

**POPULATION OF *Aspergillus* section *Flavi* AND AFLATOXIN CONTAMINATION IN
MAIZE FROM FIELDS TREATED WITH ATOXIGENIC *Aspergillus flavus*
(AFLASAFE KE01) IN LOWER EASTERN KENYA**

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DECLARATION

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DEDICATION

I dedicate this thesis to my late dad, mum, siblings and all farmers affected by the aflatoxin menace.

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TABLE OF CONTENTS

| | |
|---|------|
| DECLARATION | ii |
| DEDICATION | iii |
| ACKNOWLEDGEMENT | iv |
| TABLE OF CONTENTS..... | v |
| LIST OF TABLES | ix |
| LIST OF FIGURES | x |
| LIST OF APPENDICES..... | xi |
| LIST OF ABBREVIATIONS AND ACRONYMS | xii |
| ABSTRACT..... | xiii |
| CHAPTER ONE: INTRODUCTION..... | 1 |
| 1.1 Background information | 1 |
| 1.2 Problem statement..... | 2 |
| 1.3 Justification | 3 |
| 1.4 Study objectives | 4 |
| 1.5 Hypotheses | 5 |
| CHAPTER TWO: LITERATURE REVIEW..... | 6 |
| 2.1 Maize production in Kenya..... | 6 |
| 2.2 Factors limiting maize production in Kenya..... | 7 |
| 2.3 Aflatoxin and its effects on human and animal health..... | 7 |
| 2.4 Aflatoxin effects on trade..... | 8 |
| 2.5 <i>Aspergillus</i> section <i>Flavi</i> | 9 |
| 2.5.1 <i>Aspergillus flavus</i> | 9 |
| 2.5.2 <i>Aspergillus parasiticus</i> | 10 |

| | |
|--|----|
| 2.5.3 <i>Aspergillus tamarii</i> | 11 |
| 2.6 Conditions that favor aflatoxin development on maize | 11 |
| 2.7 Methods of aflatoxin detection | 12 |
| 2.8 Aflatoxin management strategies..... | 13 |
| 2.9 Biological control of aflatoxigenic <i>Aspergillus</i> species..... | 13 |
| CHAPTER THREE: MATERIALS AND METHODS | 15 |
| 3.1 Description of the study area | 15 |
| 3.2 Preparation of culture media..... | 16 |
| 3.3 Determination of population of <i>Aspergillus</i> section <i>Flavi</i> in soils | 17 |
| 3.3.1 Determination of maize production practices in lower Eastern Kenya | 17 |
| 3.3.2 Collection, handling and processing of soil samples | 18 |
| 3.3.3 Isolation and identification of <i>Aspergillus</i> section <i>Flavi</i> from soil samples..... | 18 |
| 3.4 Effect of atoxigenic <i>Aspergillus flavus</i> (Aflasafe KE01) on the population of toxicogenic <i>Aspergillus</i> section <i>Flavi</i> and aflatoxin contamination | 20 |
| 3.4.1 Application of atoxigenic <i>A. flavus</i> (Aflasafe KE01) | 20 |
| 3.4.2 Assessment of sporulation of atoxigenic <i>A. flavus</i> (Aflasafe KE01) in maize fields..... | 20 |
| 3.4.3 Collection, handling and processing of maize grain samples | 20 |
| 3.4.4 Isolation and identification of <i>Aspergillus</i> section <i>Flavi</i> from maize samples..... | 21 |
| 3.4.5 Extraction and quantification of aflatoxin levels in maize samples | 21 |
| 3.5 Determination of aflatoxin producing ability of <i>Aspergillus</i> section <i>Flavi</i> isolates | 22 |
| 3.5.1 Inoculation and culture of <i>Aspergillus</i> section <i>Flavi</i> for aflatoxin production in vitro..... | 22 |

| | |
|---|----|
| 3.5.2 Extraction and quantification of aflatoxin from the fermentation cultures..... | 23 |
| 3.6 Statistical data analyses..... | 24 |
| CHAPTER FOUR: RESULTS | 25 |
| 4.1 Maize production practices in Lower Eastern Kenya | 25 |
| 4.2 Diversity of <i>Aspergillus</i> section <i>Flavi</i> in soil and maize samples | 28 |
| 4.3 Population of <i>Aspergillus</i> section <i>Flavi</i> in soils | 30 |
| 4.4 Correlation between the population of <i>Aspergillus</i> section <i>Flavi</i> in soil and maize production practices in lower Eastern Kenya..... | 32 |
| 4.5 Efficacy of Aflasafe KE01 in reducing toxigenic <i>Aspergillus</i> and aflatoxin in treated maize fields..... | 33 |
| 4.5.1 Sporulation of atoxigenic <i>A. flavus</i> L-strain (Aflasafe KE01) in treated maize fields..... | 33 |
| 4.5.2 <i>Aspergillus</i> section <i>Flavi</i> isolated from maize samples | 34 |
| 4.5.3 Aflatoxin levels in maize samples | 36 |
| 4.5.4 Correlation among sporulation of Aflasafe KE01, population of <i>Aspergillus</i> section <i>Flavi</i> and aflatoxin levels in maize..... | 36 |
| 4.6 Aflatoxin producing potential of <i>Aspergillus</i> section <i>Flavi</i> isolates..... | 38 |
| CHAPTER FIVE: DISCUSSION..... | 41 |
| 5.1 Maize production practices and population of <i>Aspergillus</i> section <i>Flavi</i> in soil in Lower Eastern Kenya | 41 |
| 5.2 Efficacy of atoxigenic <i>A. flavus</i> (Aflasafe KE01) in managing toxigenic <i>Aspergillus</i> and aflatoxin in maize fields | 44 |
| 5.3 Aflatoxin production by <i>Aspergillus</i> section <i>Flavi</i> isolates from soil and maize..... | 46 |

| | |
|--|----|
| CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS | 48 |
| 6.1 Conclusions..... | 48 |
| 6.2 Recommendations..... | 49 |
| REFERENCES | 50 |
| APPENDICES | 62 |
| Appendix I: Preparation of stock solutions..... | 62 |
| Appendix II: Annual average temperature (°C), precipitation (mm) and relative humidity (%) in lower Eastern Kenya in 2013 and 2014..... | 64 |
| Appendix III: Questionnaire on maize production practices in lower Eastern Kenya | 65 |

LIST OF TABLES

| | | |
|-----------|---|----|
| Table 1: | Proportion (%) of crops intercropped with maize in four districts in lower Eastern Kenya..... | 26 |
| Table 2: | Proportion (%) of farmers using different soil amendments in maize production in lower Eastern Kenya..... | 27 |
| Table 3: | Proportion (%) of farmers using different types of structures to store maize in lower Eastern Kenya..... | 28 |
| Table 4: | Proportion (%) of <i>Aspergillus</i> section <i>Flavi</i> in soil sampled from four districts in lower Eastern Kenya..... | 31 |
| Table 5: | Correlation among the population of <i>Aspergillus</i> section <i>Flavi</i> in soil and maize production practices in lower Eastern Kenya..... | 33 |
| Table 6: | Sporulation (%) of Aflasafe KE01 in farmers' maize fields in four districts in lower Eastern Kenya..... | 34 |
| Table 7: | Proportion (%) of <i>Aspergillus</i> section <i>Flavi</i> in maize samples from Aflasafe KE01 treated and untreated fields in lower Eastern Kenya..... | 35 |
| Table 8: | Total aflatoxin levels (ppb) in maize samples from Aflasafe KE01 treated and untreated fields in four districts of lower Eastern Kenya..... | 37 |
| Table 9: | Proportion (%) of maize samples from four districts of lower Eastern Kenya that conformed to different aflatoxin standards..... | 37 |
| Table 10: | Correlation among, the population of <i>Aspergillus flavus</i> L-strain, <i>A. flavus</i> S-strain aflatoxin levels and sporulation of Aflasafe KE01..... | 38 |
| Table 11: | Aflatoxin production (ppb) by <i>Aspergillus</i> section <i>Flavi</i> isolates from soil samples collected in lower Eastern Kenya..... | 39 |
| Table 12: | Aflatoxin production (ppb) of <i>Aspergillus</i> section <i>Flavi</i> isolates from maize fields..... | 40 |

