus flavus* and *parasiticus* agar |
| **BecA** | Biosciences eastern and central Africa |
| **CTAB** | Cetyl Trimethyl Ammonium Bromide |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **ICFM** | International Commission on Food Mycology |
| **ILRI** | International Livestock Research Institute |
| **MC** | Moisture Content |
| **MEA** | Malt Extract Agar |
| **PCoA** | Principal Coordinates Analysis |
| **PCR** | Polymerase Chain Reaction |
| **PDA** | Potato Dextrose Agar |
| **RPM** | Revolutions per Minute |
| **Sq. Km** | Square Kilometers |
| **SSRs** | Simple Sequence Repeats |
| **UV** | Ultra Violet radiation |
| **UPLC** | Ultra Performance Liquid Chromatography |
| **µg/kg** | micrograms per kilogram |

## ABSTRACT

Aflatoxin contamination of maize in Africa poses a major threat to food security and the health of many African people. In Kenya, aflatoxin contamination of maize is high due to the environmental, agricultural and socio-economic factors. Many studies have been conducted to understand the scope of the problem especially at pre-harvest level, but few of them have been concentrated to the post-harvest level. This research was carried out to gather scientific information on the fungi population, genetic diversity and aflatoxin levels during post-harvest period. The study was conducted in three geographical locations of; Kitui, Trans-Nzoia and Nakuru Counties. Samples were collected from storage structures of farmers and transported to the Biosciences eastern and central Africa (BecA), International Livestock and Research Institute (ILRI) hub laboratories. Mycoflora were recovered using the direct plating method. A total of five fungal genera (*Aspergillus, Penicillium, Fusarium, Rhizopus* and *Bsyochlamys spp.*) were isolated from the stored maize samples. The most common fungal species that were isolated from the three study sites included *A. flavus* at 82.03 % followed by *A. niger* and *F. solani* at 49 % and 26 % respectively. The aflatoxin producing fungi *A. flavus* was recovered in 82.03 % of the samples. Aflatoxin levels were analysed on both the maize samples and *in vitro*. Most of the *A. flavus* isolates recorded a high level of aflatoxin when they were analysed for presence of aflatoxin $B_1$ using ELISA. In Kitui, all the samples had aflatoxin levels above 10 ppb with a total aflatoxin mean of 219.2 ppb. In Trans-Nzoia, only 3 samples (n=39) had their aflatoxin levels less than 10 ppb while in Nakuru, the total aflatoxin mean level of this region was 239.7 ppb. When individual samples were analysed using Vicam fluorometer method, aflatoxin analysis revealed that most of the samples (58.4 %) had been contaminated. The means were significantly different ($p \leq 0.05$) in all the three locations. Genetic relationships of *A. flavus* isolates were determined using 13 Simple Sequence Repeats (SSRs) markers. The results were used to generate a phylogenetic tree using DARwin5 software program. A total of 5 distinct clusters were revealed among the genotypes. The isolates appeared to cluster separately according to the geographical locations. Principal Coordinates Analysis (PCoA) of the genetic distances among the 91 *A. flavus* isolates explained over 50.3 % of the total variation when two coordinates were used to cluster the isolates. Analysis of Molecular Variance (AMOVA) showed high variation of 87 % within populations and 13 % among populations. This research has shown that *A. flavus* is the main fungal species infecting maize grains in the three sampled sites in Kenya. The effects of aflatoxins on human populations in Kenya demonstrates a clear need for tools to manage contamination of locally produced maize. Food basket surveys for aflatoxin contamination should be conducted on a regular basis. This would assist in obtaining reliable data on aflatoxin incidence in different food crops. This would go a long way in defining the most appropriate and long-lasting control strategies for this menace that would suffice to the entire Kenyan population.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the study

Aflatoxins are potent carcinogenic and mutagenic metabolites that are produced mainly by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus* (Banu, 2010). These two fungal species are mostly responsible for contamination of foods such as cereals, nuts, cotton among other crops. Aflatoxin poisoning has been a problem of major concern in most parts of Africa posing a threat to food security and also as a major risk factor to animals and humans. Maize (*Zea mays* L.) is the most important staple food for the majority of the Kenyan population (EPZA, 2005; Kimanya *et al.,* 2008) and it is consumed as a staple at an average intake of about 400 gm per person per day (Muriuki and Siboe, 1995; Shepherd, 2008). Genotype, soil types, drought and insect activity are important factors that determine the likelihood of pre-harvest contamination (Cole *et al.,* 1995). Poor harvesting practices like improper storage or keeping cereals in less than optimal conditions during transportation can also contribute to fungal growth and proliferation of mycotoxins (Bhat and Vasanthi, 2003; Betran and Isakeit, 2003;Wagacha and Muthomi, 2008).

Aflatoxins have been detected in many countries of the world especially in the tropical and subtropical regions where the weather is warm and humid thus provides optimal growth conditions for the development of aflatoxigenic moulds (Odoemelam and Osu, 2008). This therefore means agricultural crops and animal feed in both tropical and sub-tropical regions are more prone to aflatoxin contamination compared to the temperate regions (Odoemelam and Osu, 2008). Although fungi are ubiquitous and cosmopolitan in nature, the problem of mycotoxicoses is more pronounced in tropical developing countries as a result of their higher humidities,

temperatures and generally poor pre and post harvest agricultural practices (Fandohan *et al.,* 2003; Hell *et al.,* 2003). People living in tropical areas are therefore often exposed to aflatoxin before and after birth and throughout their lives posing a serious impact on their health (Williams *et al.,* 2004; Wild and Gong, 2010). Kenya is one of the countries in tropical regions that have favourable conditions of 37 °C and 85 % humidity (Diener *et al.,* 1987) for fungal growth n most locations.

Histories on outbreaks of acute aflatoxicoses have also been reported (Probst *et al.,* 2007). For instance, the first outbreak of aflatoxicosis in Kenya was reported in 1978 followed by 1984-1985 where a large number of dogs and poultry were reported dead due to aflatoxin poisoning (Manwiller, 1986). In 1978, aflatoxin contamination in dog meal exceeded 150 ng/g with the highest being 3000 ng/g (Manwiller, 1987). Another outbreak of aflatoxin poisoning was reported in the period between January and July 2004. A total of 125 people were reported dead with over 300 others being hospitalized (Lanyasunya *et al.,* 2005). These cases confirm that consumption of food or feed that contains aflatoxin could lead to serious detrimental effects to the health of both humans and animals due to inadequate pre-harvest and post-harvest techniques in prevention of mould growth (Odoemelam and Osu, 2008). Human exposure to aflatoxins results from direct ingestion of contaminated foods or indirectly through consumption of meat or animals previously exposed to aflatoxins in their feeds (Rustom, 1997). This study was therefore carried out to determine the level of aflatoxin contamination in stored maize and determine the diversity of post-harvest fungi in three agro-ecological zones in Kenya. The gathered information from this study was aimed at educating farmers from these regions on risks associated with

aflatoxin and their management; a strategy that could be used in controlling this frequent recurring challenge.

## 1.2 Problem statement and justification

Aflatoxin contamination in foodstuffs has been a recurrent problem in Africa (Shepherd, 2003). Chronic exposure to aflatoxin, which has far reaching effects, has also not been well documented (Williams *et al.,* 2004). As a cereal crop, maize is a very important commodity, both as food and animal feed. However, contamination of maize by mycotoxins represents a widespread problem. Maize is easily contaminated by toxigenic moulds such as *Aspergillus* and *Fusarium* species that are usually found in the field and are the source of mycotoxin contaminants during storage. In many parts of Africa, proper storage of foodstuff is a challenge hence predisposing these foods to fungal contamination. Food safety is therefore minimal and food-borne toxications have become a serious problem (Bankole and Adebajo, 2003).

FAO (2002) has documented maize as one of the most important dietary foods in the world. Most communities in Kenya rely on maize as their main source of food. Conducive environmental parameters and poor grain storage practices heighten the level of aflatoxin contamination. Many strategies, including biological control, control of insect pest and development of resistant cultivars have been investigated to manage aflatoxins in crops. However, limited scientific work has been conducted at post-harvest level to understand the scope of the prevalent fungi and aflatoxin accumulation. There was therefore need to evaluate the quality and levels of fungal contamination at post-harvest level and understand the problem before advising on implementing some control measures.

## 1.3 Research questions

i. What is the toxigenic *Aspergillus* species diversity across the three study sites of Kitui, Trans-Nzoia and Nakuru Counties?

ii. What is the level of aflatoxin contamination in stored maize in the three geographical locations of Kitui, Trans-Nzoia and Nakuru Counties?

## 1.4 Hypotheses

i. Toxigenic *Aspergillus* species colonizing stored maize do not differ genetically from one study site to another in Kenya.

ii. Aflatoxin levels in stored maize do not vary among the three study sites of Kitui, Trans-Nzoia and Nakuru Counties.

## 1.5 Objectives

### 1.5.1 General objective

i. To assess the toxigenic *Aspergillus* species, its diversity and aflatoxin level in stored maize in Kitui, Trans-Nzoia and Nakuru Counties.

### 1.5.2 Specific objectives

i. To assess the total mycobiota and fungal profile in stored maize in Kitui, Trans-Nzoia and Nakuru Counties.

ii. To characterize the toxigenic *Aspergillus* species across the three geographical regions of Kitui, Trans-Nzoia and Nakuru Counties.

iii. To determine the aflatoxin levels in stored maize collected from of Kitui, Trans-Nzoia and Nakuru Counties.

## 1.6 Significance of the study

These results will be used to create awareness to the farmers on proper post-harvest practices that will minimize the chances of their maize being contaminated by post-harvest fungi. This study will also be used through these findings to educate farmers in the study areas on the risk factors that enhance aflatoxin contamination of maize and how they can take caution while preserving their maize after harvest. It is anticipated that these findings will go a long way in reducing aflatoxin contamination hence enhancement towards food security in Kenya.

# CHAPTER TWO

# LITREATURE REVIEW

## 2.1 Economic importance, cultivation and storage practices of maize in Kenya

In sub-Saharan Africa, maize is the most important cereal crop. An estimated 50 % of the population relies on it as their staple food. Africans consume maize in a wide variety of ways such as porridge, pastes and beer. Green maize, fresh on the cob, is eaten when it is baked, roasted or boiled. In sub-Saharan Africa, maize is mostly grown by small-scale farmers, generally for subsistence as part of mixed agricultural systems (Figure 1). In Kenya, maize is the major staple crop for both the rural and urban population. It constitutes 21 % of the total value of primary agricultural commodities (GoK, 1998). It is cultivated by both small-scale farmers and large scale farmers on an estimated 2.3 million hectares (Okoth *et al.,* 2012). It thus contributes an effective food security system in any society especially in Africa (Agoda *et al.,* 2011). The small-scale farmers contribute 75 % of the total maize produced in Kenya while large scale farmers contribute the remaining 25 % (Okoth *et al.,* 2012).

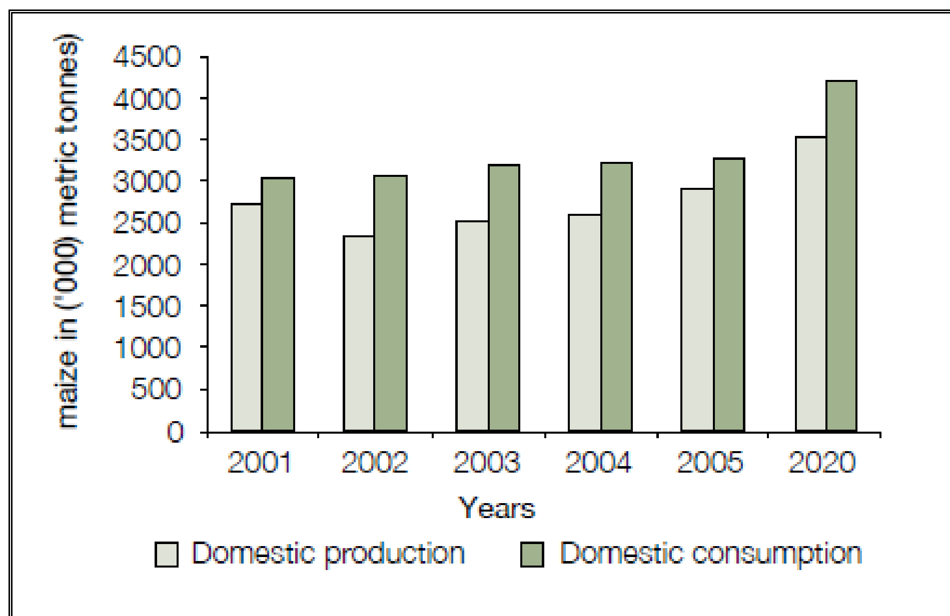


**Figure 2.1:** Maize production and consumption in Kenya from 2001- 2020
*Source:* Republic of Kenya, Economic Survey, 2006

In Kenya, almost every family has a garden where they normally cultivate maize mostly for their own consumption and this signifies the importance of this crop to the Kenyan population (Okoth *et al.,* 2012). Both cereals and legumes are important since they constitute primary sources of food for man. Cereals are rich in methaionine that is limited in legumes (Aworh, 1999). Legumes on the other hand are rich sources of lysine and tryptophan amino acids which are insufficient in cereals (Agoda *et al.,* 2011). When maize and beans are combined in a meal, they provide better nutrition than when eaten separately (Agoda *et al.,* 2011). Maize flour which is obtained by grinding maize is used to manufacture bread, biscuits, dough and pap (Agoda *et al.,* 2011).

During storage, maize grains can undergo quantitative and qualitative losses mainly due to improper storage. Grain storage is a crucial component of the post-harvest chain. It is practiced by farmers, traders and governments to facilitate marketing and ensure food security. Different communities in Kenya practice varied storage methods for maize preservation. Drying of maize in the open sun is one technique practiced by almost all communities. However, if maize is not properly dried before it is stored; the risk of aflatoxin contamination is very high. Fungal growth and aflatoxin contamination can occur within a few days if maize is not properly dried before storage (Hell *et al.,* 2008). It can increase up to ten times in three days if the maize was not properly dried (Tanboon-ek, 1989; Hell *et al.,* 2008). Aflatoxins have no colour, are odorless and tasteless thus, they are difficult to detect. Since aflatoxins mostly develop on grains and legumes during storage, they must be stored correctly to limit this problem. Most storage structures such as granaries, silos or sisal sacks also favour mycotoxin production and proliferation especially if there is poor ventilation. Most farmers in Africa use polythene bags for storage which are poorly aerated and hence encourage fungal growth and aflatoxin contamination (Udoh *et al.,* 2000; Hell *et al.,* 2000b).

## 2.2 Fungi as agents of food spoilage

### 2.2.1 Food spoilage

Most fungi are able to grow on a variety of foods such as cereals, nuts, fruits, vegetables, fats, meat, milk as well as their by-products (Filtenborg *et al.,* 1996). During their growth, these moulds produce a number of enzymes such as proteases, carbohydrases and lipases that lead to deterioration in sensorial properties (Bigelis, 1992; Filtenborg *et al.,* 1996). This leads to reduction of food quality that is normally characterized by off-flavours, discoloration, rotting and production of toxins (Chelkowski, 1991; Bigelis, 1992; Gravesen *et al.,* 1994: Tipples, 1995). The enzymatic activities give rise to stale odours in wine or dried fruits (Tindale *et al.,* 1989; Whitfield *et al.,* 1991; Illy and Viani, 1995) caused by fungal transformation of 2,4,6 trichlorophenol to trichloroanisol (TCA) by *Aspergillus flavus* and some *Penicillium* species (Filtenborg *et al.,* 1996). Other toxic compounds are produced in species specific combinations of different genera that include *Aspergillus, Penicillum* and *Fusarium* (Larsen and Frisvad. 1995a, b).

### 2.2.2 Life cycle, distribution and transmission of *Aspergillus flavus* fungus

The haploid fungus *Aspergillus flavus* belongs to the genus *Aspergillus* Division *Ascomycota* and class *Eurotiomycetes* (Kaaya and Warren, 2005). It is the second most common species after *Aspergillus fumigatus. A. flavus* and the closely related subspecies *Aspergillus parasiticus* have a worldwide distribution and normally occur as saprophytes in the soil and on many types of decaying organic matter (Dvorockova, 1990). These two species, *A. flavus* Link and *A. parasiticus* Speare are the most predominant fungi responsible for aflatoxin contamination of food crops either prior to harvest or during storage (Creepy, 2002; Yu *et al.,* 2004). These fungi grow on a wide variety of agricultural commodities like dried corn, peanuts, groundnuts, cotton

seed, millet and tree nuts (Micheal and Ensley, 2007). They have also been found growing on water damaged carpets. Other *Aspergillus* species such as *A. niger, A. carbonarius* and *A. ochraceus* that colonize cereals and cereal-based products produce ochratoxin A in tropical parts of the world (Accensi *et al.,* 2004; Magnoli *et al.,* 2006). *A. flavus* grows saprophytically on different parts of the maize plant like the cob, leaf or kernels (Figure 2). Maximum decomposition caused by this fungus occurs when there is sufficient nitrogen, phosphorus and other essential inorganic nutrients. The main reservoir for these filamentous fungi is agricultural soils (Horn and Dorner, 1998) where it forms the primary innocula. *A. flavus* produces prodigious amounts of conidia which are spread by wind, water, insects and birds.