LIST OF FIGURES

| | |
|--|----|
| Fig 1: Map of lower Eastern Kenya showing experimental sites: Kitui, Machakos and Makueni Counties..... | 16 |
| Fig. 2: Percentage of farmers growing different maize varieties in lower Eastern Kenya during 2013/2014 cropping season..... | 26 |
| Fig. 3: Proportion of farmers using different tillage methods in lower Eastern Kenya..... | 27 |
| Fig 4: <i>Aspergillus</i> section <i>Flavi</i> species isolated from soil and maize samples in lower Eastern Kenya..... | 29 |
| Fig 5: Conidial heads of <i>Aspergillus</i> section <i>Flavi</i> from random maize and soil samples from lower Eastern Kenya..... | 30 |
| Fig 6: Population (CFU/g) of <i>Aspergillus</i> section <i>Flavi</i> in soil sampled from four districts in lower Eastern Kenya..... | 32 |
| Fig 7: Incidence (%) of atoxigenic and toxigenic members of <i>Aspergillus</i> section <i>Flavi</i> from Aflasafe KE01 treated and untreated fields..... | 40 |

LIST OF APPENDICES

| | |
|---|----|
| Appendix I: Preparation of stock solutions..... | 62 |
| Appendix II: Annual average temperature, precipitation and relative humidity in lower Eastern Kenya in 2013 and 2014 | 64 |
| Appendix III: Questionnaire on maize production practices in lower Eastern Kenya..... | 65 |

LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|--------|--|
| ASDSP: | Agricultural Sector Development Support Programme |
| ELISA: | Enzyme Linked Immunosorbent Assay |
| EC: | European Commission |
| EU: | European Union |
| FAO: | Food and Agriculture Organization |
| FDA: | Food and Drug Administration |
| HPLC: | High Performance Liquid Chromatography |
| IARC: | International Agency for Research on Cancer |
| IITA: | International Institute of Tropical Agriculture |
| KALRO: | Kenya Agricultural and Livestock Research Organization |
| LC: | Liquid Chromatography |
| MC: | Moisture content |
| mRBA: | modified Rose Bengal Agar |
| PACA: | Partnership for Aflatoxin Control in Africa |
| TLC: | Thin Layer Chromatography |
| UV: | Ultra Violet |
| VCG: | Vegetative Compatibility Group |

ABSTRACT

Maize, the staple food for 96% of Kenya's population is highly prone to aflatoxin contamination. The objective of this study was to understand shifts in baseline populations within *Aspergillus* section *Flavi* in soils and associated contamination of maize with aflatoxin as a result of application of atoxigenic *A. flavus* (Aflasafe KE01) in aflatoxin hotspot areas in lower eastern Kenya. The study was conducted during the short rains of 2013/2014 cropping season in four districts distributed across three Counties of lower Eastern Kenya which were worst hit by aflatoxicosis in 2004: Kathiani district (Machakos County), Nzambani district (Kitui County), Wote and Kaiti districts (Makueni County). Twenty four farms were randomly sampled in each district. A structured questionnaire was used to collect data on maize production practices. Soil samples were collected at the seventh leaf growth stage of maize, ground using a motor and pestle, sieved through a 2 mm mesh screen, sun dried for five days and stored at room temperature ($23 \pm 2^\circ \text{C}$) in Kraft bags until microbial analysis was undertaken. Aflasafe KE01 was applied when maize was at the seventh leaf growth stage by broadcasting at the rate of 10 kg/ha. Maize cobs were sampled at harvest, sun dried for seven days, shelled manually and oven dried at 45°C for 48 hours. Moisture content (MC) of the maize grains was measured and samples that had $>13\%$ MC were oven dried again for 12 hours at 45°C . *Aspergillus* section *Flavi* from soil and maize samples were isolated on modified rose Bengal agar. One gram of each ground maize and soil samples were dissolved in 10 ml of sterile distilled water and incubated for three days at 31°C . *Aspergillus* species were identified on 5/2 agar based on cultural and morphological characteristics after incubation for five days at 31°C . Aflatoxin level in maize samples was determined using the Enzyme Linked Immunosorbent Assay (ELISA) based Accuscan Pro-reader method. Aflatoxin produced by *Aspergillus* section *Flavi* isolates *in vitro* was measured by thin layer chromatography. Farmers in lower Eastern Kenya were mainly

found to grow the local variety of maize and were intercropping it with pigeon peas. They were also found to use oxen plough to till their land and were applying manure as the main soil amendment. After harvesting the maize, the farmers were mostly found to store their maize in granaries. Four morphotypes of *Aspergillus* section *Flavi* were commonly isolated from the soil: *A. flavus* S-strain (56.9%), *Aspergillus flavus* L-strain (26.2%), *A. parasiticus* (13.6%) and *A. tamarii* (3.2%). The population of atoxigenic *A. flavus* L-strain was significantly ($p \leq 0.05$) higher (84%) in maize samples from Aflasafe KE01 treated fields than the other members of *Aspergillus* section *Flavi*. On the other hand, the population of *A. flavus* S-strain was significantly ($p \leq 0.05$) higher (62%) in samples from untreated maize fields. The mean aflatoxin level in maize grains from treated fields was 44.8ppb while in untreated fields the mean was 84.3ppb. Aflasafe KE01 reduced aflatoxin level in maize grains by 47% across the four districts. Aflatoxin B1, B2, G1 and G2 were detected in the isolates of *Aspergillus* section *Flavi*. Isolates of *A. flavus* S-strain produced the highest level of aflatoxin B1 (Mean = 3964ppb). Both *A. flavus* S-strain and *A. parasiticus* produced aflatoxin B1, B2, G1 and G2 while *A. flavus* L-strain only produced aflatoxin B1 and B2. Aflasafe KE01 is a promising biocontrol product in shifting the population of toxigenic strains of *Aspergillus* section *Flavi* and subsequently reducing aflatoxin levels in maize.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Maize is the staple food that dominates food security considerations in Kenya (Probst *et al.*, 2010). Its production in Kenya has not kept pace with consumption and population growth. Maize production is estimated at 30 million bags (90kgs) annually (Olwande, 2012) with a consumption rate estimated at 98 kilograms per person per year, which translates to about 35 million bags per year.

Maize is prone to colonization by toxigenic fungal species resulting in production of mycotoxins. Out of the more than 400 mycotoxins known, the most important in terms of commercial impact and risk to human and animal health are aflatoxins, fumonisins, ochratoxins, deoxynivalenol, zearalenone and patulin (Reddy *et al.*, 2010). These mycotoxins are produced as secondary metabolites during growth of the fungi. Toxigenic fungi can attack maize prior to harvest and further decay the crop during storage (Atehnkeng *et al.*, 2008a). The Food and Agriculture Organization (FAO) estimates that 25% of the world's crops are prone to mycotoxins contamination (Reddy *et al.*, 2010).

Most African staples are prone to aflatoxin due to colonization by *Aspergillus* species. *Aspergillus* section *Flavi* is widely known to contain the most dangerous aflatoxin contaminants in agricultural products (Perrone *et al.*, 2014). There is concern on the effects of aflatoxin from *Aspergillus* section *Flavi* (Soares *et al.*, 2011). Aflatoxin is one of the most potent, naturally occurring, compounds known to man (Machida and Gomi, 2010). An earlier study by Probst *et al.* (2007) implicated *A. flavus* S-strain as the causative agent of the major aflatoxicosis outbreak

in Kenya in 2004. Other species in *Aspergillus* section *Flavi* include *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae*, and *A. tamaraii* (Rodriguez *et al.*, 2009).