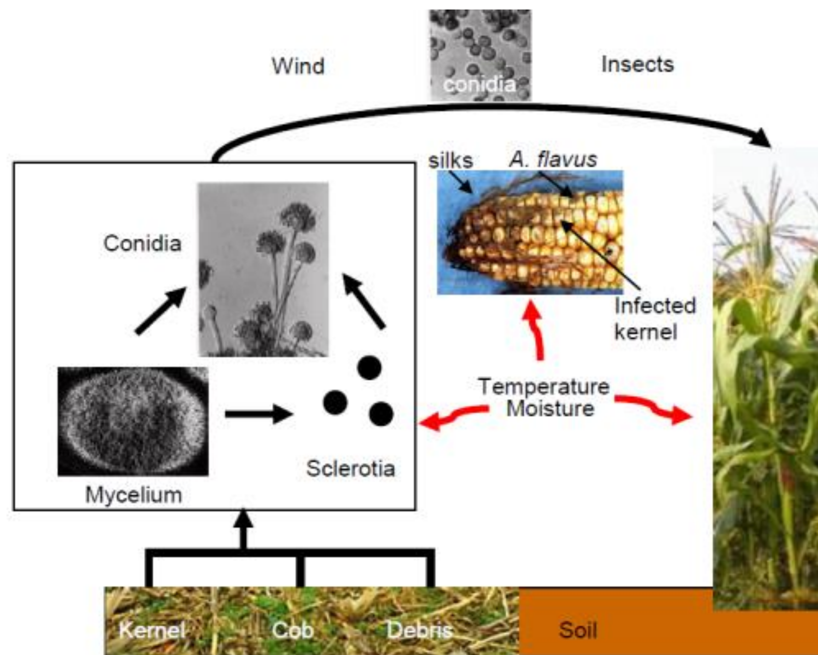


**Figure 2.2:** Life cycle of *A. flavus* and how it infects corn.
Source: Image adapted from http://www.aspergillusflavus.org/images/cycle.jpg. Fungal images from: http://www.cbs.knaw.nl. Date accessed: 22.02.2013.

In the laboratory, *A. flavus* is known to reproduce asexually with some potential for nonsexual recombination in highly similar isolates (Papa, 1986). Colonies of *A. flavus* grow rapidly and will reach a diameter of 6 - 7 cm in 10 - 14 days. *Aspergillus* spores are common components of

aerosols where they drift on air currents, dispersing themselves over both short and long distances depending on environmental conditions. When the spores come in contact with a solid or liquid surface, they are deposited and if conditions of moisture are right, they germinate (Kanaani *et al.,* 2008). The most important aspect of food spoilage by fungi is the production of mycotoxins due to their detrimental effects on human health (Filtenborg *et al.,* 1996). These mycotoxins can be present in the fungi itself but most of them are excreted into the foods. In liquid foods such as pears, peaches, tomatoes and most fruits, these mycotoxins diffuse very fast leaving the entire fruit contaminated. For solid foods however, diffusion is much slower hence mycotoxin contamination occurs to a much lesser extent (Filtenborg *et al.,* 1996). Toxicity of these mycotoxins varies with the most important toxic effects being immune suppression and carcinogenic effects (Miller, 1991; Pestka and Bondy, 1994: Prelusky *et al.,* 1994).

### 2.2.3 Mycotoxins and responsible fungi

Mycotoxins are small, molecular weight compounds produced by fungi that are toxic to animals and humans when introduced through a natural route (Richard and Payne, 2003). They are produced by toxigenic fungi during both pre- and post-harvest periods (Gnonlonfin *et al.,* 2013). Mycotoxins are very resistant to physical and chemical treatments. Once they occur in food, they continue to persist even during processing and storage (Scott, 1991). Individual mycotoxins are produced by a limited number of species.

## 2.3 Mycotoxins and their causal agents

Mycotoxins commonly occur in agricultural commodities including human food and animal feed (Pande *et al.,* 2002). When introduced to animals or humans in low concentrations, they are

known to cause toxic responses (Smith *et al.,* 1994). Contamination of food or animal feed by mycotoxins can occur during both pre-harvest and post-harvest periods (Cardwell and Cotty, 2002; Magan and Olsen, 2004 ). Field fungi are responsible for pre-harvest mycotoxin contamination while storage fungi mainly cause post-harvest contamination (Ghiasian and Maghsood, 2011). There are more than 400 different types of mycotoxins that are produced by various species of filamentous fungi. Most of the mycotoxins of concern are those produced by three fungal genera: *Aspergillus, Penicillium and Fusarium* (Wogan, 1999). These species occur worldwide in air and soil in form of spores and when they occur in food, they lead to food spoilage and biodeterioration. It is estimated that about 25 % of the world's food crops gets contaminated with mycotoxins causing substantial economic losses to national economies according to FAO (2003). Mycotoxin contamination is known to cause serious health effects including liver damage, immunosuppression, nephrotoxicoses, neurotoxicoses, hepatotoxicoses, reduced egg and milk production in poultry and dairy animals (IARC, 2002). If animals are fed with mycotoxin contaminated feed, their products such as meat, milk and eggs subsequently become contaminated (Fink-Gremmels, 2008). The presence of mycotoxins in maize often leads to a lowered market value that posses a direct effect on maize production among farmers (Cardwell *et al.,* 2001).

### 2.3.1 Aflatoxins

Aflatoxins are the most widely studied group of mycotoxins (Hussein and Brasel, 2001). The first report was that of the 1960s in England during which the turkey X disease caused more than 100,000 deaths of turkey (Klich *et al.,* 2000; Papp *et al.,* 2002; Kuhn and Ghannoum, 2003). They are the most important mycotoxins with respect to their occurrence, effect on human health, toxicity and trade (Gnonlonfin *et al.,* 2013). Aflatoxins are toxic and carcinogenic

metabolites produced by species of *Aspergillus* fungi, especially the strains *A. flavus*, *A. parasiticus* and *A. nomius.* Other *Aspergillus* species that produce aflatoxin to a lesser extent include *A. bombycis, A. ochraceoroseus* and *A. pseudotamarii* (Goto *et al.,* 1996; Klich *et al.,* 2000; Peterson *et al.,* 2001). *A. flavus* strains are classified according to those that produce large sclerotia (L-strains) and those that produce small sclerotia (S-strains) (Mohale and Allotey, 2011). Both strains produce only type B aflatoxin ($B_1$ and $B_2$). Although the S-strain is not very commonly isolated, it is a potent producer of aflatoxin as it produces numerous sclerotia (Mohale and Allotey, 2011).

Aflatoxins are difuranocoumarin derivatives of which more than 20 different types are known to occur (Hussein and Brasel, 2001; Papp *et al.,* 2002). Of the 20, there are four major types of aflatoxins; $B_1$, $B_2$, $G_1$ and $G_2$, produced primarily by the closely related fungi; *A. flavus* and *A. parasiticus* (Figure 2.3) (Muro-Cacho *et al.,* 2004). These four compounds are separated by the color of their fluorescence under long-wave ultraviolet illumination (B-Blue, G-Green) (Dutton and Heathcoat, 1966). The subscripts relate to their relative chromatographic mobilities. Of the four, $B_1$ is often the highest in concentrations followed by $G_1$ and $G_2$. *A. flavus* produces only $B_1$ and $B_2$ while *A. parasiticus* produces the same metabolites along with $G_1$ and $G_2$. Two additional metabolic products, aflatoxin $M_1$ and $M_2$ are found in feed of lactating animals fed on aflatoxin contaminated feed. Aflatoxin $G_1$ and $M_1$ are both hydroxylated metabolites of aflatoxin $B_1$. Cyclopiazonic acid is another unrelated mycotoxin produced by *A. flavus* but not by *A. parasiticus* (Horn *et al.,* 1996). A third species, *A. nomius* has a mycotoxin profile that is similar to that of *A. parasiticus* although it morphologically resembles *A. flavus* (Kurtzman *et al.,* 1987; Peterson *et al.,* 2001).

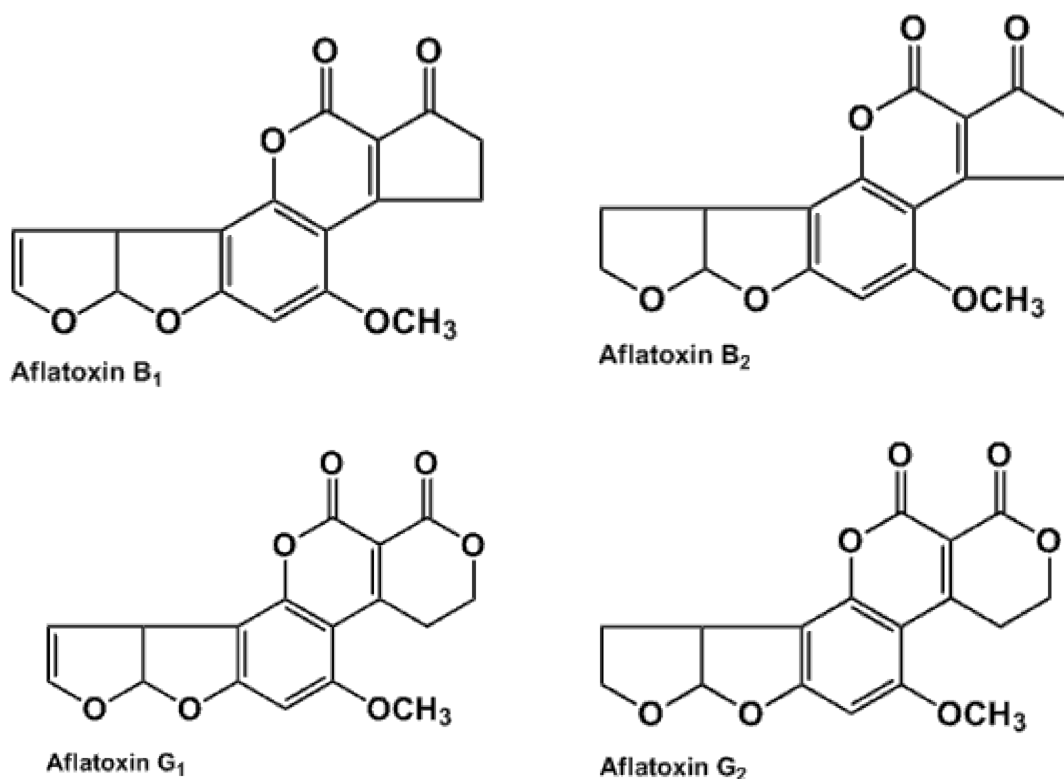


**Figure 2.3 :** Structures of the common types of aflatoxins.

### 2.3.2 Health hazards caused by aflatoxin consumption

Aflatoxin contamination is a worldwide problem that has devastating effects to the agricultural commodities, economy, industries and regions where it occurs (Dorner, 2004). Exposure to aflatoxins in many African countries has been shown to occur even before birth (Montesano *et al.,* 1997). Aflatoxin consumption by animals and humans results in diseases known as aflatoxicoses. These diseases can occur in two forms of either chronic or acute aflatoxicoses. Chronic aflatoxicoses causes slow metabolism, stunted growth, immune suppression, cancer and other “slow” pathological conditions (Hsieh, 1988). Acute aflatoxicoses causes impaired digestion, liver damage, hemorrhage and eventually death. As such, outbreaks of aflatoxin poisoning occur when there is consumption of aflatoxin contaminated food (Gnonlonfin *et al.,*

2013). The largest outbreak of aflatoxicoses to have been recorded occurred in Western India in 1974 where 106 deaths were reported and another 397 cases were recognized (Krishnamachari *et al.,* 1975).

In Kenya, various cases of aflatoxin poisoning have been reported. From March to June 1981, several people were admitted in Eastern Kenya due to aflatoxicoses with 50 % of them reported to have succumbed to death (Moturi, 2008). In the following year, there was another outbreak with a 60 % mortality being reported (Nagindu *et al.,* 1982). Similar cases of acute aflatoxicoses have been reported in Eastern Kenya in the years; 2001, 2004, 2005 and 2006 during which many people especially children died (Aziz *et al.,* 2005; WHO 2006). The severest outbreak took place in the year 2004 in Kenya with 39 % mortality in which 317 total number of cases were reported (Nyikal *et al.,* 2004; Onsongo *et al.,* 2005). Out of the 317 cases reported, 89 % of the patients resided in Kitui and other districts in Eastern and Central Kenya. The maize products in these regions had aflatoxin levels above the Kenyan regulatory limit of 10 ppb. About 90 % had levels greater than 100 ppb and 7 % had levels greater than 1,000ppb (CDC, 2004). These findings were however limited to medical facilities and health centers. Due to the extensive geographical area and remote nature of the affected villages, some people were not able to reach health facilities for diagnosis and medication. As such, the true magnitude of the outbreak was most probably greater than that which was reported (CDC, 2004; Probst *et al.,* 2007).

These cases have continued to recur in the drought stricken counties in Kenya. Aflatoxins $B_1$ $B_2$ $G_1$ and $G_2$ are hepatocarcinogens and they have been classified as group I human carcinogens while $M_1$ is classified as a group 2B probable human carcinogen (Krishnamurthy and Shashikala, 2006). Epidemiological studies reveal that people who are continually exposed to diets

contaminated with aflatoxin stood a higher risk of contracting liver cancer (Turner *et al.,* 2002). Hepatitis B can act synergistically with aflatoxins leading to an increase in contracting hepatocellular carcinoma (Turner *et al.,* 2000). In sub-Saharan Africa, about one in every ten individuals is infected with either Hepatitis B or C (Gnonlonfin *et al.,* 2013). If these individuals are exposed to aflatoxin, they raise the risk of liver cancer by more than ten times as compared to exposure of both hepatitis alone (Turner *et al.,* 2003). HIV and esophageal cancer related deaths have been shown to be significantly related to maize consumption (Gnonlonfin *et al.,* 2013). However, the infection rate was shown to be minimized when maize consumption was reduced in the sub-Saharan region. This shows an interesting interaction between aflatoxin and HIV in immunosuppression.

According to Jiang *et al.* (2005), presence of aflatoxin in the immune system facilitates HIV immune hyperactivity and thereby leading to severity of the disease. Due to these immunosuppressive effects of aflatoxin, the individual is predisposed to secondary infections owing to the presence of other bacteria, viruses and fungi (McLean, 1995). In the reproductive system, aflatoxins have been associated with infertility in men according to a survey conducted by Uriah *et al.,* (2001). In animals, aflatoxin poisoning has been blamed for reduced appetite and feeding especially in pigs fed with contaminated feed (Harvey *et al.,* 1988). In Kenya, aflatoxin contamination in dairy feed has been reported to occur (Lanyasunya *et al.,* 2005). If the level of aflatoxin $M_1$ in dairy products like milk is above the legal limit of 20 µg/kg, it becomes a serious health concern (Lanyasunya *et al.,* 2005). In addition to the toxin, the *Aspergillus* fungi itself is hazardous and can affect humans and animals in different ways. The disease that results from *Aspergillus* infections is known as aspergillosis. This mould affects humans or animals that are

allergic to the mould and elicit chronic or acute reactions. Immunocompromised individuals are also at risk of contracting aspergillosis which is a serious disease that is at times fatal. This disease invades the lungs and other vital organs (Figure 2.4). Due to the severity of this disease, it is advisable that dust masks should be worn by grain handlers to minimize exposure to aflatoxin contaminated dust and the responsible fungi (Stack and Carlson, 2006).

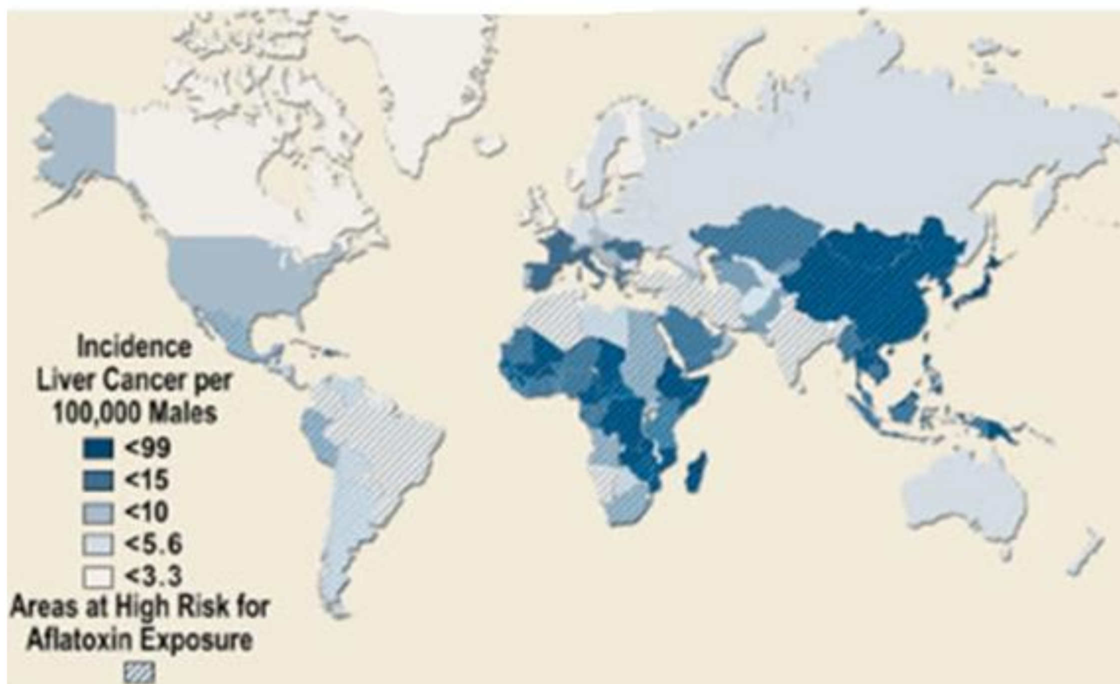


**Figure 2.4 :** Global occurence of aflatoxins.
Source: Aflatoxin data from Williams *et al,* (2004). Human aflatoxicoses in developing countries. Liver data from GLOBOCAN 2002 Database.

### 2.3.3 Aflatoxin contamination of food commodities

Aflatoxins are extremely durable during all the stages in the food transport chain. They can persist during storage, handling and processing of foods originating from contaminated seeds. Aflatoxins are also more prevalent during drought thereby raising the issue of food safety. This is because high temperatures combined with low amounts of rainfall favour growth and continued survival of mycotoxic moulds. During the dry season, food crops become stressed by these unfavourable parameters and may also become weakened by insect damage. When the aflatoxin producing fungi *A. flavus* colonizes maize, it forms a green mould on the kernel surface. This mould can also develop on the corn ear but it is mostly observed at the base of the ear. However, mould growth is at times not visible. It should also be noted that colonization of maize kernels by *A. flavus* does not automatically lead to aflatoxin contamination. Aflatoxins are therefore economically important due to their ability to contaminate a wide variety of human food and animal feed (Kaaya and Warren, 2005).

A lot of studies have focused on aflatoxin contamination of major food crops like maize, peanut, cottonseed, groundnuts, tree nuts among other crops. However, a few studies have paid attention to aflatoxin contamination in beans. In a study conducted in Benin by Houssou *et al.* (2009), there was no aflatoxin contamination in cowpea even though *A. flavus* was frequently isolated. Rahimi *et al,* (2010) reported the presence of aflatoxin $M_1$ in raw milk of cows and buffalos in Iran at high concentrations that exceeded the maximum tolerance limit of 50 ng/l according to the European Union. The same toxin, aflatoxin $M_{1,}$ was found in cow's breast milk from Australia, Thailand and Victoria (El-Nezami *et al.,* 1995).

### 2.3.4 Other mycotoxins commonly isolated from maize

#### 2.3.4.1 Fumonisins

Fumonisins were first identified and characterized in 1988 (Bezuidenhout *et al.,* 1988; Gelderblom *et al.,* 1988). They are mainly produced by *Fusarium* fungi which contaminate cereals and grains leading to reduction in crop yields (SCEC, 2003). Most *Fusarium* species are plant pathogens and are responsible for many diseases including root rot and seedling blight in cereals and *Fusarium* wilt in bananas. Maize diseases caused by *Fusarium* fungi have been reported in different places in the world since 1980s (Palazon *et al.,* 1982). The main mycotoxin producing *Fusarium* fungi are *F. verticilliodes, F. proliferatum, F. oxysporum, F. graminierum* and some strains of *F. subglutinans* (Eckard *et al.,* 2011) *F. verticilliodes* was formally known as *F. moliniforme* and it reproduces sexually and at times this fungus is referred to by its sexual stage name*, Gibberella moliniformis. F.verticilliodes* has been known as the major colonizer of cereals and grains where it produces fumonisins (SCEC, 2003, Shepherd, 1996). *F. proliferatum* has also been reported to occur in stored maize from Benin (Fandohan *et al.,* 2005). In small grain cereals, infection by *Fusarium* fungi such as *F. graminearum, F. avenaceum*, *F. culmorum*, *F. poae* and *F. crookwellense* leads to Fusarium head blight (FHB) (Parry *et al.,* 1995; Bottalico *et al.,* 2002). In growing maize, *Fusarium* infection leads to stalk and ear rot (Eckard *et al.,* 2011). There are about 12 types of fumonisins (Gelderblom *et al.*, 1991; 1992; 1993; Musser and Plattner, 1997; Sweeney and Dobson, 1998) with Fumonisin B1 (FB1) being classified as a possible carcinogen to humans (IARC, 2002). The maximum tolerable daily intake of fumonisin has been set at 2 µg/kg according to the European Commission Scientific Committee of Food (SCEC, 2002).

#### 2.3.4.2 Deoxynivalenol

Deoxynivalenol (DON) belongs to the trichocethenes group of mycotoxins that are produced by the *Fusarium* genera. DON mainly produced by the fungal species *F. graminearum*, *F. crookwellense* and *F. culmorum.*. This mycotoxin affects maize leading to Gibberella ear rot (JECFA, 2001). Infection of cereals by these fungi results in decrease in grain size and protein content and also affects seed germination. The maximum limit of intake of DON in animal feed for dairy cows has been set at 1 μg/kg. Zearalenone is another mycotoxin produced by these species in addition to *F. equiseti and F.oxysporum* (Leslie and Summerell, 2006). DON has been known to cause serious health effects including pulmonary oedema in pigs, leucoencephalomalacia (LEM) in equine species and oesophageal cancer in humans (Visconti *et al.,* 1996).

### 2.4 Factors influencing growth of toxigenic fungi and subsequent toxin production

Many soil micro-organisms rely on fungi as their food source (Carroll *et al.,* 1992). Production of mycotoxins by fungi is mainly influenced by biotic and abiotic factors (Gnonlonfin *et al.,* 2013). Biotic factors include micro-organisms, insects and available substrate while abiotic factors include temperature, moisture content, relative humidity and soil composition. Insects have been showed to play a major role in the plant infection and contamination process. They act as vectors transporting conidia that adhere on their bodies to other parts of a plant (Diener *et al.,* 1987). The *A. flavus* fungus grows best at moisture content levels of between 18 - 18.5% (Wrather, 2008). As the fungus grows, it respires releasing heat and moisture into the immediate environment of the grain. This translates to increased temperature and moisture of the surrounding grain mass causing a hot spot. Therefore, as these two factors continue to rise, the environment becomes favourable for *A. flavus* growth.

If the moisture content continues to rise and goes above 20%, other fungi grow better and crowd out *A. flavus* (Wrather, 2008). Aflatoxin contamination has been reported to be more pronounced in years that recorded below average rainfall. Cracks in maize kernels provide easy entry sites for *A. flavus* hyphae during drought stress (Wrather, 2008). This shows strong evidence that drought stress alone is a single contributor to elevated aflatoxin levels. Aflatoxin production is favoured at temperatures of 17- 42 °C with optimum temperatures of 25-32 °C. However temperatures above 30 °C can stress the plant giving the invading fungus a greater advantage (Chen *et al.,* 2004). Plant stress can also occur if the essential nutrients are limited. Maize kernels that undergo nitrogen stress produce higher levels of aflatoxin compared to those receiving the nutrient at the required amount (Payne *et al.,* 1992). Low amounts of nitrogen can be brought about by conditions of drought stress.

### 2.4.1 Environmental factors

Aflatoxin contamination is mostly associated with the tropical region which has warmer and drier climate (Shepherd *et al.,* 1996; Hell *et al.,* 2003). Climatic patterns influence contamination with direct effects on the causative fungi (Gnonlonfin *et al.,* 2013). As the climate changes, the aflatoxin producing fungi also shift. These changes are reflected in the environment and altered communities of the fungal population structure. Certain environmental factors favour aflatoxin contamination of maize by *A.flavus* while it is still in the field. This includes high soil and air temperatures, drought and nitrogen stress, crowding of plants and other conditions that speed up the dispersal of conidia (Diener *et al.,* 1987). Physical factors such as light, temperature, pH, moisture, aeration and level of atmospheric gases are known to influence aflatoxin production. Optimum temperature and water activity are vital factors during mycotoxin production.

Aflatoxins are only produced between temperatures of 12 $^{o}$C and 42 $^{o}$C and the optimal temperatures is 25 $^{o}$C to 35 $^{o}$C (Diener and Davis, 1996). Maximum moisture content for aflatoxin production in corn kernels is 25 % at 30 $^{o}$C and the minimum relative humidity for aflatoxin production varies between 83 % and 88 %. Moisture content levels in various food commodities are directly related to the resultant aflatoxin contamination. This has been well documented in foods like maize, cowpea and groundnut (Hell *et al.,* 2000b; Kaaya *et al.,* 2006; Houssou *et al.,* 2009). Presence of both $CO_2$ and $O_2$ positively influences production of aflatoxin. Decreasing $O_2$ concentration in the air to 10 % depresses aflatoxin production, but only at $O_2$ levels of less than 1 % are growth and aflatoxin production completely inhibited (Diener *et al.,* 1967). This shows that aflatoxin production is favoured by aerobic environments. Environmental conditions can be controlled in storage facilities but this comes with a high additional cost (Gnonlonfin *et al.,* 2013). This strategy can however reduce the problem of aflatoxin contamination at post-harvest level.

### 2.4.2 Pre-harvest and post-harvest operations

Contamination of maize with aflatoxin begins while maize is still growing in the field and continues even after harvest. Various agricultural commodities are affected depending on the type of food. Geographical location and conditions, production and farming practices are some of the factors that determine the level of aflatoxin contamination while crops are still in the field. Pre-harvest practices employed during crop production such as tillage methods, application of fertilizer, plant population, date of planting and irrigation techniques are significantly associated with aflatoxin levels if they are not properly managed (Campos *et al.,* 2008). Delayed harvest also increases the chances of fungi to attack the maize kernels. This is because insects and birds may continue to feed on the maize while it is still in the field. During this period, the moisture

content in the grains remains high enough to allow continued fungal development and subsequent toxin production. This damages the kernels making them more vulnerable to toxigenic post-harvest fungi. In Africa, crops are cultivated during the rainy season using low amounts of fertilizer and little pesticide use. These conditions normally enhance *A. flavus* infection of fertility stressed plants (Hell *et al.,* 2008). The rate of fungal infection can be influenced by factors such as microbial interactions and presence of other toxigenic strains (Horn, 2003). Certain agricultural practices like crop rotation and eradication of crop residues can reduce *A. flavus* infection in maize while still in the field (Hell *et al.,* 2008).

Post-harvest contamination of aflatoxin in maize is a serious health constraint for the African population (Hell *et al.,* 2008). Aflatoxin levels may decline during the storage period but they may still be present even after 7 years (Abbas, 2005). Post-harvest practices such as harvesting, drying and time of harvest greatly determine the extent of post-harvest contamination (Hell *et al.,* 2008). In most parts of Kenya, maize is usually air dried and may therefore take a longer time to dry depending on the weather. The storage form of maize can also influence contamination by toxigenic fungi. Maize that is shelled immediately after harvest was found to contain higher aflatoxin levels as compared to maize that was left drying on the cob according to Mora and Lacey (1997). Fandohan *et al* (2006) found that damaged maize kernels and maize cobs that are shelled mechanically are more prone to aflatoxin contamination compared to those that are manully shelled. This becomes a threat to food security to millions of people who are struggling to meet their dietary needs. FAO (1996) explains that food security can only exist when people have met their nutritive and dietary needs for a healthy and active life. Of all the mycotoxins, Aflatoxin still remains the major threat compromising food security in sub-Saharan Africa.

### 2.4.3 Fungi interactions

Interactions between *A. flavus* and other fungi influence fungal infection and the resultant mycotoxin production. For instance interaction of *A. niger* and *A. flavus* can inhibit aflatoxin production in groundnuts (Hill *et al.,* 1983). In the tropics, harvested maize already contains spores and mycelia of the three commonly encountered fungal genera *Aspergillus, Fusarium* and *Penicillium.* If the environmental conditions are favourable, the fungi come into contact with food where it grows and competes with other species (Fandohan *et al.,* 2003). Some *Fusarium* species have been shown to be competitive and dominate against *A. flavus* when the water activity is greater than 0.96 (Marin *et al.,* 1998). This inhibition can lead to a significant reduction of aflatoxin contamination in infected maize grains (Zummo and Scott, 1992).

### 2.4.4 Role of insects in fungi infection and mycotoxin development

Among the various agricultural constraints, insect pests are major bottlenecks in obtaining higher crop yields. Insect infestation has been related to aflatoxin contamination both at pre-harvest (Setamou *et al.,* 1997) and post-harvest (Hell *et al.,* 2000a). Many species of insects facilitate the entry of mycotoxin producing fungi in commodities like maize, cotton seed, figs and nuts (Dowd, 2003). Insects enhance the fungal infection process by carrying the inoculum hence causing damage that permits entry of the fungus (Dowd, 2003). They therefore act as vectors by transporting fungal spores on their bodies thereby contaminating grains during their movement (Gnonlonfin *et al.,* 2013). The extent of insect damage is normally correlated to mycotoxin contamination. Aflatoxin and fumonisin are the major mycotoxins associated with insect damage. Dowd (2003) showed a number of insects that sometimes had a significant positive correlation with *A. flavus* and aflatoxin levels. These included insects such as beetles,

caterpillars, sugar cane borer, stem borer (*Sesamia calamistis*), large grain borer and the maize weevil. The relative importance of insect damage depends on the insect population, resistant mechanisms of plants to a given fungus and environmental factors favouring fungal growth (Dowd, 2003). An earlier study conducted in Benin showed that several insect species facilitated the presence of both *A. flavus* and *F. verticillioides* (Dowd, 2003) with the most consistent insect being the caterpillar ear borer, *Mussidia nigrivenella.* A two year survey conducted in the same country indicated that *M. nigrivenella* was significantly correlated with aflatoxins (r =0.36 and 0.52) in both years (Setamou *et al.,*1997). It also showed that this insect was the major source of maize damage in both years (Setamou *et al.,*1998). Both the percent grain damage and the percent incidence were significantly correlated with aflatoxin $B_1$ in both years (Setamou *et al.,* 1998). Another insect *Eldana saccharina* has been shown to be more important in promoting aflatoxin contamination in areas where they occur in high density (Setamou *et al.,* 1998).

## 2.5 Regulations of mycotoxins: Focus on aflatoxins

Aflatoxins are potent hepatotoxins and carcinogens (Cullen and Newberne, 1994; Roebuck and Maxuitenko, 1994) and their quantity in food and feed is closely monitored and regulated in most countries (Egmond and Jonker, 2005). Most countries and organisations have established regulations in order to protect consumers from harmful effects of mycotoxins (Van Egmond, 2002). For instance, the European Union has the most rigorous regulations regarding mycotoxins in food. For aflatoxin in foods, the limit has been set at 2 µg/kg for aflatoxin $B_1$ while it is 4 µg/kg for total aflatoxin (ECRC, 2005). It has also established maximum acceptable levels of aflatoxin $B_1$ in cereals, peanuts and dried fruits intended for direct human consumption. The levels have been set at 4ng/g for total aflatoxins ($AFB_1$, $AFG_1$, $AFB_2$ and $AFG_2$ ) and 2 ng/g for $AFB_1$ alone (Ricci *et al.,* 2007). The limits of total aflatoxin and aflatoxin $B_1$ in foods have been

set at 10 µg/kg and 5 µg/kg respectively in almost 80 countries world over (Herzallah, 2009). The food and drugs administration (FDA) in the United States has set the maximum limit of aflatoxin in food at 20 µg/kg (FAO, 1996). The level in milk is much lower at 0.5 ppb (Grybauskas *et al.,* 2000). In some European countries the regulated limit are as low as 5 ppb (Grybauskas *et al.,* 2000). In Kenya, the regulated limit of aflatoxin in food and feed has been set at 10 µg/kg (GoK, 2012; WFP, 2012). In countries without strict controls, aflatoxin certainly remains a significant public health problem (Wogan, 1992). However, the costs associated with the monitoring of aflatoxins and the rejection of the crops in the market is enormous (Robens and Cardwell, 2005). Consequently, aflatoxin contamination in maize is a major constraint to trade in the African region (Lubulwa and Davis, 1994). Due to these serious health effects associated with aflatoxin contamination in food and feedstuffs, various analytical methods have been developed for their quantification and detection.