Aflatoxins are highly mutagenic and toxic to human and animal health (Othman and Al-Delamiy, 2012). They are of great concern due to their carcinogenic, immune-suppressing and growth retardation effects in humans and animals (Diedhiou *et al.*, 2011). Probst *et al.* (2007) among others reported the most severe aflatoxicosis episode which claimed more than 125 lives in Kenya in 2004. Global review of aflatoxin contaminated food items revealed that virtually majority of foods are vulnerable to *Aspergillus* colonization (Oluwafemi *et al.*, 2010). Many methods to contain toxigenic *Aspergillus* fungi and aflatoxin contamination have been explored. One of these is the use of atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers and thereby reduce aflatoxin contamination in crops (Probst *et al.*, 2011).

1.2 Problem statement

Kenya has repeatedly experienced epidemics of acute aflatoxicosis for several decades, which has been associated with maize produced in the country (Probst *et al.*, 2010; 2012). Re-current aflatoxicosis outbreaks in Kenya have been attributed to among other factors, small scale maize production is a primary avenue through which human exposure occurs particularly in Africa (Probst *et al.*, 2010). Maize is also one of the richest substrates for aflatoxin elaboration (Wagacha and Muthomi, 2008). Deadly epidemics due to lethal aflatoxicosis in both animals and humans have been reported in Kenya severally since discovery of aflatoxin in 1960s (Blount, 1961). Most severe episode of lethal aflatoxicosis in the world claimed 125 lives in Makueni,

Kitui, Machakos and Kiambu Counties (Probst *et al.*, 2010). A total of 317 cases of aflatoxicosis were reported by July 2004 (Probst *et al.*, 2007).

Aflatoxins are a major cause of food insecurity in Kenya's growing population. Reduced crop value is a significant component of the losses caused by mycotoxins. In 2010, 2.3 million bags of maize grown in the Eastern and Coastal regions of Kenya were declared as being unfit for human consumption by the Ministry of Public Health and Sanitation due to high levels of aflatoxin contamination (FAO, 2011). Moreover, Partnership for Aflatoxin Control in Africa (PACA) (2012) has indicated a reduction in marketable volumes for aflatoxin contaminated crops and rejection of products at international markets. Aflatoxins are potent hepatocarcinogens and immunosuppressants to humans (Ezekiel *et al.*, 2014b). Furthermore losses in animal productivity have been observed from mycotoxin-related health problems (Schmale and Munkvold, 2009).

1.3 Justification

There are no effective fungicides to combat aflatoxin contamination of food crops. The governments of Kenya and other agencies have been conducting education campaigns on aflatoxin to create awareness among stakeholders and encourage good postharvest activities including proper storage. However, farmers lack appropriate storage and drying facilities. Moreover, extended periods of field drying are related to increased fungal contamination. Postharvest practices like sorting may reduce aflatoxins in stored maize but the proportion of cobs sorted will depend on farmer's judgment and choice (Hell *et al.*, 2008). Post-harvest interventions will also not arrest pre-harvest contamination.

Aflasafe KE01 is a formulated biological control product composed of four atoxigenic *A. flavus* L-strains on a sorghum carrier. The four strains are native to Kenyan soils, abundant and can displace toxigenic strains. The use of atoxigenic *A. flavus* L-strain strains has been shown to reduce aflatoxin contamination of staples by modifying the *Aspergillus* community structure through competitive exclusion (Bandyopadhyay and Cotty, 2013). Moreover, Probst *et al.* (2011) reported the ability of atoxigenic strains to reduce aflatoxin level by more than 80% in field trials in Kenya. Use of this technology will be necessary in the background of a report y FAO (2009) that projected the world's population to hit nine billion by 2050 which will require raising food safety standards and production by 70% by then.

1.4 Study objectives

The broad objective of this study was to understand shifts in baseline populations within *Aspergillus* section *Flavi* in soils and associated contamination of maize with aflatoxin as a result of application of atoxigenic *A. flavus* (Aflasafe KE01) in maize in lower eastern Kenya.

The specific objectives were:

- i. To evaluate maize production practices and their effect on the population of *Aspergillus* section *Flavi* in soils in aflatoxin prone regions of lower Eastern Kenya.
- ii. To determine the effectiveness of field application of atoxigenic *Aspergillus flavus* (Aflasafe KE01) in reducing the population of toxigenic *Aspergillus* section *Flavi* and aflatoxin contamination.
- iii. To determine aflatoxin producing ability of *Aspergillus* section *Flavi* isolates.

1.5 Hypotheses

- i. Maize production practices do not affect the population of *Aspergillus* section *Flavi* in soils in aflatoxin hot spots of lower Eastern Kenya.
- ii. Atoxigenic *Aspergillus flavus* (Aflasafe KE01) is not effective in reducing the population of toxigenic *Aspergillus* section *Flavi* and aflatoxin contamination in maize.
- iii. There is no variation in the aflatoxin producing ability of *Aspergillus* section *Flavi* isolates.

CHAPTER TWO: LITERATURE REVIEW

2.1 Maize production in Kenya

Maize is the staple food in Kenya planted on 90% of farms (Mbithi and Huylenbroeck, 2000). Maize production was averaged at 2.7 million tons per year and 2.8 million tons per year (Nyoro *et al.*, 2004). In recent years there has been an expansion of land used for maize production from 1.7 million hectares in 2008 to 1.8 million hectares in 2009 (Kang'ethe, 2011). Over time, national maize production has not kept pace with consumption. Production has not increased as fast as demand driven mainly by population growth (Nyoro *et al.*, 2004). With the country's population projected to reach 43.1 million by the year 2020, the demand for maize is then likely to be 5 million metric tonnes. This means based on the prevailing maize production rates that the maize deficit will be around 1.2 million metric tonnes in 2020 (Kang'ethe, 2011). Improving the competitiveness of Kenyan maize production is of importance in resolving the food price dilemma and can ensure greater profitability to producers at lower prices and consequently improve poor consumers' access to food. Reducing the cost of production also allows domestic producers to compete more effectively with imports from other countries (Nyoro *et al.*, 2004).

Maize production in Kenya is dominated by small scale farmers who account for 75% of the overall production; the other 25% is grown by large scale farmers (Kang'ethe, 2011). High-potential maize zones like Trans Nzoia, Uasin Gishu, Nakuru, Bomet, and Kakamega Counties have higher maize prices which help small-scale farmers where roughly 70% of households sell maize.

2.2 Factors limiting maize production in Kenya

Kenya has lost its competitiveness in maize production to the neighbouring countries due to the high cost of maize production (Nyoro, 2004). Moreover pests account for 50-80% losses in maize production. Fungal pathogens mainly account for 11% of maize diseases hampering the harvest (Oerke and Dehne, 2004). Furthermore, most of the maize produced and consumed in Africa comes from smallholder rural farms. Production takes place under difficult conditions characterized by poor soils; low-yielding varieties; inadequate access to yield-enhancing inputs such as fertilizers and improved seeds; inadequate access to finance by producers, suppliers and buyers; and variable climatic and environmental conditions. There are also heavy post-harvest losses due to poor storage, processing facilities and technologies (Kimeli, 2013).

2.3 Aflatoxin and its effects on human and animal health

Aflatoxins are highly carcinogenic mycotoxins frequently produced by *Aspergillus* species. Contamination of maize with aflatoxins imposes both economical and health burdens in many regions (Probst *et al.*, 2012). Aflatoxins B1 are group one human carcinogens. Research on aflatoxins over the last 40 years has made it one of the best studied fungal secondary metabolites (Scheidegger and Payne, 2003). Generally health effects of aflatoxins include immune suppression, impaired child growth, abnormal fetal development, hepatitis, jaundice abdominal swelling and death (Probst *et al.*, 2010). Exposure to high level of aflatoxin can cause acute aflatoxicosis and chronic aflatoxicosis may occur with low levels of aflatoxins taken for a long period (Mehan *et al.*, 1991).

Presence of toxigenic *Aspergillus* in poultry feeds is a leading cause of low productivity and mortality in poultry birds (Ezekiel *et al.*, 2014a). Aflatoxins are teratogenic for animals leading

to diminished health (Probst *et al.*, 2010). Thus strict regulations are necessary to monitor aflatoxin levels in both feeds and food (Probst *et al.*, 2010). When aflatoxin B1 is ingested by cows, it is transformed into its hydroxylated product, aflatoxin M1 and M2. Such aflatoxins are secreted in the milk and are relatively stable during milk pasteurization, storage, and preparation of various dairy products (Stroka and Anklam, 2002).

2.4 Effects of aflatoxin on trade

Different countries have different aflatoxin thresholds for different food product use for instance the European Union (EU) threshold is 4ppb, Kenya Bureau of Standards (KEBS) threshold is 10ppb and Food and Drug Administration (FDA) threshold is 20ppb. The regulatory thresholds may impose trade barriers and result to reduced marketable volume thus trade between two countries may undergo several costs to meet the standards: cost of testing, possibility of shipment rejection (PACA, 2013). Countries with stringent thresholds like the EU (4ppb) pose a challenge in importing from aflatoxin prevalent areas. A study by Otsuki *et al.* (2000) demonstrated reduction in importation in Europe from African countries that have had by USD\$670 million which translated to 64% reduction in cereal exportation due to the stringent threshold set. Losses due to aflatoxin contamination range up to hundreds of million Dollars in the USA (Wu, 2015). However, most countries in Africa do not implement regular surveillance of aflatoxins on food crops (Warth *et al.*, 2012). Even countries with stringent aflatoxin regulatory thresholds still have many subsistence farmers consuming maize that has not undergone surveillance (Wu, 2015).

2.5 *Aspergillus* section *Flavi*

Aspergillus section *Flavi* is a group of species with conidial heads in shades of yellow-green to brown and dark sclerotia (Varga *et al.*, 2011). *Aspergillus* section *Flavi* is composed of both aflatoxigenic and non aflatoxigenic species (Ehrlich, 2014). *Aspergillus flavus* and *A. parasiticus* are the most commonly implicated species in aflatoxin contamination of crops (Probst *et al.*, 2007; 2010). According to Kumeda and Asao (1996), the atoxigenic species in *Aspergillus* section *Flavi* include *A. oryzae*, *A. sojae* and *A. tamaritii*.

2.5.1 *Aspergillus flavus*

The surface of *A. flavus* is yellow-green in color and it is gold or red brown underneath, it has a particular affinity for nuts and oilseeds as substrates (Othman and Al-Delamiy, 2012). *Aspergillus flavus* has received considerable attention due to its ability to produce aflatoxins, a secondary metabolite that is both immunosuppressive and carcinogenic to animals and humans (Probst *et al.*, 2010). *Aspergillus flavus* is the predominant species comprising 98% of *Aspergillus* section *Flavi* isolated from maize samples in Eastern province of Kenya (Probst *et al.*, 2010).

Aspergillus flavus exists in complex communities that vary widely in both strain and vegetative compatibility group (VCG) composition and aflatoxin producing ability (Probst *et al.*, 2010). Aflatoxin production is more similar within VCGs than among VCGs (Probst *et al.*, 2011). Previous phylogenetic studies of *A. flavus* have shown that it consists of two subgroups, called groups I and II, and morphological studies indicated that it consists of two morphological groups based on sclerotium size, called “S” and “L (Geiser *et al.*, 2000). According to Cotty (1989), *A.*

flavus L-strains form large sclerotia greater than 400 µm in diameter and S-strains form sclerotia of less than 400 µm diameter. The S-strain and L-strain differ in aflatoxin producing ability (Probst *et al.*, 2011). The *A. flavus* S-strain produces copious amount of aflatoxin B1 compared to the L-strain and a recent study by Probst *et al.* (2014) has projected *A. flavus* S-strain found in Kenya to further produce G type aflatoxin although previously *Aspergillus flavus* in Kenya were known to produce aflatoxin B only. The lack of aflatoxin G production is a consequence of a 0.8- to 1.5-kb deletion near the 5' end of the gene cluster for aflatoxin biosynthesis (Ehrlich *et al.*, 2004). *Aspergillus flavus* was said to only produce aflatoxin B1 and aflatoxin B2 while *A. parasiticus* produces these same metabolites along with aflatoxin G1 and aflatoxin G2 (Bennett, 1981). Aflatoxin B and G fluoresce blue and green respectively during excitation with 365nm wavelength Ultra Violet (UV) light (Espinosa-Calderón *et al.*, 2012). *Aspergillus flavus* is composed of toxigenic and atoxigenic strains. Atoxigenic strains lack the ability to produce aflatoxins due to deletion in their gene structure. Atoxigenic *A. flavus* L-strain is a promising candidate for the development of competitive displacement strategies (Klueken *et al.*, 2009).

2.5.2 *Aspergillus parasiticus*

Almost all isolates of *A. parasiticus* are capable of producing aflatoxin (Al- Hmoud *et al.*, 2012). Conidial wall ornamentation is regarded as the primary morphological diagnostic character or separation of *A. flavus* and *A. parasiticus*. Conidia of *A. flavus* have relatively thin walls which are finely to moderately rough. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose (Rodriguez *et al.*, 2009). *Aspergillus parasiticus* has been indicated to produce dark green colonies on 5/2agar and rough conidia (Atehnkeng *et al.*, 2008a). Highly toxigenic isolates of *A. parasiticus* have been isolated in maize kernels in Nigeria (Atehnkeng *et al.*, 2008a). *Aspergillus parasiticus* is

predominant in peanuts and studies by Pildain *et al.* (2008) showed that *A. parasiticus* from Argentinean peanuts can produce aflatoxin B1, B2, G1 and G2, aspergillic acid, kojic acid, parasiticolides, oryzaechlorin, paspaline, paspalinine, aflavinines and other sclerotial metabolites.

2.5.3 *Aspergillus tamarii*

Aspergillus tamarii is a filamentous fungus isolated from soil which has a yellowish brown colour and rough conidia (Ezekiel *et al.*, 2014b). The double walled spores make it morphologically similar to *A. caelatus*. Occurrence of atoxigenic isolates of *A. tamarii* has been reported in Sub-Saharan Africa (Ezekiel *et al.*, 2014b). Recent studies have documented the occurrence of *A. tamarii* in peanuts in Kenya (Nyirahankizimana *et al.*, 2013; Mutegi *et al.*, 2012; Wagacha *et al.*, 2013). *Aspergillus tamarii* has also been isolated from chicken feeds in Nigeria (Ezekiel *et al.*, 2014b). *Aspergillus tamarii* produces tannase and gallic acid in submerged and solid state culture (Da costa *et al.*, 2013). Xylanase enzyme from *A. tamarii* has economic importance in food, chemical and pharmaceutical industries (Gouda and Abdel-Naby, 2002). Other members of *Aspergillus* section *Flavi* include *A. arachidicola*, *A. bombycis*, *A. flavus*, *A. minisclerotigenes*, *A. nomius* and *A. parvisclerotigenus*.

2.6 Conditions that favor aflatoxin development on maize

Colonization of maize by toxigenic *Aspergillus* species leads to the production of aflatoxin in maize. Environmental factors such as high temperature (Jaime-Garcia and Cotty, 2007) and humidity may facilitate the growth of *Aspergillus* hence increase production of aflatoxin. Post-harvest practices such as improper drying, poor storage, and mechanical shelling cause damage to the grain hence provide an avenue for *Aspergillus* colonization (Diedhiou *et al.*, 2011).

Climate may also cause stress to the crops hence increasing its susceptibility to *Aspergillus* colonization and aflatoxin production (Jaime- Garcia and Cotty, 2007).