### 2.6 Methods of aflatoxin analysis in food commodities

These methods normally involve extraction, separation and quantification and depending on the commodity being analyzed, these steps can be modified (Gnonlonfin *et al.,* 2013). It is advisable to use at least two methods for quantifying mycotoxins in order to reduce quantification errors (Trucksess and Pohland, 2002). Aflatoxin analysis in food is mainly performed using immunosorbent assays, UV absorption, spectrophotometry, fluorescence and chromatography. The difference in these methods lies in their limits of detection (Shepherd, 2008). The principal immunochemical assay used for aflatoxin analysis is Enzyme Linked Immunosorbent Assay (ELISA). The sample to be analyzed is mixed with a known portion of enzyme-conjugate in a micro-titer plate holder. These two substances compete for active binding sites on the antibody. The unbound aflatoxins and conjugated enzymes are washed away after which the substrate is

added to each well. This reaction catalyses a color change whose intensity depends on the amount of aflatoxins bound to antibody. This colour change can be evaluated either visually or by use of an absorbance plate reader (Shepherd, 2008). ELISA is very common in sub-Saharan Africa since it is inexpensive but it is also unreliable as it sometimes gives false positives (Gnonlonfin *et al.,* 2013).

The most common chromatography techniques are Gas Chromatography (GC), Liquid chromatography (LC), High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). Among these, HPLC and LC are the most widely used in routine analysis of aflatoxins (Cavaliere *et al.,* 2006; Vosough *et al.,* 2010). TLC is mainly used in agricultural and plant analysis. It is preferred due to simplicity of operation, cost effective and many samples can be analyzed on one plate at a shorter time (Sherma, 2000; Fuch *et al.,* 2010). Quantification of aflatoxins using TLC is based on immununoaffinity columns. This avoids compounds that may interfere with the assay thereby allowing quantification of aflatoxins less than 1 ng/g (Stroka *et al.,* 2000). This method also permits easy aflatoxin determination since they can be used for automated sample clean-up (Stroka *et al.,* 2002). HPLC is also a common method used to quantify aflatoxins and is used together with other techniques like UV absorption, fluorescence and mass spectrometry. It is possible to detect the four major types of aflatoxin ($B_1$, $B_2$, $G_1$ and $G_2$) with up to 5 ng being detected (Elizalde-González *et al.,* 1998). Fluorescence can also be used to detect presence of aflatoxins which have a maximum absorption of 360 nm (Akbas and Ozdemir, 2006). Aflatoxins of B type fluoresces blue at 425 nm while G type fluoresces at 450 nm under UV radiation. The fluoresce emission of B toxins is almost 10 times greater than that of G toxins (Alcaide-Molina *et al.,* 2009).

Vicam fluorometer method is quite commonly used for mycotoxin analyses. Immunoaffinity columns are used to quantify aflatoxin. Samples are extracted using appropriate solvents, filtered and diluted with water after which they are passed through affinity columns that contain monoclonal antibodies which are specific for each mycotoxin (Gnonlonfin *et al.,* 2013). The Near –Infra Red (NIR) technique is the most recent technique for aflatoxin detection but is underutilized due to calibration requirements (Tripathi and Mishra, 2009). NIR is fast, has good accuracy and precision and the equipment is easy to use. The principle involves measuring absorbance of the sample to light whose wavelength varies within the NIR region of the spectrum. Tripathi and Mishra (2009) used this technique to quantify aflatoxin $B_1$ in chilli powder. The results obtained were good compared to those of HPLC and TLC since it can detect up to 500 mg/kg of toxin present.

## 2.7 Molecular markers used in *A. flavus* genetic diversity studies

DNA-based molecular markers are developed using simple Mendelian genetic traits. Molecular markers are more stable and are not influenced by environmental or developmental markers unlike morphological markers (Jaccoud *et al.,* 2001). A molecular marker is an allele or chromosomal landmark that allows a genetist to trace a specific region of DNA. The difference between individuals occurs within the nucleotide sequences of their DNA.Various types of DNA based molecular markers such as Amplified Fragment Length Polymorphism (AFLPs) (Vos *et al.,* 1995), Random Amplified Fragment Polymorphism (RAPDs) (Williams *et al.,* 1990) and Simple Sequence Repeats (SSRs) (Litt and Lutty, 1989; Tautz *et al.,* 1989; Weber and May, 1989; Jacob *et al.,* 1991) have been developed. These markers have been used in various genetic diversity studies. SSRs are DNA sequences with repeat motifs of few base pairs (Tautz *et al.,* 1989) or short tandem repeats (Edwards *et al.,* 1991). The repeat units can be either mono, di, tri,

tetra or penta nucleotides like AA, AG, ACG, ACGT, ACCGG respectively (Haussmann *et al.,* 2002). When the repeats are long enough and uninterrupted, they exhibit a high level of polymorphism making them excellent genetic markers (Powel *et al.,* 1996). SSR markers are co-dominant meaning they can show both homozygote and heterozygote individuals. They combine several desirable properties including high polymorphism levels, being informative, high reproducibility and rapid, simple genotyping assays (Powel *et al.,* 1996).

Genotyping using SSRs markers was first described by Litt and Luty (1989). It is performed through a PCR reaction with defined oligonucleotide primers. After PCR amplification, the size of the amplicons is analyzed using either agarose or polyacrylamide gel electrophoresis systems or the laser detection method (Schuelke, 2000). For the laser detection method, the primers are normally labeled with a fluorescent dye which can be either 6-FAM, PET, NED or VIC. Analysis is then done using the automated fluorescent- based capillary detection system such as ABI 3130 or ABI 3730 from Applied Biosystems. These analysis systems allow one to co-load, whereby the PCR products are labeled with different fluorescent probes if the size of alleles is different. However care should be taken while co-loading to avoid overlapping of alleles.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study area

The study was carried out in three different geographical locations of Kitui, Trans-Nzoia and Nakuru Counties (Figure 3.1). These areas have been documented as having recently experienced outbreaks of aflatoxin contamination (Okoth *et al.,* 2012). Kitui County is situated in Eastern Kenya. It is specifically located along geographical co-ordinates of 1° 22' 0" South and 38° 1' 0" East. The County is estimated to have a population of 1,012,709 people and an area of 20,402 sq. km. This part of Kenya forms an environmental gradient of increasing altitude from 440 m to 2,100 m above sea level. The average rainfall in this area ranges from 500 mm to 1050 mm annually with 40 % reliability. The precipitation pattern is bimodal and the long rains occur between March and May while short rains are experienced during October and December (Jaetzold *et al.,* 2006). The preceding periods of this rainy seasons are usually dry i.e. June to September, and January to March. Temperatures are normally high with the mean ranging from 26 °C to 34 °C.

Residents of Kitui County rely on subsistence farming as their major source of livelihood. After the harvest period, farmers store their maize in varied storage structures. Trans-Nzoia County is an agricultural town situated in Western Kenya at an altitude of 2,100 m above sea level. Temperatures in this region range from a low of 9.05 °C to a high of 26.85 °C. The average rainfall ranges from 950 mm to 1500 mm annually. Nakuru County is located in the Rift Valley of Kenya on the Northwest of Nairobi. Its geographical coordinates are 0° 17' 0" South and 36° 4' 0" East. The rainy season occurs from April to June and sometimes during August. Due to this weather pattern, March is usually the warmest month before onset of long rains. Temperatures in

Nakuru range from 9.1 °C to 26.8 °C.

**Figure 3.1 :** Map of Kenya showing the study areas. Source: Google Maps.

## 3.2 Administration of questionnaires

During sample collection, questionnaires were used as a data collection tool (Appendix I). Each farmer was asked questions regarding cultivation of maize, storage structures, pest problems in storage and storage duration. The responses were used to evaluate storage techniques used after harvest. The collected information was used to draw conclusions basedon the experiments and results of the study.

## 3.3 Sample collection

Maize grains were collected from storage structures of farmers from the three study sites, Kitui, Trans-Nzoia and Nakuru Counties. The Cochran formula (1977) was used to calculate the sample size at 95% confidence level and a desired level of precision at 5%.

$$n = \frac{Z^2 pq}{e^2}$$

where n=sample size, z = confidence level at 95% (1.96), p= estimated proportion of the sample population, q= (1-p) and e= desired level of precision at 5% with a standard value of 0.05. Based on this formula of 5% precision and a Z value of 1.96, the sample size was

$$n = \frac{1.96^2(200)(1-200)}{(0.05)^2} = 130$$

The samples were collected from different storage structures that included granaries, wooden structures, polypropylene bags and containers. The storage duration of the maize ranged from between 1 to 6 months. Sampling was carried out by picking the maize grains multiple times from the same storage item or structure using the farmers' respective tools. The samples were then mixed to form one homogenous sample that was then labeled appropriately. The samples were packed in brown bags and transported to the Biosciences eastern and central Africa (BecA), International Livestock Research Institute (ILRI) Hub laboratories Nairobi where they were stored at 4 $^{o}$C for subsequent mycological, molecular and aflatoxin analyses.

## 3.4 Laboratory analyses

### 3.4.1 Determination of moisture content

The moisture content (MC) was determined by drying 5 g of each sample for 2 hours at 105 $^{o}$C

to constant weight in an oven (ADP21/31 Yamato Scientific, America). The samples were then allowed to cool for 30 minutes in a dessicator after which dry weight was then recorded. The difference in weight was used to calculate moisture content, expressed (dry weight) as a percentage, according to the following formula (ISO, 1980):

$$\% \ MC = \frac{(A - B)}{B} \times 100$$

Where:

% MC = percentage of moisture content in the sample

A = wet weight of sample (gm)

B = dry weight of sample (gm)

### 3.4.2 Mycoflora isolation

Quantitative enumeration of fungi was determined by use of direct plating technique as described by Pitt and Hocking (1997). Maize kernel surface was decontaminated using 10 % sodium hypochlorite for 2 minutes and rinsed twice in sterile distilled water to remove all external contaminants. The kernels were blotted to dry using sterile Whatman filter papers and directly plated on Potato Dextrose Agar (PDA) media. The prepared plates were incubated at a temperature of 28 $^{o}$C up to 7 days with a photoperiodicity of 12 hours. During the incubation period, the cultural characteristics of the fungi were recorded and followed up to seven days of incubation (Samson *et al.*, 2010). This isolation procedure was carried out in three replicates for each sample.

### 3.4.3 Fungal identification

#### 3.4.3.1 Morphological identification

Identification of all the recovered fungal isolates was achieved morphologically based on their macroscopic and microscopic features according to Samson *et al.* (2010). Macroscopic features that were observed include the colour of the colony, size of the spores, their texture and pattern. The microscopic features such as elevation of the philiades, the size of the conidiohores and protrusion of the hyphae were observed through a microscope mount.

#### 3.4.3.2 Molecular identification of *A. flavus* isolates

##### 3.4.3.2.1 DNA extraction

Total DNA was extracted from 91 *A. flavus* isolates according to the modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol of Doyle and Doyle 1987. Fungal mycelia grown on Malt Extract Agar for two days were used. A mycelia mass weighing approximately 2 g was placed in strip tubes containing two metal steel balls on a grinding machine. The mycelia were freeze dried with liquid nitrogen to ease the grinding process. Grinding of the fungal mycelia was carried out for 2 minutes using the Genogrinder® SPEX sample prep 2010 at a speed of 1500 rounds per minute until a fine powder was obtained. The strip tubes were then spun for about 2-3 minutes to bring the ground tissues to the bottom of the tubes. The CTAB extraction buffer (200 mM Tris, pH 7.5; 50 mM EDTA, pH 8.0; 2 M NaCl; 2 % CTAB; 1 % beta- Mercaptoethanol) was placed in a water bath and pre-heated to 65 °C and 450 µl was dispensed in each of the strip tubes. Grinding after the addition of extraction buffer served to disperse and homogenize the powdery fungal tissues with the extraction buffer.

The samples were then incubated at 65°C for one hour while continuously rocking gently and

inverting the tubes once every 10 minutes. After one hour, the samples were removed from the water bath and allowed to cool for 10 minutes in a fume hood. Centrifuging of the samples using Allegra™ 25R centrifuge (BECKMAN COULTER™) was then carried out at 3500 xg for 10 minutes at a teemperture of 24°C to spin down the debris. The aqueous phase of about 450 µl of was transferred into new eppendorf tubes. An equal volume of 450 µl of phenol: chloroform: isoamylalcohol in the ratio 25:24:1 was added to the supernatant to precipitate the proteins. Each strip tube containing the sample was mixed well by vortexing for about 30 seconds. The samples were centrifuged for 10 minutes at 3500 xg and the supernatant was transferred into fresh eppendorf tubes and 300 µl of chloroform: isoamylalcohol (24:1) was added.

Centrifuging was carried out again for 10 minutes at 3500 xg. The upper aqueous layer was transferred into fresh strips tubes and 300 µl of -20 °C chilled isopropanol (2-propanol) was added and mixed to precipitate the DNA. The samples were incubated at -20 °C for 2 hours after which they were centrifuged at 3500 xg at 4 °C for 20 min to form a pellet at the bottom of the tube. The supernatant was decanted to remain with the DNA pellet which was washed with 1 ml of 70 % ethanol in two washes of 500 ml each. Centrifuging was also carried out for 10 minutes at a temperature of 4 °C during each wash and the ethanol discarded by decantation. Any remaining alcohol smell indicated the pellet was not completely dry. After the pellet dried, it was re-suspended in 100 µl of Tris ethylenediaminetetraacetic acid (TE). To get rid of any RNA present, 5 µl of RNase A was added and mixed with gentle inversion and incubated for 1 hour at a temperature of 37 °C. The temperature was then increased to 65 °C to denature the RNase enzyme for 20 minutes. The DNA concentration was determined using the Nanodrop® 2000 spectrophotometer (Thermo Fischer Scientific) and a 1 % (w/v) agarose gel (1g agarose powder

mixed with 100 ml of TBE buffer) was run to visualize the DNA bands.

### 3.4.3.2.2 PCR amplification using SSR markers

*A. flavus* fungal DNA amplification was performed through a Polymerase Chain Reaction (PCR) in a 10 µl reaction mixture. The master mix constituted PCR water, PCR buffer solution (with 10X of 20 mM $MgCl_2$), 10mM/ml dNTP mix, 5 pmoles/µl of forward and reverse primers, 5 U/µl *Taq* DNA polymerase enzyme, fluorescent probes which were either 6-FAM, PET, VIC or NED and 20 ng/µl of the template DNA. The amplification conditions were: initial denaturation at 94 °C for 5 minutes, 40 cycles of the following three steps: denaturation at 94 °C for 20 seconds, annealing at 55 °C for 20 seconds and extension for 1 minute. The final extension was at 72 °C for 30 minutes. The PCR was performed using the GeneAmp® PCR system 9700 series thermal cycler from Applied Biosystems. The SSR markers used in this study were grouped into 4 categories according to their size range and the appropriate fluorescent dye (Grubisha and Cotty, 2008).

**Table 3.1:** List of primers and their sequences that were used for PCR and genotyping

| NAME OF MARKER | GROUP No. | PRIMER SEQUENCE (5'-3') | PROBE (DYE) | REPEAT MOTIF and SCAFFOLD | EXPECTED SIZE RANGE (BASE PAIRS) |
|---|---|---|---|---|---|
| AF8 | 1 | F: GGCTTGCAAGTCTAATCTGC<br>R:TGTGTCTTTGGGATGTATTTCG | PET | $(AAG)_{16}$-2911 | 178 |
| AF10 | 1 | F:CGTGCCATCGTAGAACTTCC<br>R: GGGACATTGGTAGTACCTTGG | 6-FAM | $(TAC)_{10}$ -2504 | 274 |
| AF11 | 1 | F:GACGGCGGTGTACAGTGATAGT<br>R: GCAGTAACGCGATTATGCAAGT | NED | $(AAG)_{12}$ – 2504 | 142 |
| AF13 | 1 | F: CGTGTTCCAAGTCAAGTCCA<br>R: TCTCCTTTGCTCCCGTTAGA | VIC | $(CTT)_{9}$ 1866 | 136 |
| AF16 | 2 | F: AGGTCGTGAAGCCGATACTG<br>R: CAAAGGCAGATCGAAGGGTA | 6-FAM | $(TTG)_{10}$ – 2541 | 179 |

| AF48 | 2 | F:CCACGTTCCACTGTCTCCTT<br>R: GCAAGTCCTCCACTGATGGT | VIC | $(AAG)_{12}$ – 2802 | 352 |
|---|---|---|---|---|---|
| AF54 | 2 | F: GAGAGGTATGCCTTCATGCTTT<br>R: AGTGTGTCGACATGGATTGC | VIC | $(ACAT)_{8}$ – 1918 | 173 |
| AF53 | 3 | F: -TCCTCCAAAGTGACCAAAGC<br>R: TGCGATTGCTCAGGACATAG | 6-FAM | $(TCT)_{8}$ – 1918 | 147 |
| AF55 | 3 | F: TCATGATCAACCCAGTCCAA<br>R: TGGGCAGAATATCCACGTCT | PET | $(GT)_{10}$- 1739 | 169 |
| AF64 | 3 | F: ACTGAGCATTCACCTGCTTG<br>R: ACCTAGCGGGAGGTTCTAGG | VIC | $(AC)_{16}$ – 2856 | 177 |
| AF18 | 4 | F: CCGCCTCCGAGTGTACTTA<br>R: CAATAAGGATCGCAATCGTACA | NED | $(TTC)_{29}$ – 1918 | 198 |
| AF25 | 4 | F: GTGAGAGCAATTGGGAAACC<br>R: TGACCAATATGCTGGAGGTG | VIC | $(TAC)_{7}$<br>TCC(TAC)- 2504 | 304 |
| AF43 | 4 | F: GTGAGAGCAATTGGGAAACC<br>R: TGACCAATATGCTGGAGGTG | VIC | $(GAG)_{13}$ – 2634 | 392 |

#### 3.4.3.2.3 Gel electrophoresis

The PCR products were separated in a 2 % (w/v) agarose gel (2 g of agarose powder mixed in 100 ml of TBE buffer) stained using 2.5 µl of gel red dye. The bands were visualized using GEL doc 2000 transilluminator.