2.7 Methods of aflatoxin detection

Different methods of detection and quantification of aflatoxins have been used in various studies ranging from traditional methods such as solid phase extraction to analytical techniques. These include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography (LC) and ELISA (Food Safety and Standard, 2012).

Recent studies have used ELISA to quantify concentrations of aflatoxins and other mycotoxins (Probst *et al.*, 2014). This method is less costly, simple, and rapid and is preferred to HPLC which is expensive (Yang, 2014). However, ELISA is the most common and widespread technique although it requires well equipped laboratories, trained personnel and use of harmful solvents (Espinosa-Calderón *et al.*, 2012). Accu scan Pro method is an ELISA based method that uses antibody- antigen coagulation. It is rapid, easy, cheap and reliable method of analysing mycotoxin level. The Accu scan machine is portable and can be used in the fields. Furthermore it requires minimal training and equipment (Neogen, 2007). However it can only analyse a single sample at a time.

Quantitative methods preferred in research and routine analysis of aflatoxins include LC, TLC and HPLC because they offer excellent sensitivities (Vosough *et al.*, 2010); but their use is limited because they frequently require skilled personnel, extensive sample pre-treatment and are expensive.

2.8 Aflatoxin management strategies

Use of resistant varieties, crop rotation, well-timed planting, weed control, pest control and avoiding drought and nutritional stress through fertilization and irrigation can be used to minimize pre harvest aflatoxin contamination of crops (Hell and Mutegi, 2011). According to FAO (2011) capacity development will enable self-management of aflatoxin and adoption of good agricultural practices. Another approach is consuming a more varied diet, and diversifying the diet into less risky staples like sorghum and millet (Hell and Mutegi, 2011). Other methods like storage in oxygen deficient fridges and use of mold inhibiting chemicals could also inhibit mold growth and consequent mycotoxin production (Raeker *et al.*, 1992). Timely harvesting before onset of short rain can prevent moisture accumulation in grains. Proper drying of maize below 13% moisture level is important to prevent mold colonization. A recent approach has been the use of biological control. Atoxicogenic *A. flavus* has been used in the U.S to prevent aflatoxicosis (Probst *et al.*, 2007). Similar technology is being adopted in Kenya to test efficacy of the technology to reduce aflatoxin contamination.

2.9 Biological control of aflatoxigenic *Aspergillus* species

Biological control plays an important role in aflatoxin management by modifying the fungal community in favor of the biocontrol strains thus protecting the crop from harvest to consumption. Competitive exclusion principle has been used in the development of Aflasafe KE01, a biocontrol product which contains four native atoxicogenic strains of *A. flavus* on a sorghum carrier which is applied in maize fields two to three weeks before tussling. Together with ongoing programs for better postharvest handling and storage, biocontrol with atoxicogenic *A. flavus* may provide sufficient aflatoxin reduction to significantly reduce human aflatoxicosis in

the Kenya. Atoxigenic isolates of *A. flavus* can reduce aflatoxin contamination in crops by direct interference with aflatoxin production during co-infection where the atoxigenic strain competes for nutrients with toxigenic hence the toxigenic strain is deprived of substrate because the atoxigenic has a greater colonization ability (Probst *et al.*, 2011).

Probst *et al.* (2011), indicated that even within highly toxic communities, there reside atoxigenic isolates of potential value in mitigating aflatoxin outbreaks in Kenya. The atoxigenic strains activity can be used to minimize aflatoxin contamination of vulnerable crops (Cotty *et al.*, 2007). According to Cotty *et al.* (2007), the atoxigenic strains prevent colonization of crops by toxigenic strains therefore reducing aflatoxin contamination. This technology has led to up to 99.9% reduction in aflatoxin contamination in maize in Nigeria (Atehnkeng *et al.*, 2008b). In the US, similar technology has been used in maize resulting in up to 80% reduction in aflatoxin contamination in trial fields (Probst *et al.*, 2011).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Description of the study area

The study was carried out in Kathiani, Kaiti, Nzambani and Wote districts in lower Eastern region of Kenya which are aflatoxin hot spots (Fig. 1). The regions recorded majority of the fatalities resulting from the aflatoxicosis outbreak in 2004 (Probst *et al.*, 2007). Lower Eastern Kenya experiences two rainy seasons: long rain season from March to May and short rain season from October to December (Recha *et al.*, 2012).

Kathiani district in Machakos County is mostly semi-arid with hilly terrain with an altitude of 1000 to 2100 meters above sea level (Wikipedia, 2016). The average annual rainfall received is 500-700 mm in two seasons with a temperature range between 16 to 27 °C as in appendix II. The area is characterized by sandy loam soils that can be used for agricultural activities. Wote and Kaiti districts in Makueni County lies in an altitude of 600 to 1900 meters above sea level (Agricultural Sector Development Support Programme (ASDSP), 2016) with an average annual rainfall of 150-650 mm. Temperature ranges between 16- 28 °C as indicated in appendix II. The soils are sandy with low water holding capacity. Nzambani district in Kitui County lies in the low lands and has an altitude of between 400 to 1800 meters above sea level (Kitui County government, 2014) with an annual average rainfall of between 500-1050 mm with temperature ranging between 17 to 28 °C as indicated in appendix II. The County is covered with a mixture of coarse sands and fine alluvial soil.

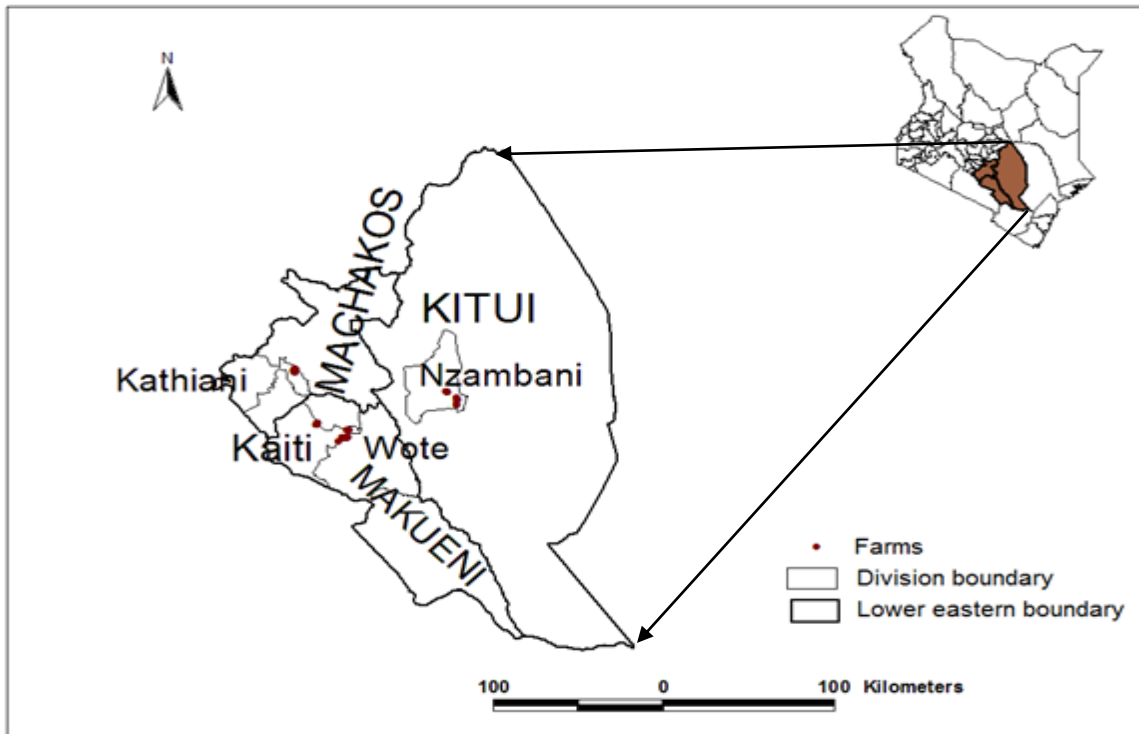


Fig 1: Map of lower Eastern Kenya showing the study sites in Kitui, Machakos and Makeni Counties.