#### 3.4.3.2.4 Capillary electrophoresis and Genotyping

The amplicons were separated by capillary electrophoresis using the ABI 3730 genotyping machine (Applied Biosystems) which is a fluorescent-based detection system. The PCR products were co-loaded using the appropriate fluorescent dye label. Depending on the intensity of the bands observed on the 2 % agarose gel, 1.5 – 2.0 µl of the PCR product was co-loaded with 8.0-8.5 µl of GeneScan LIZ 500, an internal molecular weight size standard (which is orange) and HiDi formamide from PE-Applied Biosystems which served to separate the double strands of the

DNA.The co-loaded products were first denatured for 5 minutes at 95 °C on the GeneAmp® PCR system 9700 series thermal cycler from Applied Biosystems after which they were analyzed by the ABI 3730 genotyping machine.

## 3.5 Aflatoxin producing ability

### 3.5.1 Total aflatoxin quantification by Vicam aflatest fluorometer method

#### 3.5.1.1 Sample extraction

This was carried out for all the 130 samples. At least 5g of previously ground samples was weighed and placed in a falcon tube. This was then mixed with 0.5 g of NaCl to give an extraction ratio of 1:10 w/w. A volume of 10 ml of 80 % methanol extraction solvent was added to each falcon tube and shaken well by hand to ensure thorough mixing of the extraction solvent and the ground sample. Aflatoxin was extracted by shaking at 225 rpm for 4 minutes at 25 ºC in an orbital shaker (New Brunswick Co.Inc, Edison, USA). The extract was filtered through a 24 cm Vicam fluted filter paper (VICAM Watertown, MA) and the filtrate collected in a clean 15 ml falcon tube.

#### 3.5.1.2 Extract dilution

A volume of 2 ml of the filtered extract was put into a 15 ml falcon tube and diluted with 8 ml of distilled deionized water to make 1:4 v/v ratio. This was mixed thoroughly on a linear mixer (DENLEY Spiramix 5, Sussex, England) for 2 minutes. The diluted extract was again filtered through a 1.5 µm -11 cm microfiber filter paper and collected in a clean 50 ml falcon tube.

#### 3.5.1.3 Column chromatography

The 2 ml of the diluted sample extract was passed through the aflatest®-P immunoaffinity column at the rate of 2 drops per second until air came out of the column (2 ml = 0.2 g of sample equivalent). The column was then washed twice with 5ml of distilled deionised water at the rate of two drops per second. The procedure was repeated once until air came out of the column. The coming out of air was determined through observation and also by the release of a hissing sound. The aflatest affinity column was eluted by passing 1.0 ml of absolute methanol at the rate of 4 drops per second. Approximately 1 ml of the eluent was collected in a glass cuvette. Aflatest® developer measuring 1 ml (45 ml of aflatest developer and 5 ml of distilled water) was added to the eluent in the cuvette and mixed well. The cuvette was placed in a fluorometer previously calibrated using three aflatoxin standards of known concentrations. Aflatoxin concentration was read after 60 seconds using the Vicam 4-EX series fluorometer from Source Scientific, USA. This procedure has a detection limit of 2 ppb and can detect total aflatoxin ($B_1$+$B_2$ + $G_1$ + $G_2$). (AOAC,1999)

### 3.5.2 Aflatoxin B1 (AFB1) ELISA quantitative assay

#### 3.5.2.1 Extraction procedure

The assay was carried out on the 91 positive *A. flavus* isolates that were previously inoculated on aflatoxin inducing media, Yeast Extract Sucrose Agar Media (YESA). The isolates were plated in duplicate and incubated at a temperature of 28 $^{o}$C for 7 days. After incubation, a portion of each representative YESA sample media was cut and a 5 g weighed agar placed in a clean 50 ml falcon tube. A volume of 25 ml of 70 % methanol extraction solvent was added to each tube and mixed well at the extraction solvent ratio of 1:5 w/v. The falcon tubes were then shaken well by hand and placed in an orbital shaker to mix thoroughly for 2 minutes. The suspension was then

allowed to settle and filtered through a Whatman no.1 filter paper and the filtrate collected in another clean 50 ml falcon tube (AOAC, 1999).

#### 3.5.2.2 Assay procedure

AFB1 was quantified from the *A. flavus* isolates using the Helica Aflatoxin B1 solid phase direct competitive Enzyme Linked ImmunoSorbent Assay (ELISA). The procedure was performed according to the Helica Biosystems Inc. (Fullerton, Carlifornia). One dilution well was placed in a microwell holder for each standard and the sample to be tested. An equal number of antibody coated microtiter wells were then placed in another microwell holder. In each dilution well, 200 µl of the HRP-conjugate was dispensed. A volume of 100 µl for each standard and sample was then added to the appropriate dilution well containing the conjugate. The contents of each well were mixed thoroughly by priming the pipette at least three times. Using a new pipette tip for each reaction, 100 µl of these contents were transferred from each dilution well into a corresponding antibody coated microtiter well. The ELISA plate was then incubated for 15 minutes at room temperature. The contents from the microwells were decanted into a discard basin and washed 5 times with distilled deionised water. For the final wash, the microwells were tapped facing down on a layer of absorbent paper towels to remove residual water. The required volume of 100 µl substrate reagent was measured and added to each microwell. The ELISA plate was incubated for 5 minutes again at room temperature. A volume of 100 µl of an acidic stop solution was then added to each microwell. The optical density (OD) of each microwell was read using a BIOTEK microtiter plate reader at an absorbance of 450 nm from Biotek Instruments, Inc. (AOAC,1999).

### 3.5.3 AFPA Toxigenicity tests

*Aspergillus flavus* and *parasiticus* agar (AFPA) was used in differenting *A. flavus* and *A.parasiticus* in seven days incubation. *A. flavus* grows on it releasing aspergillic acids that change the media from its brown colour to orange. The colour varies according to amount of aflatoxin producing fungus present from moderate to high.

## 3.6 Data analysis

All data was subjected to Analysis of Variance (ANOVA) using Genstat (1998, version 4). The treatments and means of the incidence of fungal isolation and aflatoxin contents in the samples was compared using the Student Newman-Keul's test (SNK) test and Fisher's Protected Least Significant Difference (LSD) test (Steel and Torrie, 1981). Analysis of fragments from ABI 3730 DNA genotyping machine was done using GeneMapper® Software version 4.0. Genotyping analysis was done using Molecular Evolutionary Genetics Analysis (MEGA) version 5.1 software (Kumar *et al.,* 2008). The number of alleles per locus and haploid diversity were calculated using GenALex version 6.41 and Power marker v3.25 software (Liu, 2004). Genetic distance matrix was used to perform Principle Coordinates Analysis (PCA) and Analysis of Molecular Variance (AMOVA) (Peakall and Smouse, 2006). The dendrogram was generated using unweighted pair wise method by Neighbour Joining using (Dissimilarity analysis and representation for windows) DARwin 5 software program (Perrier, 2006). The resulting phylogenetic tree was viewed using Tree View (Page, 2001).

# CHAPTER FOUR

# RESULTS

## 4.1 Post-harvest practices of maize farmers

### 4.1.1 Storage systems

The storage systems were evaluated as they were important in relating them to the mycoflora recovered. The methods of storage for harvested maize varied from one geographical region to another. Most farmers first put their maize in polypropylene bags locally known as "gunias" after which they are stored in the granary. This was the most common method of storage across the three locations with a mean of 44.33 % (Table 4.1). Farmers who did not have granaries stored their maize within the family house. Few of the farmers (3 %) stored their maize in plastic bags. The storage methods were shown to differ significantly from each other. ($p \leq 0.05$).

**Table 4.1:** Different storage systems used by farmers to store harvested maize in three sites in percentage.

| | Site | | | |
|---|---|---|---|---|
| **Storage System** | **Kitui n=30** | **Trans-Nzoia n=40** | **Nakuru n=60** | **Mean** |
| Polypropylene bags in granary | 16 | 52 | 65 | 44.33 [a] |
| Polypropylene bags inside the house | 50 | 22 | 23 | 31.67 [a] |
| Containers | 27 | 25 | 11 | 21.00 [b] |
| Plastic bags | 7 | 1 | 1 | 3.00 [c] |

[a,b,c] Means within a column followed by different superscripts are significantly different ($P<0.05$). The means and p-values were calculated using SPSS p-significant tests.

The maize samples used in this study were sampled from different maize storage structures commonly used by local farmers (Figures 4.1 to 4.4).

**Figure 4.1:** Maize kept on top of the roof to dry and also as the temporary storage place

**Figure 4.2:** Maize stored in one room inside the family house

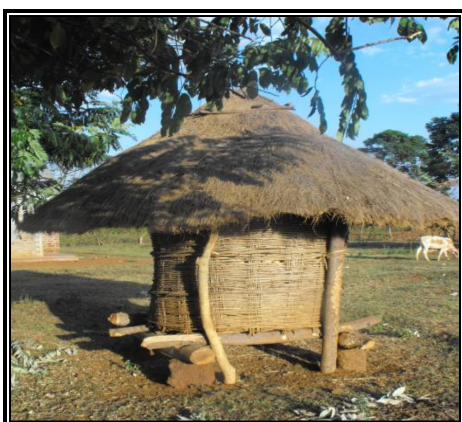

**Figure 4.3 :** Sisal-woven grass thatched granary for storing maize

**Figure 4.4**: All round mud-walled granary for maize storage

### 4.1.2 Drying methods for maize

Different farmers used different methods to dry their maize after harvest. Drying the maize under direct sunlight was the most common method (Table 4.2). Some farmers in Trans-Nzoia preferred to dry their maize on tarmac roads (25 %) especially when they are not busy. They argued that the combined heat from the sun and the tarmac would make the cereal dry faster. A minority of farmers (1.9 %) smoked their maize which served a dual purpose of drying and preservation. Therefore the methods used to dry maize differed significantly from each other ($p=0.01<0.05$).

**Table 4.2:** Drying methods used by farmers from three geographical locations

| | Site | | | |
|---|---|---|---|---|
| | % farmers practicing the method | | | |
| **Drying Method** | **Kitui** n=30 | **Kitale** n=40 | **Nakuru** n=60 | Mean |
| Direct sun drying | 66.67 | 75 | 66.67 | 69.4[a] |
| On roof tops | 16.67 | 0 | 16.67 | 11.1[b] |
| Bare ground | 10.84 | 0 | 16.67 | 9.2[b] |
| On the road | 0 | 25 | 0.00 | 8.3[b] |
| Smoking inside the kitchen | 5.82 | 0 | 0.00 | 1.9[c] |

[a,b,c] Means within a column followed by different superscripts are significantly different ($P<0.05$). The means and p-values were calculated using SPSS p-significant tests.

## 4.2 Moisture content of stored maize

Moisture content levels were assessed so as to correlate them to the mycoflora recovered. Most of the samples analyzed had their moisture content (MC) levels above 13.5 %. In Kitui, only 1 sample (n=30) had the MC below 13 %. The MC in this region ranged from a low of 13 % to a high of 25 %. In Kitale however, 80 % of the samples (n=40) had their MC above 13 %. The moisture content here ranged from 6 % to 34 %. This region recorded the highest level of MC at 34 % (Table 4.3). In Nakuru, 83 % (n=60) of the samples had a MC above 13 %.The MC ranged from 8 % to 30 %. In total, 85 % of the samples had their MC above 13 %. The moisture content observed in each sample correlated to the mycoflora that was recovered. There was no significant difference observed among MC values of the three sites.

**Table 4.3:** Moisture content levels of maize collected from three different geographical locations

| Sample Site | Agro- ecological Zone | N | MC range (% ) | Samples >13.5% | Samples <13.5% | Mean MC between each study site (p=0.23) |
|---|---|---|---|---|---|---|
| Kitui | Semi humid- semi arid | 30 | 13 -25 | 29 | 1 | 19.33[a] |
| Trans-Nzoia | Sub humid – semi humid | 40 | 6-34 | 32 | 8 | 16.39[a] |
| Nakuru | Semi-humid | 60 | 8 - 30 | 50 | 10 | 17.23[a] |

Means within a column followed by the same superscripts are not significantly different ($P \leq 0.05$). The means and p-values were calculated using SPSS p-significant tests.

## 4.3 Morphological identification

### 4.3.1 Isolation and identification of fungi from maize samples

The isolates of *A. flavus* formed green colonies with a few samples having yellow colonies when cultured on PDA (Figure 4.5). Morphological features observed on most PDA plates revealed that *A. flavus* formed white mycelia with spreading dark green, green or yellow colonies. The conidia crowns were olive green with some being overlaid by olive yellow colonies. The fungus produced brown sclerotia in some samples. Microscopic characteristics of *A. flavus* showed smooth globose conidia. The conidiophores appeared wide and roughened and are oftenly uncoloured. The cells were septate and born on hyaline hyphae. The sub-culture of *A. flavus* on PDA is shown on figure 4.6.

**Figure 4.5 :** *A. flavus* isolated on PDA

**Figure 4.6 :** Sub-culture of *A. flavus* on PDA

### 4.3.2 Fungal Profile of maize samples from Kitui, Trans-Nzoia and Nakuru

Fungi grew in most of the samples that had been cultured within a 7-day incubation period. A total of five fungal genera (*Aspergillus, Penicillium, Fusarium, Rhizopus* and *Bssyochlamys spp.*) were isolated from the stored maize samples. Out of these, *Aspergillus* and *Fusarium* fungi were the most isolated with the frequency of 51.91 % and 5.0 % respectively. The most common fungal species that were isolated from the three study sites included *A. flavus* at 82.03 % followed by *A.niger* and *F.solani* at 45 % and 18 % respectively (Figure 4.7).

In some samples from Trans-Nzoia, *Rhizopus* and *Mucor* species overgrew all other fungi and inhibited colony development of others. With regard to study site, Trans-Nzoia recorded a high incidence of *A. flavus* (41 %) followed by Nakuru and Kitui at (24 %) and (17 %) respectively. Kitui which is a hotspot for aflatoxin contamination interestingly had the least amount of *A. flavus* incidence. In this region, the highest incidence of *A .niger* was recorded at 42.4 % compared to 7.5 % incidence of the same fungus in both Trans-Nzoia and Nakuru. *Fusarium poae, Fusarium graminearum* and *Fusarium culmorum* were some of the least encountered *Fusarium* species that were found to occur together with *A. flavus* from the same samples. Other fungal genera that were recovered include *Rhizopus, Byssochlamys, Cladosporium, Aureobasidium, Acremonium* and *Exophiala.*

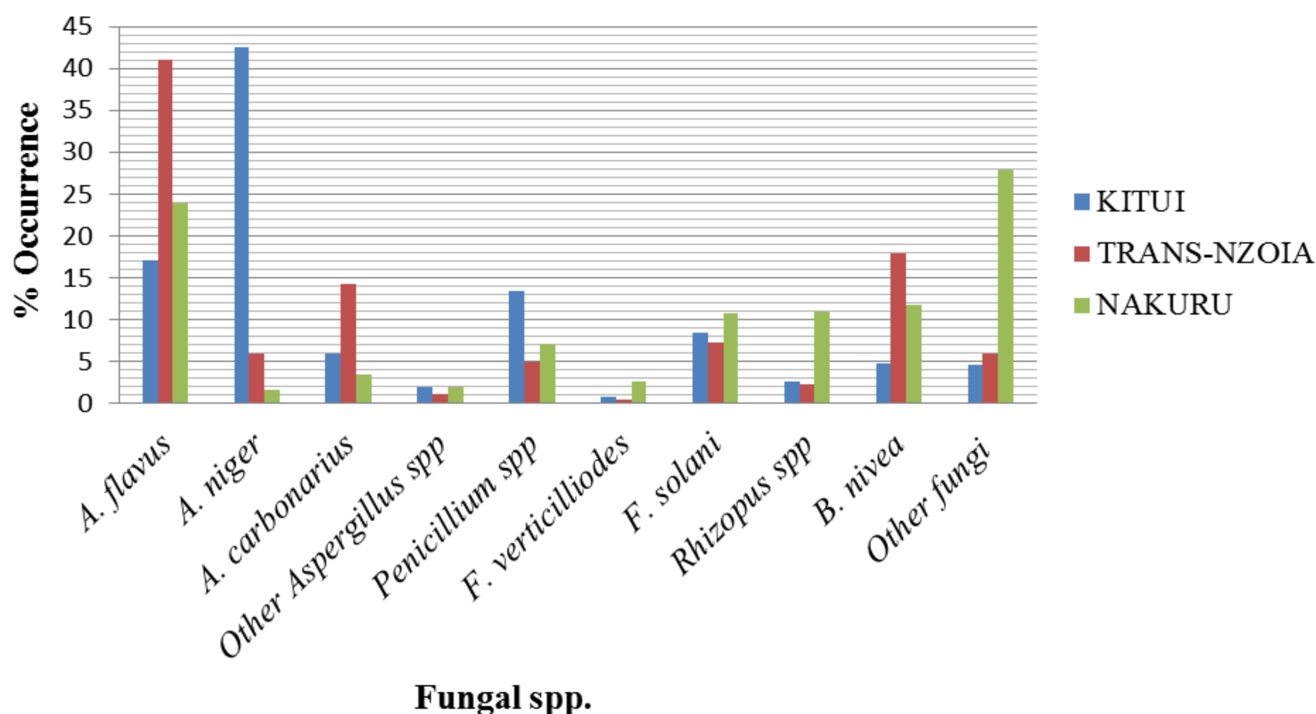


**Figure 4.7:** Fungal profile from the three geographical locations

## 4.4 Toxigenic versus non-toxigenic fungi

Mycological analyses revealed that 62 % of the fungi isolated were toxigenic while 38 % of non-toxigenic fungi. Among the toxigenic fungi isolated were *A. flavus, A. niger, A. carbonarius F. verticilliodes* and some *Penicillium* species (Figure 4.8). *F. solani, B. nivea* and *Rhizopus* species comprised the major part of the non-toxigenic fungi. *Aspergillus flavus* comprised the major proportion of toxigenic fungi at 82.03 % while *F. verticilliodes* was the least encountered toxigenic fungi at 3.7 %. In Kitui region, *A. niger* was the predominant toxigenic fungus at 42.4 % compared to a total of 6 % from the other two geographical regions. *A. carbonarius* was frequent in Kitale at 14.3 % with Kitui and Nakuru recording 5.8 % and 3.5 % of this fungus respectively. *Penicillium* species was encountered in all the three sites with the highest occurrence being in Kitui at 13.4 %.

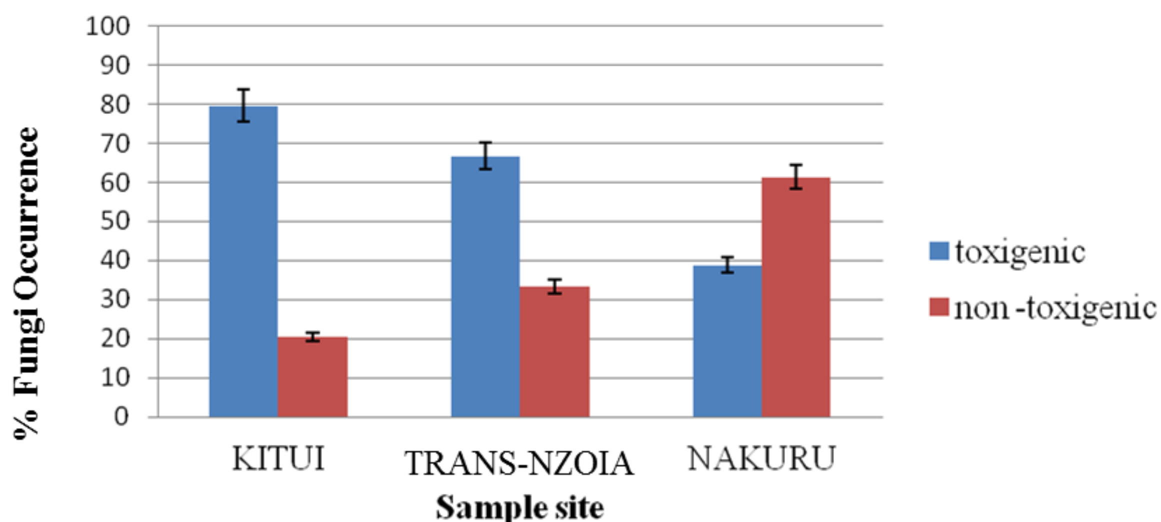


**Figure 4.8:** Relationship between toxigenic and non-toxigenic fungi isolated from stored maize grains in three geographical locations.

### 4.5 Differentiation of *A. flavus* and *A. parasiticus* using AFPA toxigenicity test

Isolates of *A. flavus* were differentiated from those of *A. parasiticus* according to the colour pigmentation of the undersides (Figure 4.9 to 4.12) after 7 days of incubation. The reverse colour of the AFPA plates ranged from weak orange colour to a bright cadmium orange colour.

**Figure 4.9:** No orange colour (-). (isolates that are not *A. flavus*).

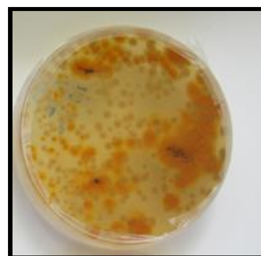

**Figure 4.10:** Low orange colour intensity (+). (*A. flavus* isolates that are low aflatoxin producers).

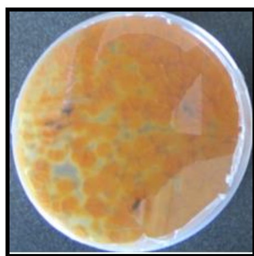

**Figure 4.11:** Moderate orange colour intensity (++). (*A. flavus* isolates that are moderate aflatoxin producers).

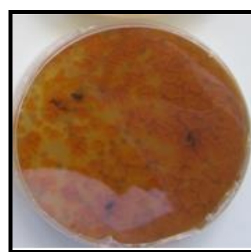

**Figure 4.12:** Bright orange colour intensity (+++). (*A. flavus* isolates that are high aflatoxin producers).

All the three sites had more than 60 % of their samples showing cadmium orange colour on the reverse (Table 4.4). Of the three study sites, Kitale recorded the highest intensity of orange reverse color on AFPA (38 %, n=39) followed closely by Kitui and Nakuru at (36 %, n=11) and (304 %, n=32) respectively.

**Table 4.4:** Differentiation in percentage occurrence of *A. flavus* and *A. parasiticus* as observed on the reverse side of AFPA plates.

| Population | Cadmium orange reverse colour intensity | % Occurrence |
|---|---|---|
| KITUI | - | 4/11 - 36 |
| | + | 2/11 - 18 |
| | ++ | 1/11 - 10 |
| | +++ | 4/11 - 36 |
| TRANS-NZOIA | - | 4/39 - 10 |
| | + | 8/39 - 21 |
| | ++ | 12/39 -31 |
| | +++ | 15/39 -38 |
| NAKURU | - | 7/32 - 22 |
| | + | 7/32 -22 |
| | ++ | 7/32 - 22 |
| | +++ | 11/32 -34 |

Key : (-) No orange colour, (+) Low orange colour intensity, (++), Moderate orange colour intensity, (+++) Bright orange colour intensity

### 4.6 *In vitro* toxigenicity of *A. flavus* isolates

Most of the *A. flavus* isolates recorded a high level of aflatoxin when they were cultured *in vitro*. In Kitui, all the samples (100 %) had aflatoxin levels above 10 ppb with a total aflatoxin mean of 219.2 ppb. In Kitale, only 3 samples (n=39) had their aflatoxin levels less than 10 ppb while in the remaining samples it was above 10 ppb giving a total aflatoxin mean of 234 ppb. In Nakuru, only 11 samples (n=32) were below 10 ppb with the total aflatoxin mean levels of this region being 239.7 ppb. From these results, samples from Kitui region had significantly different aflatoxin levels compared to Trans-Nzoia and Nakuru. However, there was no significant difference in the aflatoxin levels between Trans-Nzoia and Nakuru.

### 4.7 Toxigenicity profile of the maize samples

Aflatoxin analysis revealed that most of the samples (58.4 %) had been contaminated with aflatoxins. Nakuru (83.3 %) had the highest level of aflatoxin contamination followed by Trans-Nzoia (57.5 %) with Kitui (10 %) being the lowest (Table 4.5). Out of samples that tested positive for aflatoxin, 88 % (115/130) had aflatoxin levels below the legal limit of 10 ppb. The highest aflatoxin level recorded was from a Trans-Nzoia sample at 72 ppb. Samples from this region had an aflatoxin mean of 9.68 ppb with the aflatoxin levels ranging from 0ppb to 72 ppb. At least 70 % of samples from this region had their aflatoxin levels less than the legal limit of 10 ppb. Averagely, the aflatoxin levels were o.70 ppb and 4.18 ppb for Kitui and Nakuru respectively. These two regions also recorded 97 % aflatoxin levels that were less than 10 ppb. (Table 4.5).

**Table 4.5:** Mean aflatoxin levels calculated on basis of the legal limit of 10 ppb

| Sample site | AEZ | (N) | No. of aflatoxin contaminated samples | Mean aflatoxin levels (ppb) | Range (ppb) | Category of total aflatoxin (ppb) |
|---|---|---|---|---|---|---|
| Kitui | Semi-humid-semi arid | 30 | 3 | 0.70[a] | 0-13 | < 10ppb 29/30 (97%) |
| | | | | | | > 10ppb 1/30 (3%) |
| Trans-Nzoia | Sub-humid - semi humid | 40 | 23 | 9.68[b] | 0-72 | < 10ppb 28/40 (70%) |
| | | | | | | > 10ppb 12/40 (30%) |
| Nakuru | Semi-humid | 60 | 50 | 4.18[a] | 0-13 | < 10ppb 58/60 (97%) |
| | | | | | | > 10ppb 2/60 (3%) |

[a, b,] Means within a column followed by different superscripts are significantly different (P<0.05). The means and p-values were calculated using SPSS p-significant tests.

A comparison was also done to determine the relationship between the incidence of *A. flavus* and the aflatoxin levels from the stored maize (Figure 4.13). There was a significant and positive correlation between aflatoxin levels in the three study sites and the percentage incidence of *A. flavus* (p=0.01, r=0.785).

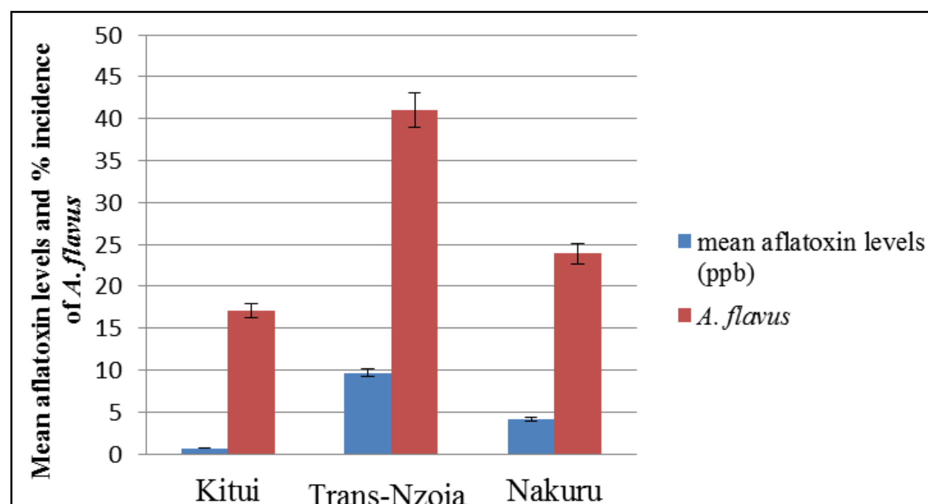


**Figure 4.13:** Correlation between incidence of *A. flavus* and aflatoxin levels in the three study sites.

## 4.8 Molecular characterization

### 4.8.1 DNA quality and quantity determination

All the 91 isolates of *A. flavus* were used in this molecular study. They all showed that their DNA had a high molecular weight that ranged from 21.3 ng /µl (Kitui 26), 1028 ng/µl (Trans-Nzoia 3) to 1082.3 ng/µl (Nakuru 39). This DNA was of good quality since all isolates had their DNA normalized to 20 ng/µl to ensure uniformity of results. On agarose gel electrophoresis, the isolates showed good quality DNA were indicated by a clear band while the poor quality ones had a smear (Figure 4.14).

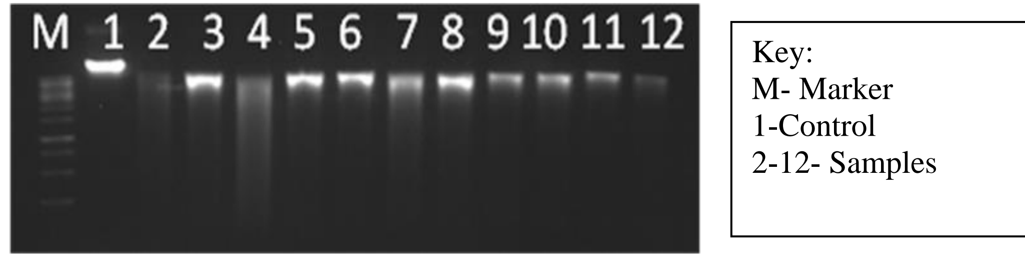


**Figure 4.14:** 0.8% genomic DNA agarose gel.

### 4.8.2 SSR characterization of *A. flavus* strains

Genetic diversity of *A. flavus* population was determined on 91 isolates originally isolated from maize grains. Most of the markers showed good amplification after gel electrophoresis (Figure 4.15).

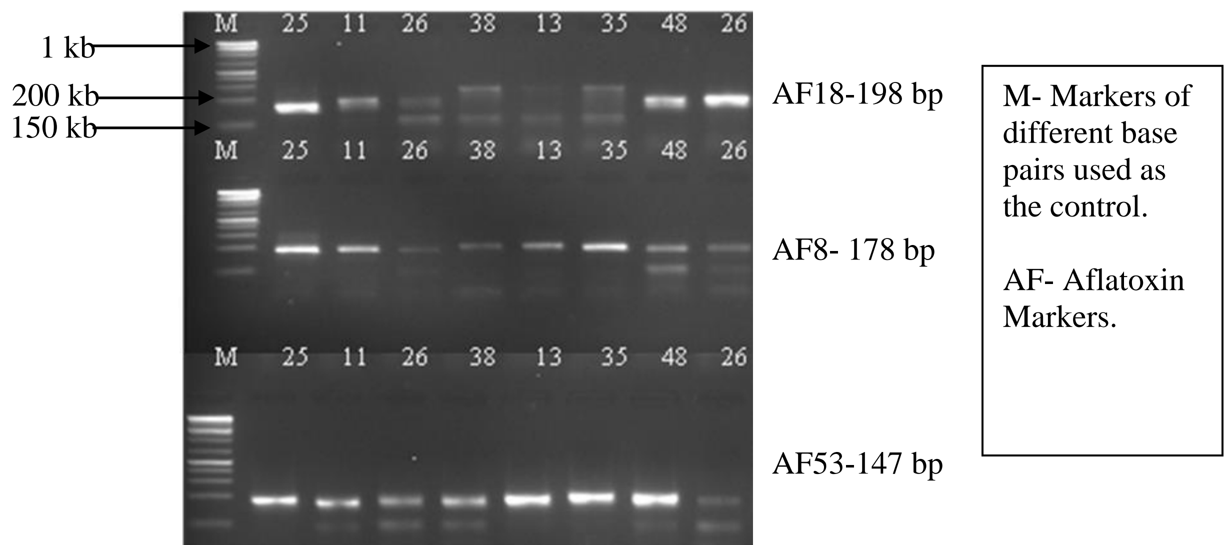


**Figure 4.15:** 2% agarose gel showing good amplification in a few selected markers and their expected band sizes.

### 4.8.3 Informativeness of SSR markers used in the genetic diversity study

The major allele frequency ranged from 0.2381 to 0.7267 with a mean of 0.4529. The gene diversity ranged from 0.4533 to 0.8790 with a mean of 0.6983 (Table 4.6). The heterozygosity

ranged from 0.0000 to 0.0595 with a mean of 0.0137. The low heterozygosity mean value of 0.0137 correlates to the haploid nature of the *A. flavus* fungus.