Sampled farms indicated by the red points.

3.2 Preparation of culture media

Modified Rose Bengal Agar (3g sucrose, 3 g NaNO_3 , 0.75 g KH_2PO_4 , 0.25 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KCl, 10 g NaCl, 10 g agar, 1ml A and M micronutrients, 1000 ppm Rose Bengal, 2500 ppm streptomycin, 2500 ppm chloramphenicol, 5000 ppm dichloran, 1000 ml distilled water) also known as clean up medium was prepared according to Garber *et al.* (2012). Details on preparation of the stock solutions of A and M micronutrients, antibiotics and dichloran are described in Appendix I. The media was prepared by suspending the ingredients,

except the antibiotics and dichloran in distilled water and dissolving by heating for 15 minutes in a microwave. The pH was adjusted to 6.5 and 2.5 ml of chloramphenicol solution added followed by autoclaving for 20 minutes at 121 °C and 15 Pa. After cooling to 45-50 °C, 5 ml dichloran and 2.5 ml streptomycin solution were added to the media which was subsequently dispensed in aliquots of approximately 15 ml into sterile Petri dishes in a biological safety cabinet.

The 5/2 Agar containing 10 g (2%) Bacto™ agar, 50 ml V-8™ Juice (Campbell Company, Camden, USA) and 950 ml of purified water was prepared as described by Garber *et al.* (2012). The pH was adjusted to 6.0 after all ingredients had dissolved. Thereafter, 500 ml of the solution was heated for 15 minutes in a microwave and then autoclaved for 20 minutes at 121 °C and 15 Pa. The media was subsequently dispensed in aliquots of 10 ml into sterile Petri dishes in a biological safety cabinet.

3.3 Determination of population of *Aspergillus* section *Flavi* in soils

3.3.1 Determination of maize production practices in lower Eastern Kenya

A field survey was conducted in Kathiani district (Machakos County), Nzambani district (Kitui County) and Wote and Kaiti districts (Makueni County) in lower Eastern Kenya during the short rains of 2013/2014 cropping season. Twenty four farmers with more than two acre under maize, in these regions and with a low chance of maize crop failure were selected in each district with help from agricultural extension officers. A structured questionnaire was used to collect data on maize production practices including maize varieties grown, types of crops intercropped with maize, tillage methods, soil amendments and maize storage method used.

3.3.2 Collection, handling and processing of soil samples

Ninety six soil samples were collected two weeks before tussling of maize by scooping approximately 5 g of the top 2 cm soil using a sterile spoon. The soil samples were collected from different parts of the field in a zigzag manner before application of Aflasafe KE01. The subsamples from each field were mixed thoroughly to make a composite sample of approximately 200 g. The samples were transported to the regional Mycotoxin Laboratory in Kenya Agricultural Livestock Research Organization (KALRO) Katumani for analysis within three days of sampling. Soil samples were ground using a motor and pestle, sieved through a 2 mm mesh screen, sun dried for five days and then stored at room temperature (23 ± 2 °C) in Kraft bags in readiness for microbial analysis.

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

The method described by Garber *et al.* (2012) was adopted where one gram of soil sample was obtained from a thoroughly mixed sample and suspended in 10 ml of sterile distilled water. The mixture was then homogenized for two minutes and aliquots of 10, 20 and 40 μ m were inoculated on modified Rose Bengal Agar (mRBA) in a biological safety cabinet. The inoculated plates were incubated without illumination at 31 °C for three days. Distinct colonies with a typical greenish-yellow appearance resembling members of *Aspergillus* section *Flavi* were counted and recorded. Colony forming units of *Aspergillus* section *Flavi* per gram of soil were calculated. Colonies of *Aspergillus* section *Flavi* were transferred to 5/2 media (5% V-8 juice and 2% agar pH 5.0) from plates with 1-10 colonies for further characterization.

Identification of *Aspergillus* section *Flavi* was based on cultural and morphological characteristics of the morphotypes where isolates with greenish-yellow colonies and large or no sclerotia on 5/2 agar were identified as *A. flavus* L-strain. Isolates with numerous small sclerotia were identified as *A. flavus* S-strain. Those that appeared dark green on 5/2 agar were identified as *A. parasiticus* while those that appeared brownish were identified as *A. tamaritii*. Twelve isolates of *Aspergillus* section *Flavi* were stored in 2 ml sterile distilled water in 4 ml Wheaton clear vials and stored at room temperature (23 ± 2 °C) in storage boxes for aflatoxin production test.

Riddell slides were prepared to confirm the identity of *Aspergillus* section *Flavi* isolates based on morphological characteristics. A 10 mm diameter culture block growing on 5/2 agar media was placed on a microscope slide and a cover slip placed on top of the agar block. The slides were incubated for five days at 31 °C in a sterile 90 by 15 mm Citotest plastic Petri dish where the microscope slide was placed on a V-shaped glass rod which had been aseptically placed on a sterile filter paper. The filter paper was wet with sterile distilled water and the Petri plate closed in biological safety cabinet. A drop of water was put on the sterile glass slide and a coverslip with the isolate was put on top carefully to prevent formation of air bubbles. *Aspergillus* morphology was observed using a light microscope (LEICA DM 500, Leica Microsystems, Wetzler, Germany) fitted with a camera (LEICA ICC 50, Leica Microsystems, Wetzler, Germany). Members of *Aspergillus flavus* producing numerous sclerotia less than 400 µm in diameter were identified as S-strains while those with few large sclerotia and more green smooth conidia were identified as L-strain. Those with dark green rough conidia were identified as *A. parasiticus* while those with large brown coarse conidia were identified as *A. tamaritii*.

3.4 Effect of atoxigenic *Aspergillus flavus* (Aflasafe KE01) on the population of toxigenic

***Aspergillus* section *Flavi* and aflatoxin contamination**

3.4.1 Application of atoxigenic *A. flavus* (Aflasafe KE01)

The Aflasafe KE01 is made up of an inoculant comprising four atoxigenic *A. flavus* L-strains which are native to Kenya: KN00A, KN001, KN011 and KN012, a polymer, a blue dye and sorghum grains as carrier material. The product was made by culturing the strains on sterile sorghum grains at the Plant Pathology unit, International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria. Of the twenty four farms sampled in each of the four districts, twelve farms were treated with Aflasafe KE01 while twelve were left untreated to act as control farms. The distance between each treated and control farm was a maximum of 100m. Twelve farms were treated with 10 kg/ha of Aflasafe KE01 which was applied by broadcasting the product by hand on the soil.

3.4.2 Assessment of sporulation of atoxigenic *A. flavus* (Aflasafe KE01) in maize fields

Sporulation of the atoxigenic *Aspergillus flavus* (Aflasafe KE01) was assessed 10-14 days after application. Four quadrants measuring 1m² were tossed randomly in each of the inoculated fields and the number of Aflasafe KE01 sorghum grains per quadrant that had sporulate were counted and expressed as a percentage of the total number of sorghum grains in the quadrant.

3.4.3 Collection, handling and processing of maize grain samples

Twenty five to thirty dry maize cobs were picked at harvest from each farm in a zigzag manner at an interval of approximately five meters and transported to the Regional Mycotoxin Laboratory in KALRO Katumani for analysis. The maize samples were sun dried for seven days,

shelled manually and dried in hot air oven (Memmert, United Kingdom) at 45 °C for 48 hours, until the moisture content was 13% and below. Moisture content was determined using a moisture meter (Intratec 1241 analyzer, Foss, Denmark) with a temperature range of 0-42 °C. After attaining the requisite moisture content, samples were ground in a coffee mill (Bunn-omatic Corporation, Springfield, Illinois, USA). Each maize sample was divided into two subsamples for microbial and aflatoxin analysis. The sub-samples for microbial analysis were stored in zip-lock bags at 4 °C while those for aflatoxin analysis were stored at -20 °C until analysis was done.

3.4.4 Isolation and identification of *Aspergillus section Flavi* from maize samples

The method described by Garber *et al.* (2012) was adopted. Isolation and identification was done as described in Section 3.3.3

3.4.5 Extraction and quantification of aflatoxin levels in maize samples

Aflatoxin levels in the maize samples were analyzed using Accuscan Pro reader following the manufacturer's instructions (Neogen, 2007). The sample was first homogenized by shaking for one minute. Five grams of each maize sample was mixed for three minutes with 25 ml of 65% ethanol: water (v/v) using an orbital shaker (HS501 IKA-WERKE, Germany). The mixture was filtered through Watmann No. 4 filter paper and filtrate obtained in a Tripor beaker. Red sample dilution cups and clear sample dilution cups were placed in the sample cup rack and labeled. To each red sample dilution cup, 500 µl of sample diluents was added. A hundred micro liters of sample extract was added to the red dilution cup with sample diluents and was mixed by pipetting up and down seven times. A hundred micro liters of diluted sample extract was

transferred into a new clear sample cup. A reveal Q+ strip was placed into the clear sample cup and left for six minutes and the test strip was read within one minute. Aflatoxin levels were read in parts per billion (ppb) with a lower detection limit of 2 ppb and a higher detection limit of 150 ppb. Samples with more than 150 ppb were further diluted in 65% ethanol in the ratio of 1:9. For every 100 µl of sample, 900 µl of 65% ethanol was added. Then 100 µl of sample mixture was mixed with 500 µl of diluent and level of aflatoxin determined.

3.5 Determination of aflatoxin producing ability of *Aspergillus* section *Flavi* isolates

3.5.1 Inoculation and culture of *Aspergillus* section *Flavi* for aflatoxin production in vitro

To determine the aflatoxin producing ability of *Aspergillus* section *Flavi* isolates aflatoxin-free maize samples were sourced from a highland maize producing region at the foot of Aberdare ranges in Central Kenya (0.8212 °S, 036.8114 °E). The samples were confirmed to be free of aflatoxin by testing using Accuscan Pro method as described in Section 3.4.5. A given batch of maize was designated as aflatoxin free when no aflatoxin was detected after five tests.

Five grams of aflatoxin free maize samples were weighed and put in 40 ml clear vials, soaked overnight in 20 ml of sterile distilled water to adjust moisture content to 25%. The maize was washed thrice with tap water to remove any fermentation product and thereafter autoclaved for 20 minutes at 121 °C and 15 Pa. The sterile grains in each vial were inoculated with 500 µl spore suspension of *Aspergillus flavus* isolate containing approximately 10⁶ conidia/ml. The cultures were incubated without illumination at 31 °C for seven days. Vials containing only sterile grains were used as control. After seven days of incubation, the cultures were ranked on a scale of 1 to

5 based on the extent of grain colonization, where 1 = very poor colonization, 2 = poor colonization, 3 = average colonization, 4 = good colonization and 5 = very good colonization.

3.5.2 Extraction and quantification of aflatoxin from the fermentation cultures

Five grams of inoculated maize was ground using 70% methanol for three minutes using a high speed blender (Waring commercial, Springfield, MO, USA). The mixture was filtered using Whatman No. 4 filter paper into a 250 ml separating funnel and 25 ml of distilled water added to ease separation. Thereafter, 8.75 ml of methylene chloride was added to the extract. The extract was passed through a bed of anhydrous sodium sulphate into Tripor beaker and evaporated to dryness in the dark in a fume hood chamber. The dried extract was dissolved in 1 ml dichloromethane and poured into a 1.5 ml Eppendorf tube. The tubes were stored at 4 °C in darkness for stabilization of the toxin waiting spotting.

The aflatoxin extracts and standard were separated on TLC plates. The plates were later developed in Diethylene: methanol: water (96:3:1) solution. The plates were read in a TLC Scanner 3 (CAMAG, Muttenz, Switzerland) and Win-CATS 1.4.2 software (Camag AG, Muttenz, Switzerland). Extracts that fluoresced were determined to have aflatoxin and the isolate from which the extract was produced was considered as aflatoxigenic. Aflatoxin B1, B2, G1 and G2 were detected by fluorescence at different wavelengths. Levels of the aflatoxin were measured based on fluorescence in the extract and were recorded in nanograms per gram.

3.6 Statistical data analyses

Data were analyzed using Genstat version 14 software. Means of the population of *Aspergillus* section *Flavi* and the means of aflatoxin levels in treated and untreated maize fields were separated and compared using student t-test at 95% confidence level. Population of *Aspergillus* section *Flavi* in soil and maize samples was analyzed using ANOVA and means separated using Fischer's protected Least Significant Difference (LSD) value at 95% confidence level. Percentage data that were not normally distributed were transformed through Arcsine transformation while aflatoxin levels were transformed through \log_{10} .

CHAPTER FOUR: RESULTS

4.1 Maize production practices in Lower Eastern Kenya

Maize farmers in lower Eastern Kenya planted both local and improved maize varieties like Duma, DH02, KDV-1, DH04, DK8031, Pioneer, Pannar and DH01. Most (36.6%) of the farmers planted the local maize variety followed by Duma (33.1%) while DH01 was the least common variety (Fig. 2). Maize in the four districts was commonly intercropped with other crops. The major crops intercropped with maize were pigeon peas, common beans, cowpeas, green grams and sorghum (Table 1). Pigeon peas was the most commonly (82.3%) intercropped crop with maize followed by common beans (75%) whereas tomato and cotton were the least common (1.0%). Coffee-maize intercrop was only found in Kathiani district.

Farmers tilled their fields using oxen plough, manually or tractors (Fig. 3). Use of oxen plough was the most common (69%) across the four districts followed by manual tillage (28.5%) while use of tractors (2.5%) was the least common method. However, in Kathiani district, most (73%) farmers did manual tillage. Most (82.3%) of the farmers applied soil amendments during planting while the rest did not use any soil amendment. Manure was the most common soil amendment (51.8%) followed by Nitrogen Phosphorus Potassium fertilizer (18.7%) while Calcium Ammonium Nitrate was the least commonly (5.5%) used amendment for top dressing (Table 2). After harvest, the farmers dried their maize on bare ground and stored maize in granaries, wooden structures in the house, in the house on canvas and on the floor in the house (Table 3). Most (63.5 %) of the farmers stored maize in the granary although in Nzambani district, most (79.2%) of the farmers stored their maize on wooden structures inside the house.

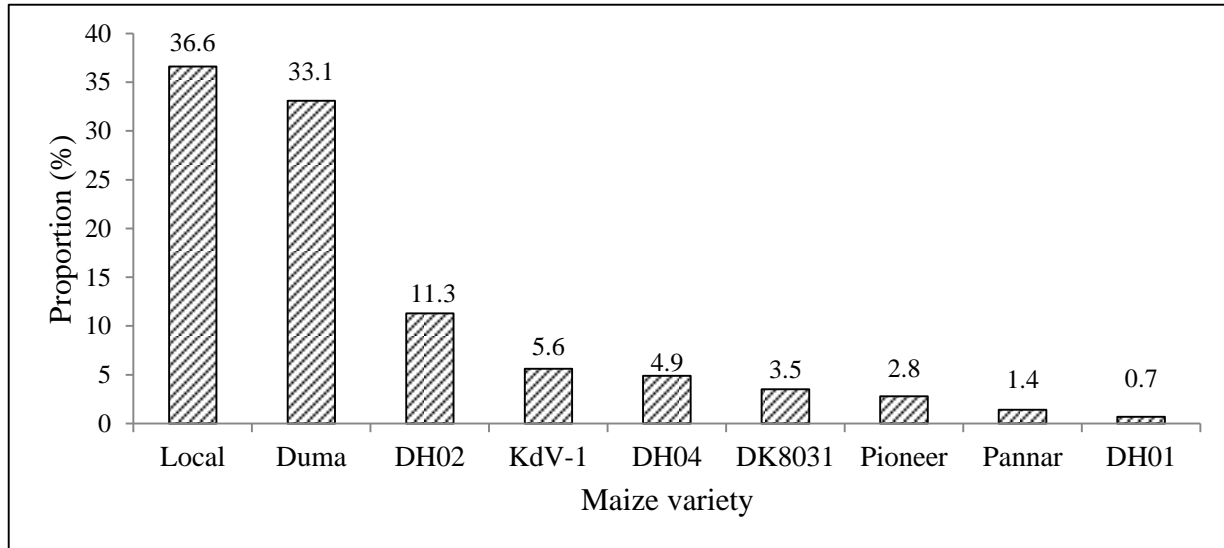


Fig. 2: Percentage of farmers growing different maize varieties in lower Eastern Kenya during the 2013/2014 cropping season