**Table 4.6:** Summary statistics showing the major allele frequency, allele number, gene diversity, heterozygosity and the Polymorphism Information Content (PIC)

| Marker | Major Allele Frequency | Allele No. | Gene Diversity | Heterozygosity | PIC |
|---|---|---|---|---|---|
| **AF8** | 0.7079 | 11.0000 | 0.4795 | 0.0449 | **0.4600** |
| **AF10** | 0.2838 | 15.0000 | 0.8616 | 0.0000 | 0.8500 |
| **AF11** | 0.5167 | 10.0000 | 0.6939 | 0.0111 | 0.6718 |
| **AF13** | 0.4615 | **7.0000** | 0.6690 | 0.0000 | 0.6153 |
| **AF16** | **0.7267** | 9.0000 | **0.4533** | 0.0116 | 0.4335 |
| **AF48** | 0.2963 | 13.0000 | 0.8134 | 0.0123 | 0.7908 |
| **AF54** | 0.5893 | 9.0000 | 0.5709 | **0.0595** | 0.5136 |
| **AF53** | 0.3767 | 8.0000 | 0.7396 | 0.0137 | 0.7005 |
| **AF55** | 0.3614 | 10.0000 | 0.7783 | 0.0000 | 0.7499 |
| **AF64** | **0.2381** | **16.0000** | **0.8790** | 0.0000 | **0.8686** |
| **AF18** | 0.3077 | 12.0000 | 0.8327 | 0.0000 | 0.8149 |
| **AF25** | 0.6463 | 10.0000 | 0.5581 | 0.0244 | 0.5375 |
| **AF43** | 0.3750 | 8.0000 | 0.7491 | **0.0000** | 0.7111 |
| **Mean** | **0.4529** | **10.6154** | **0.6983** | **0.0137** | **0.6706** |

Each SSR marker has an expected size range at which it is supposed to amplify. For all the 13 markers, amplification occurred at different positions. This was also confirmed after genotyping as shown by the different alleles at a given loci. The different alleles gave an allele frequency mean of 0.4529 (Figure 4.16).

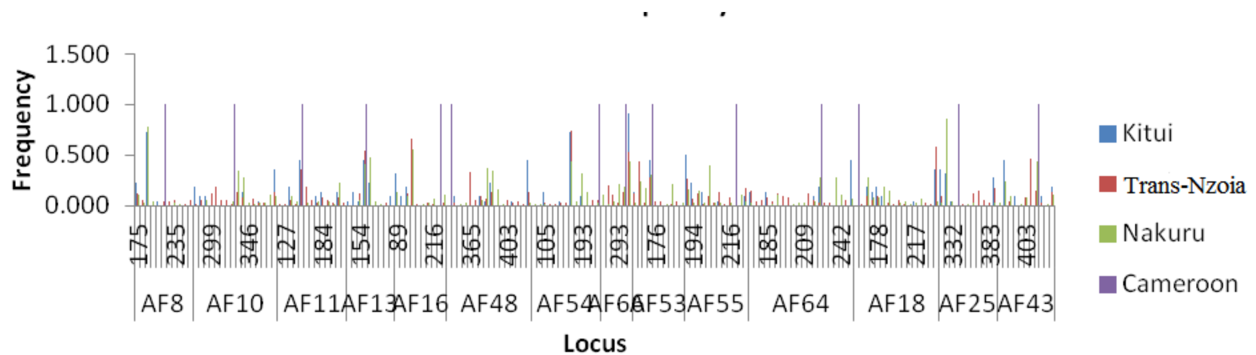


**Figure 4.16:** Allele frequencies by population over loci for codominant data.

### 4.8.4 Polymorphism of SSR markers

All the 13 SSR markers used targeted different positions on the *A. flavus* fungus that has 8 chromosomes. These markers were mapped across either the long arm or short arm of these chromosomes. The alleles revealed by SSR markers showed a high degree of polymorphism. The highest number of alleles was observed in marker AF64 which had a total of 16 alleles (Table 4.7). Markers AF25, AF55 and AF11 each had 10 alleles. The lowest number of alleles was observed in AF13 marker that had only 7 alleles. Markers AF43 and AF53 had 8 alleles each. The PIC values for the 13 SSR markers used ranged from 46 % to 87 % with a mean of 67 %.

**Table 4.7:** Polymorphism information content (PIC) of SSR loci across various *A. flavus* isolates analyzed in this study

| S/N | Primer code | Chromosome Location | Molecular weight (bps) | No. of alleles ($N_A$) | %PIC |
|---|---|---|---|---|---|
| 1 | AF8 | GGCTTGCAAGTCTAATCTGC | 178 | 11 | 46 |
| 2 | AF10 | CGTGCCATCGTAGAACTTCC | 274 | 15 | 85 |
| 3 | AF11 | GACGGCGGTGTACAGTGATAGT | 142 | 10 | 67 |
| 4 | AF13 | CGTGTTCCAAGTCAAGTCCA | 136 | 7 | 61 |
| 5 | AF16 | AGGTCGTGAAGCCGATACTG | 179 | 9 | 43 |
| 6 | AF48 | CCACGTTCCACTGTCTCCTT | 352 | 13 | 79 |
| 7 | AF54 | GAGAGGTATGCCTTCATGCTTT | 173 | 9 | 51 |
| 8 | AF53 | TCCTCCAAAGTGACCAAAGC | 147 | 8 | 70 |
| 9 | AF55 | TCATGATCAACCCAGTCCAA | 169 | 10 | 74 |
| 10 | AF64 | GCCTAAGGACGAGTCGATTG | 177 | 16 | 86 |
| 11 | AF18 | ACTGAGCATTCACCTGCTTG | 198 | 12 | 81 |
| 12 | AF25 | CCGCCTCCGAGTGTACTTA | 304 | 10 | 53 |
| 13 | AF43 | GTGAGAGCAATTGGGAAACC | 392 | 8 | 71 |

## 4.9 Genetic relationships of *A. flavus* isolates

### 4.9.1 Phylogenetic relatedness of the *A. flavus* isolates

The dendrogram produced 5 distinct clusters that were revealed among the genotypes. Cluster one comprised of isolates from two geographical regions. Isolates from Nakuru formed the major part of this cluster with some Trans-Nzoia isolates being spotted within this cluster. Cluster two also comprised mainly of Nakuru isolates with only one Kitui isolate (Kitui 6). Cluster three was a combination of isolates from all the three geographical locations in varied proportions. The most distinct cluster was cluster four that comprised of isolates exclusively from Nakuru with a few Kitui isolates appearing towards the end of the cluster (Figure 4.17). The final cluster 5 also stood out with isolates only from Kitale region. The final cluster, cluster 6, was also made up of isolates mainly from Kitale region. Apart from the major clusters, there were 4 other minor clusters that were observed. The outgroup from Cameroon appeared in these category and it clustered separately from the rest. The other three clusters were made up of isolates from Trans-Nzoia alone. The genetic distance of the in terms of the relationship was determined using a bootstrap value of 1000 (Figure 4.17).

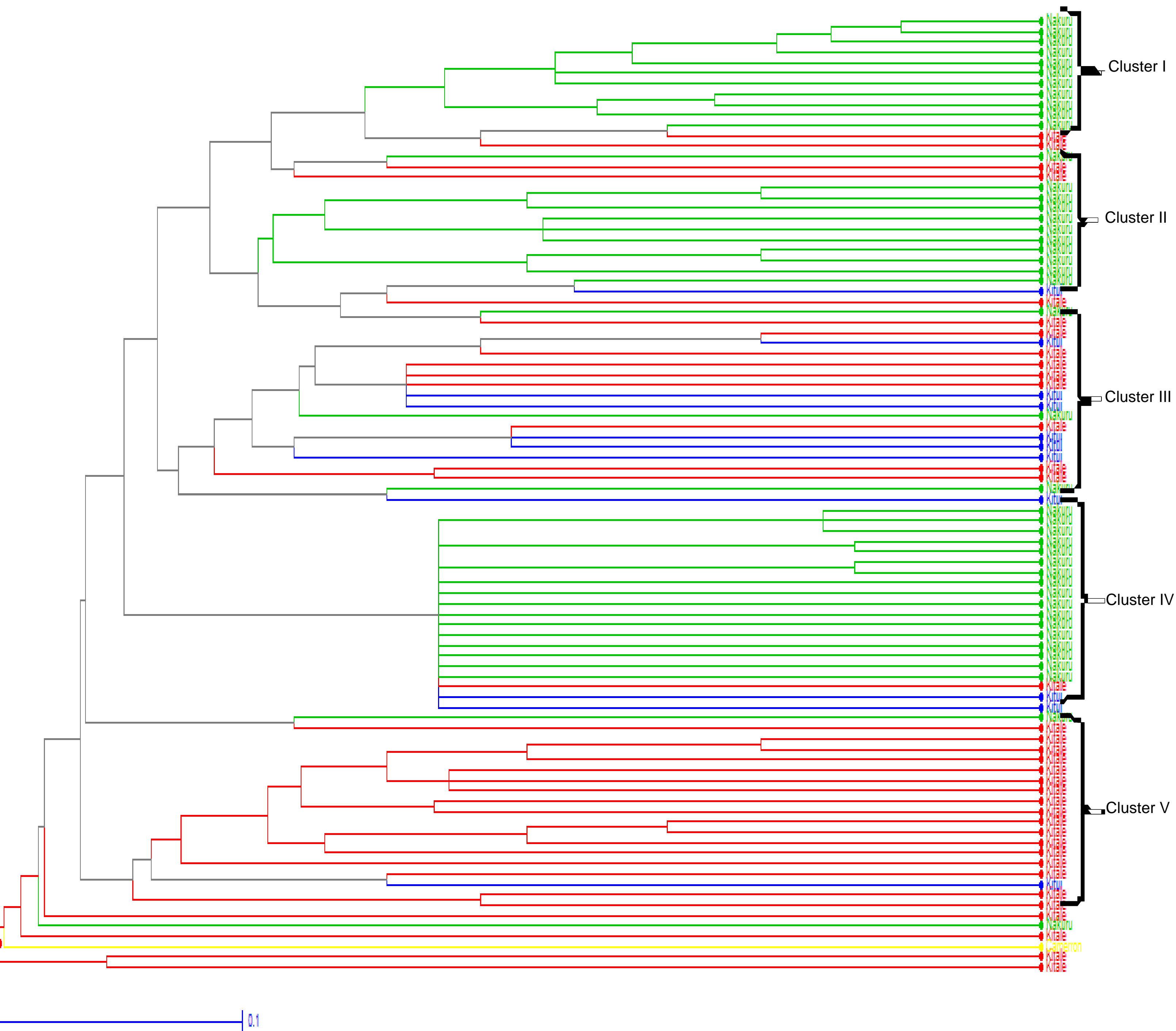


**Figure 4.17:** Phylogenetic tree showing clustering of *A. flavus* genotypes generated using UPGMA pair wise genetic distance (Nei, 1972) with a bootsrap value of 100. The isolate form Cameroon was used as an outgroup to root the tree. These data is based on genotypes of 13 SSR markers used in this study.

### 4.9.2 Principal coordinates analysis (PCoA)

Principal Coordinates Analysis (PCoA) of the genetic distances among the 91 *A. flavus* isolates explained over 50.3 % of the total variation when two coordinates were used to cluster the isolates (Figure 4.18). The first principal coordinate gave the greatest percent of variation at 30.56 % while the second principal coordinate gave 19.68 %.

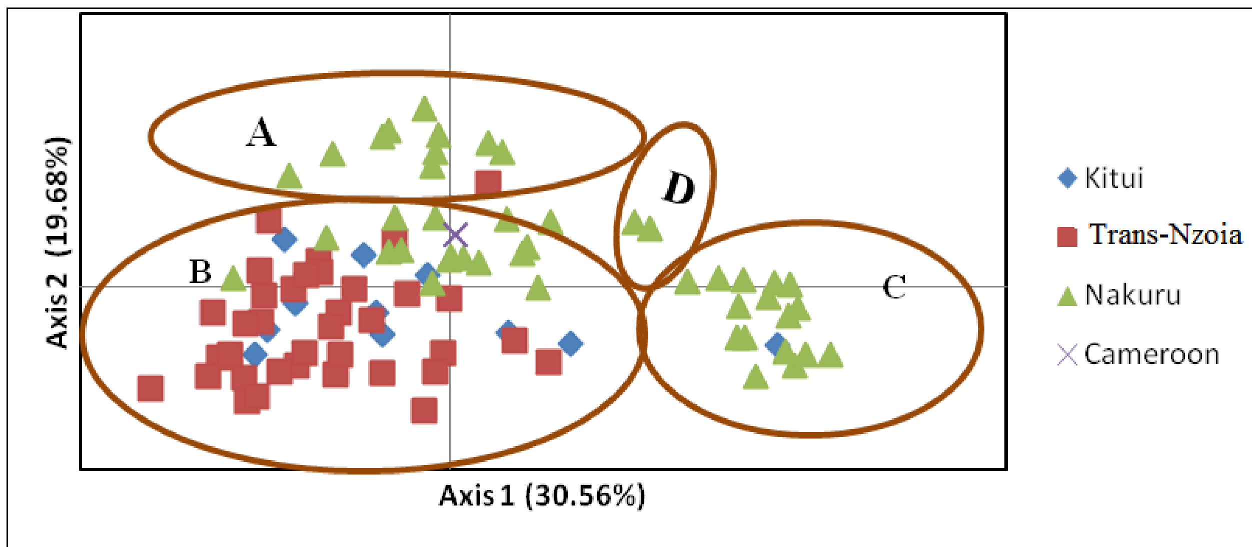


**Figure 4.18:** Principal Coordinates Analysis (PCoA) of 91 *A. flavus* isolates based on genetic distances calculated from GenALex v. 6.41.

### 4.9.3 Analysis of Molecular Variance (AMOVA)

Assessment of genetic diversity of the *A.flavus* isolates using Analysis of Molecular Variance (AMOVA) showed high variation of 87 % within the different populations analysed (Figure 4.19).Variation among the populations was 13 % indicating that the isolates also differed in terms of the geographical locations.

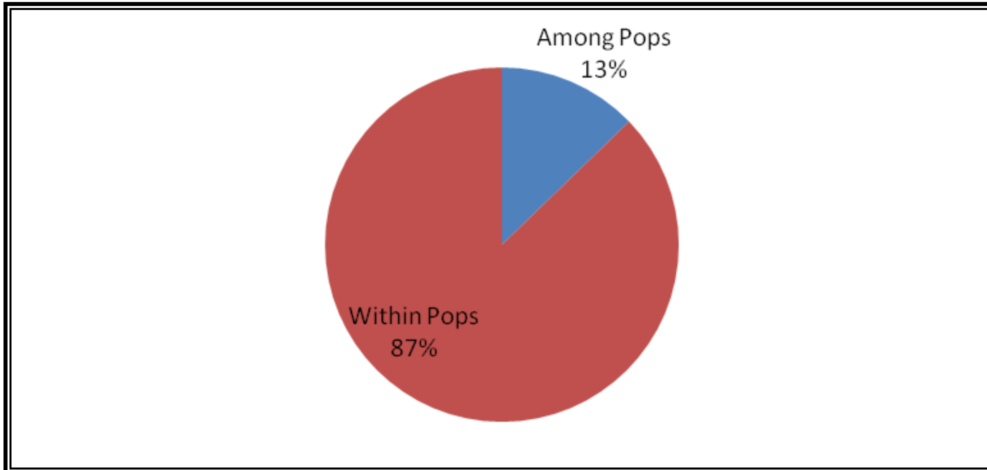


**Figure 4.19:** Analysis of Molecular Variance of 91 *A. flavus* isolates showing variation within and among populations calculated from GenALex v. 6.41.

# CHAPTER FIVE

# DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

In sub Saharan Africa, Kenya included, maize is a staple food and its presence signifies adequate food security (Shepherd, 2004). Due to this fact, a lot of emphasis has been laid on its production. However, even at harvest, aflatoxin contamination has been a challenge posing a threat to food security and consequently, the livelihoods of the Kenyan population. Nevertheless, the present study determined the level of fungal contamination, its diversity and aflatoxin levels from three agro-ecological zones in Kenya with a view of evaluating the extent of this challenge.

### 5.1.1 Fungal contamination and toxigenicity profiles

High fungal contamination was detected in samples collected from all the study regions (Figure 4.7). High moisture content levels that exceeded 13 % limit were found in these samples with the highest being up to 34 % (Table 4.3). Similar results have also been reported in a study done by by Abdullah *et al.* (1999). Usually, the standard moisture content required for most cereals should be 13 % or less in all anticipated periods of storage (Kiiru *et al.,* 1999). In this study, the detected high moisture content was positively correlated with fungal contamination with *Aspergillus* species being the most predominant contaminant. Atehnkeng *et al.* (2007) reported similar findings in a study done in West Africa. Trans-Nzoia among the sampled regions recorded the highest moisture content as well as the levels of contaminants with a high incidence of *A. flavus* of 41 % (Figure 4.7) being detected. Previous studies have shown that *Aspergillus* spp grows optimally at moisture content level of above 15 % and poor growth or none at 12 % (Abbas, 2005). From this study, all samples had all favourable conditions for these fungi. These conditions explains, the predominance of *Aspergillus* and *Fusarium* in tested samples from the

three regions (Figure 4.7). In addition, use of poor storage structures; like polypropylene bags, granaries, above fire racks in the kitchen, plastic bags and other containers as observed from study areas favoured *Aspergillus* species as the most predominant contaminant (Table 4.1). Some of these structures do not protect maize from picking up environmental moisture hence predisposing stored maize to mould infection and consequently aflatoxin contamination (Fandohan *et al.,* 2005). These findings were similar to those previous obtained in Kenya (Alakonya *et al.,* 2009), Nigeria (Bankole, 2003) and India (Janardhana *et al.,* 2011). Even though previous studies have shown differences in geographical locations and environmental conditions could be responsible for the differences in fungal distribution (Fandohan *et al.,* 2003), findings from this study are contrary. Apart from the detected fungi being responsible for toxins, they are also responsible for causing ear rot disease in maize, a disease that is also common in maize (Naidoo *et al.,* 2002; Logrieco *et al.,* 2007).

Three main *Aspergillus* species isolated in this study were; *A. flavus, A. niger* and *A. carbonarius* with *A. wentii* and *A. oryzae* being the least encountered. *A. flavus* was the most predominant. *Aspergillus* species, has been associated with high cases of food toxins in the country especially in Eastern Kenya (Mutegi *et al.,* 2010). Trans-Nzoia had the highest levels of *A. flavus,* a region where cases of maize toxification have been reported. This indicates the possible threat on the epidemiological spread and poor storage practice in a region known to supply maize within the country. Similar reported incidences of fungi in stored maize across the border in Uganda (Kaaya and Warren, 2006) and Benin (Atehnkeng *et al.,* 2008) show a possible risk posed by these fungi. *A. flavus* is the predominant aflatoxin producing fungus in the tropical regions. Similar findings were confirmed with *A. flavus* (82.03 %) as most predominant followed by *A.niger* (49 %).

These findings were similar to those reported in other parts of Kenya (Probst *et al.,* 2007) and Vietnam (Trung *et al.,* 2008).

*Fusarium* species had 15% incidence and has been previously reported to contaminate maize in Kenya (Muthomi *et al.,* 2009; Bii *et al.,* 2012) and other parts of Africa (Fandohan *et al.,* 2003; Gnonlonfin *et al.,* 2004; Ghisian *et al.,* 2006). Frequency of isolation of *Aspergillus, Fusarium* and *Penicillium* was almost the same in the three regions. The variation within each study site could be attributed to agricultural management practices at pre-harvest and post-harvest level. The presence of *A. flavus* on maize does not imply obvious occurrence of aflatoxin in maize (Muthomi *et al.,* 2008). Circumstances that favour fungal growth can also favour aflatoxin production but fungal growth can occur resulting in little or no aflatoxin production (Sheriff, 2004; Stack and Carlson, 2006).

The widespread occurrence of these fungi could be an indicator of fungal infections at the post-harvest level as they have also been isolated at similar levels of incidence in other crops such as peanuts (Adebajo *et al.,* 1994; Awuah and Kpodo, 1996; Gachomo *et al.*, 2004). Apart from *Aspergillus* and *Fusarium,* the presence of other fungal species such as *A. niger*, *Penicillium* and *Rhizopus* could indicate a likelihood of contamination by other toxins produced by these fungi (Mutegi *et al.,* 2010). Studies show that it is possible to have more than one type of toxin from co-existing fungi (Speijersa and Speijersb**,** 2004). *A. niger* and *Penicillium* species can both produce ochratoxins (Wilson *et al.,* 2002; Klich, 2002).

*A. flavus* mainly produces aflatoxins but is also capable of producing another toxin known as cyclopiazonic acid (Horn and Dorner, 1998; Vaamonde *et al.,* 2003 Horn *et al.,* 2006). Certain *Penicillium* species are known to produce patulin and citrinin (Singh *et al.,* 2008; Spadaro *et al.,*

2009; Welke *et al.,* 2009). Kitui County recorded a high incidence of *A. niger* and *Penicillium* which have been shown to produce mycotoxins. The prevalence of such fungi should be a matter of concern since such toxins could be present in stored maize from the study areas although their occurrence was not investigated in the current study. Presence of other toxigenic fungi could be a possible indicator that the stored maize could be contaminated with other mycotoxins apart from aflatoxins.

In addition to the risk of aflatoxin contamination in stored maize, bio-deterioration is also a major problem associated with fungal contamination of maize kernels (Bhattacharya and Raha, 2002). They result in physical and biochemical alterations on maize kernels rendering them unfit for human consumption (Okoth *et al.,* 2012). The internal mycoflora of stored maize sampled across the three agro-ecological zones was dominated by the species of *Aspergillus*, *Fusarium* and *Penicillium* which are capable of predisposing the maize kernels to bio-deterioration. This normally results in biochemical and physiological changes that render the maize grains unfit for human consumption (Okoth *et al.,* 2012). Higher incidence of *A. flavus* and aflatoxin contamination has been observed to be common when crop rotation was not done (Abbas *et al.,* 2004).

### 5.1.2 Aflatoxin contamination

Aflatoxin analysis that was carried out for the maize samples showed that most of them contained levels of aflatoxin that were significantly different (Table 4.5). Out of the samples analyzed, 83.3 % tested positive for aflatoxin contamination. Kitui County recorded low levels of aflatoxin and this corresponds to the low frequency of *A. flavus* isolation. The low occurrence of *A. flavus* in Kitui could be explained by variation in weather conditions that did not favour

fungal growth during the sampling period. In a survey of 350 maize products conducted in Busia County in Western Kenya, it was shown that more than 55 % of the samples exceeded the 20 µg/kg while 35 % had aflatoxin levels above 100 µg/kg (Lewis *et al.,* 2005).

Previously, it has been documented that significant correlations exist between agro-ecological zones and aflatoxin levels whereby a wet and humid climate tends to aggravate aflatoxin levels (Mutegi *et al.,* 2010). In neighboring Uganda aflatoxin levels in maize samples were reportedly higher in more humid areas compared to the drier areas (Kaaya *et al.,* 2006). Similar results were obtained in a recent survey of maize samples from Nigeria (Atehnkeng *et al.,* 2008). Kitui County located within Eastern Kenya is normally characterized by high temperatures coupled with low and unreliable rainfall. Mean temperatures in this region range from 26 °C to 34 °C annually with average annual rainfall ranging from 500mm to 1050mm. This region therefore falls within the semi-humid to semi-arid agro-ecological zone. These climatic conditions have been known to favour infection of foodstuffs with *A. flavus* (Lanyasunya *et al.,* 2005).

In this study, aflatoxin content from the maize kernels collected during the post- harvest period is documented and well contrasted across zones. High aflatoxin levels have been associated with agro-ecological zones that experience higher rainfall and temperatures (Atehnkeng *et al.,* 2008). High aflatoxin levels were recorded in Kitale and Nakuru (Table 4.5) which are agro-ecological zones that had have high reliable rainfall and temperatures. Higher levels of aflatoxin in maize have been reported previously in Kenya where an outbreak of acute aflatoxicoses was due to severe levels of aflatoxin contamination (Lewis *et al.,* 2005). This was attributed to the the climatic conditions and farming practices of people living within these areas.

Aflatoxin levels reported in this study are much lower than those previously reported in Kenya (Lewis *et al.,* 2005; Wagacha and Muthomi, 2008). The possible reasons could be related to the

climatic patternsas well as the storage practices of maize farmers from the study sites. The optimum conditions for growth and subsequent production of aflatoxins by *A. flavus* include temperatures of 28 °C to 30 °C and water activity of 0.83 to 0.97 (Mutungi *et al* ., 2008).

The aflatoxin producing fungus *A. flavus* increases in number when soil temperatures range from 35 °C to 40 °C (Riley and Norrad, 1999). The higher contamination levels in Nakuru and Kitale could be an indicator of improper production and handling practices of both pre-harvest and post-harvest maize. Aflatoxin levels have also been shown to increase if the crop is damaged by insect pests, stressed by heat or drought and after maturity when maize is exposed to high moisture content levels either before harvest or while in store (Payne, 1992; Atehnkeng *et al.,* 2008). Low-level aflatoxin exposure has been shown to increase the risk of hepatocellular carcinoma (Peraica *et al.,* 1999). Acute hepatitis resulting from consumption of mouldy maize grains has been reported to occur in Western India and Malaysia (Lanyasunya *et al.,* 2005). Ingestion of 2-6 mg of aflatoxin per day can result in acute hepatitis and eventually death within a month (Chao *et al.,* 1991).

In relation to this, residents of the three study areas could face health-related problems that could be brought about by cumulative levels of aflatoxin. (Lanyasunya *et al.,* 2005). This was illustrated by the lethal aflatoxicoses outbreak which had more than 125 mortalities in Eastern Kenya during January to July 2004 (Probst *et al.,* 2007). Apart from aflatoxins that are produced by *Aspergillus* species, the other fungi are capable of producing mycotoxins and other compounds that are lethal to human and animal health (Orsi *et al.,* 2000). The impact of aflatoxin levels in stored maize studied in this research revealed that more than half of the samples were contaminated (Table 4.5) with 36 % of the total samples reading above the legal limit of 10 ppb. This

indicates that aflatoxin contamination in maize is still a serious threat. Contamination by aflatoxin and post-harvest fungi can be reduced by focusing on improved control strategies for wet and humid zones such as planting improved maize cultivars and controlling pre-harvest and post-harvest pest damage. Conventional household maize preparation techniques should be explored as possible aflatoxin management strategies in Kenya.

### 5.1.3 Genetic diversity

Genetic diversity in any fungal population is of major importance in developing a suitable management strategy. Hence, the genetic variability among the isolates of *A. flavus* should be taken into account when *A. flavus* isolates are used in the resistance breeding programmes for evaluation or to design primers for detection of *A. flavus* in foods and feeds by PCR (Krishna *et al.,* 2002). In this study, the isolates of *A. flavus* were assessed for their genetic diversity using 13 forward and reverse primers in an attempt to characterize them across three regions.

The present study involved the use of thirteen SSR markers that were used to assess the genetic diversity of *A. flavus* isolates across three agro-ecological zones. All the microsatellite markers used were found to be highly polymorphic across all the populations studied. The occurrence of genetic polymorphism in *A. flavus* isolates has been well documented (Tran-Dinh *et al.*, 1999; Karthikeyan *et al.*, 2009). SSR markers are good tools for distinguishing different *A. flavus* isolates from different hosts and geographical regions (Diaz *et al.,* 2000; Batista *et al.,* 2008). Geiser *et al.* (1998) evaluated 11 loci of *A. flavus* from 31 isolates and identified 16 genotypes with no obvious geographical patterns. Also, two reproductive groups were found with no strong geographical or morphological basis (Wang *et al.,* 2012).

It has also been shown that there is a relationship between the amount of toxins produced and vegetative compatibility (Habibi and Banihashemi 2008). Principal coordinate analysis (PCoA) from the SSR data showed that four clusters were formed and only Nakuru isolates appeared to cluster separately from the rest. This indicates that the *A. flavus* isolates from this region are genetically divergent from the rest. (Wang *et al.,* 2012) also observed the same level of diversity while studying four different *Aspergillus* species. The largest cluster in the PCoA could be explained by the fact that maize farmers from Kitui region only produce enough for the family's consumption. When the cereal supply runs out in their stores, they are forced to buy maize from farmers in major maize growing regions, such as Trans-Nzoia County.

The phylogenetic tree from the current study showed that distinct clades were formed according to geographical locations. This indicates that the isolates of *A. flavus* are genetically divergent with respect to their origin. (Probst *et al.,* 2010) showed that *A. flavus* isolates especially those with the S strain morphology differ from other *A. flavus* isolates that are aflatoxin producers. It was also shown that these strains were restricted to Eastern Kenya where they have been known to cause great losses due to high levels of aflatoxin contamination.

Proper field management practices could serve as indispensable strategies for mitigating aflatoxin contamination of maize in Kenya. The results of this study show that there is need for continued aflatoxin awareness creation among the farmers in Kenya. After the aflatoxin poisoning outbreak in the year 2004, vigorous mycotoxin awareness campaigns were held in the country by the Food and Agricultural Organization and Kenya's Ministry of Agriculture (Strosnider *et al*., 2006). The results of these campaigns showed that individuals who received information on maize drying and storage had lower serum aflatoxin levels than those who did not

receive the information awareness (Wagacha and Muthomi, 2008). Such campaigns could use systems that are in place for disseminating information to small-scale and subsistence farmers.

## 5.2 Conclusions

1. This research has shown that *A. flavus* is the main fungal species infecting maize grains in three Counties in Kenya. With regard to economic importance, *Aspergillus* spp. was the commonly isolated fungus followed by *Fusarium* and *Penicillium* spp. Fungal infection of maize varies with households and this reflects different farm management practices, some of which adversely contribute to contamination of the maize along the food value chain.

2. Aflatoxin levels were shown to differ significantly within the three study regions. Distinct clustering of *A. flavus* isolates with high aflatoxin production was also observed throughout the study. The influence of aflatoxins in Kenya in the study areas demonstrates a clear need for tools to manage contamination of locally produced maize.

3. This study has also established that the main aflatoxin producing fungi, *A. flavus* differs with regard to molecular characterization within and between the three different populations. Phylogenetic analysis showed that five major clusters were formed with other sub-clusters with samples from the same agro-ecological zone clustering together. This indicates that there is genetic divergence of the *A. flavus* isolates. The environmental conditions that are characteristic to each agro-ecological zone could also be responsible for these observations.

### 5.3.1 Recommendations

1. The post-harvest sector is an important avenue through which food production can be increased thereby ensuring food security and protection of health of the Kenyan citizens. This can be achieved through the involvement of policy makers and educational forums in the study regions to help farmers on advising them on good post-harvest practices in prevention of aflatoxin development.

2. There should be coordinated and collaborative research efforts on aflatoxin both in Kenya and Africa so that resources can be focused on priority areas and documenting the impact of aflatoxin on health and the economy of the country.

3. Further complementary studies should be undertaken in the same field of study with a major focus on molecular characterization of *A. flavus* isolates that are atoxigenic and their potential use in mitigating aflatoxin contamination in food crops.

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# 7.0 APPENDICES

## APPENDIX I: STRUCTURED QUESTIONNAIRE

## STRUCTURED INTERVIEW GUIDE FOR MAIZE FARMERS

### I. BASIC INFORMATION

1) Date of the interview ……………………………………………..

2) Study site ……………………………………………..

3) Code of the interview ……………………………………………..

### II. STRUCTURED QUESTIONS

1. How long does it take your maize to be ready ie the duration between planting and harvesting?
   a) 4 months [ ]
   b) 5 months [ ]
   c) 6 months [ ]
   d) 7 months [ ]
   e) 8 months and above [ ]

2. When do you harvest your maize?
   f) During rainy weather [ ]
   g) During wet weather [ ]
   h) During dry weather [ ]

3. How do you dry your maize after harvesting?
   a) Drying in the sun for a few weeks [ ]

b) I store it immediately after harvest [ ]

c) Other methods (Specify) ……………………………………………..

……………………………………………………………………………

………………………………………………………………………………

…………………………………………………………………………..

4. Do you apply/sprinkle any insecticide on your maize before you store it?

    a) Yes [ ]

    b) No [ ]

5. In what form do you store your maize?

    c) Maize cobs [ ]

    d) Maize grains [ ]

6. What storage item do you use to store your maize?

    a) Sisal sacks (gunias) [ ]

    b) Paper bags [ ]

    c) Plastic containers [ ]

    d) I store them openly on the floor [ ]

7. What storage structure do you use to store your maize?

    a) Granary [ ]

    b) Others (Specify) ………………………………………………………..

8. What are the major pests that you find in your storage structure?

   a) Maize Weevils [ ]

   b) Grain borer [ ]

   c) Beetles [ ]

   d) Others ( specify) [ ]

9. How long do you store your maize before consumption?

   a) 1-3 months [ ]

   b) 3-6 months [ ]

   c) 6 and above [ ]

   d) Until the next planting season [ ]

## APPENDIX II : AMOVA TABLE

### AMOVA: Variation within and among the different populations

| **Summary AMOVA Table** | | | | | |
|---|---|---|---|---|---|
| **Source** | **df** | **SS** | **MS** | **Est. Var.** | **%** |
| **Among Pops** | 3 | 212.881 | 70.960 | 2.751 | 13% |
| **Within Pops** | 89 | 1671.947 | 18.786 | 18.786 | 87% |
| **Total** | 92 | 1884.828 | | 21.537 | 100% |