Table 1: Proportion (%) of crops intercropped with maize in four districts in lower Eastern Kenya (n=24)

| Crop | Wote | Kaiti | Nzambani | Kathiani | Mean |
|-------------------------|------|-------|----------|----------|-------------|
| Pigeon peas | 75.0 | 95.8 | 79.2 | 79.2 | 82.3 ± 4.6 |
| Common bean | 91.7 | 91.7 | 37.5 | 79.2 | 75.0 ± 12.9 |
| Cowpeas | 58.3 | 70.8 | 79.2 | 50.0 | 64.6 ± 6.5 |
| Green grams | 4.2 | 20.8 | 54.2 | 0.0 | 19.8 ± 12.3 |
| Sorghum | 4.2 | 8.3 | 41.7 | 0.0 | 13.5 ± 9.5 |
| Cassava | 8.3 | 12.5 | 0.0 | 16.7 | 9.4 ± 3.6 |
| Vegetables ^a | 0.0 | 8.3 | 0.0 | 8.3 | 4.2 ± 2.4 |
| Millet | 4.2 | 4.2 | 8.3 | 0.0 | 4.2 ± 1.7 |
| Fruits ^b | 8.3 | 4.2 | 0.0 | 0.0 | 3.1 ± 1.9 |
| Sunflower | 0.0 | 8.3 | 0.0 | 0.0 | 2.1 ± 2.0 |
| Coffee | 0.0 | 0.0 | 0.0 | 8.3 | 2.1 ± 2.0 |
| Tomato | 0.0 | 4.2 | 0.0 | 0.0 | 1.0 ± 1.0 |
| Cotton | 0.0 | 0.0 | 4.2 | 0.0 | 1.0 ± 1.0 |

^a Green leafy vegetables like spinach, kales, cabbages and amaranthas ; ^b Fruits like mangoes, watermelons, avocados

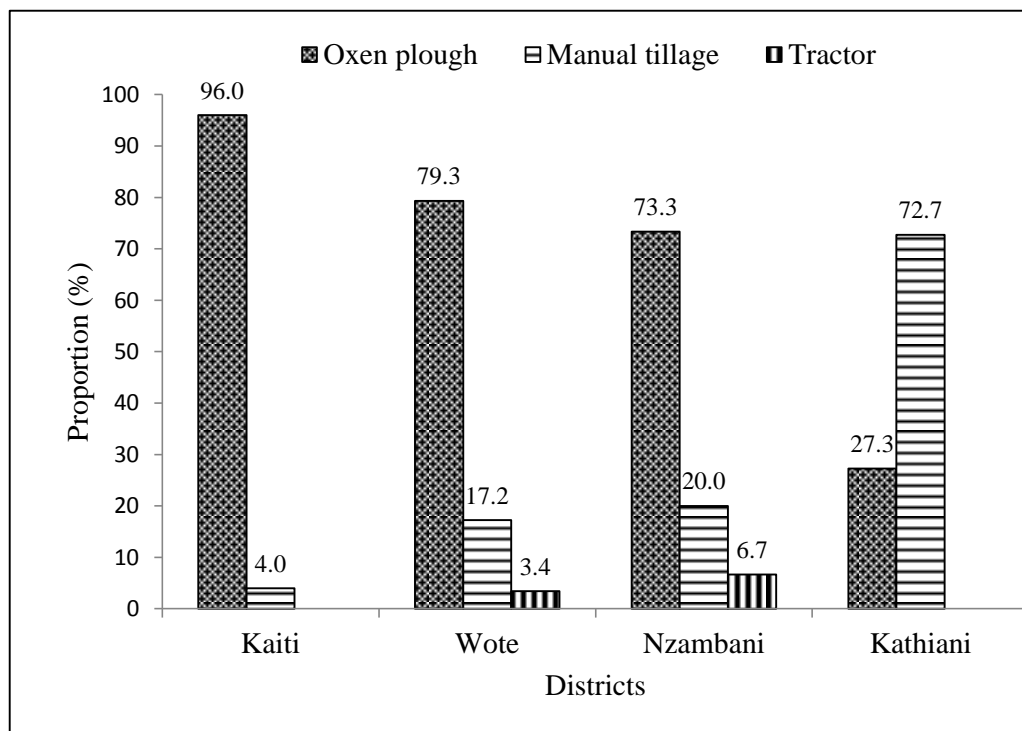


Fig. 3: Proportion of farmers using different tillage methods in lower Eastern Kenya

Table 2: Proportion (%) of farmers using different soil amendments in maize production in four districts in lower Eastern Kenya (n = 24)

| Soil amendment | Wote | Kaiti | Nzambani | Kathiani | Mean |
|----------------|------|-------|----------|----------|-------------|
| Manure | 75.0 | 58.6 | 54.2 | 19.5 | 51.8 ± 11.7 |
| NPK (20:20:0) | 0.0 | 31.0 | 0.0 | 43.9 | 18.7 ± 11.1 |
| DAP (18:46:0) | 0.0 | 10.3 | 0.0 | 14.6 | 6.2 ± 3.7 |
| CAN (26:0:0) | 0.0 | 0.0 | 0.0 | 22.0 | 5.5 ± 5.5 |
| No amendment | 25.0 | 0.0 | 45.8 | 0.0 | 17.7 ± 11.1 |

NPK- Nitrogen Phosphorus Potassium; DAP- Di-ammonium Phosphate; CAN- Calcium Ammonium Nitrate

Table 3: Proportion of farmers using different types of structures to store maize in the four districts in lower Eastern Kenya (n = 24)

| Storage method | Wote | Kaiti | Nzambani | Kathiani | Mean |
|---------------------------------|------|-------|----------|----------|-------------|
| Granary | 95.8 | 95.8 | 8.3 | 54.2 | 63.5 ± 20.9 |
| In the house on wood* | 4.2 | 0.0 | 87.5 | 29.2 | 30.2 ± 20.1 |
| Inside the house on canvas | 0.0 | 0.0 | 4.2 | 8.3 | 3.1 ± 2.0 |
| Inside the house on earth floor | 0.0 | 4.2 | 0.0 | 8.3 | 3.1 ± 2.0 |

*Wooden sticks placed on the floor

4.2 Diversity of *Aspergillus* section *Flavi* in soil and maize samples

Aspergillus flavus L-strain, *A. flavus* S-strain, *A. parasiticus* and *A. tamarii* were isolated from soil and maize samples (Fig. 4; Fig. 5). However, *A. tamarii* was recovered in very low proportion (0.8%) in maize samples from lower Eastern Kenya. *A. flavus* L-strain was most predominant (84%) in Aflasafe KE01 treated maize fields while *A. flavus* S-strain was predominant in baseline soil samples (56.9%) and untreated maize (61.8%). *A. tamarii* was more commonly isolated in soil (3.2%) compared to maize (0.8%).

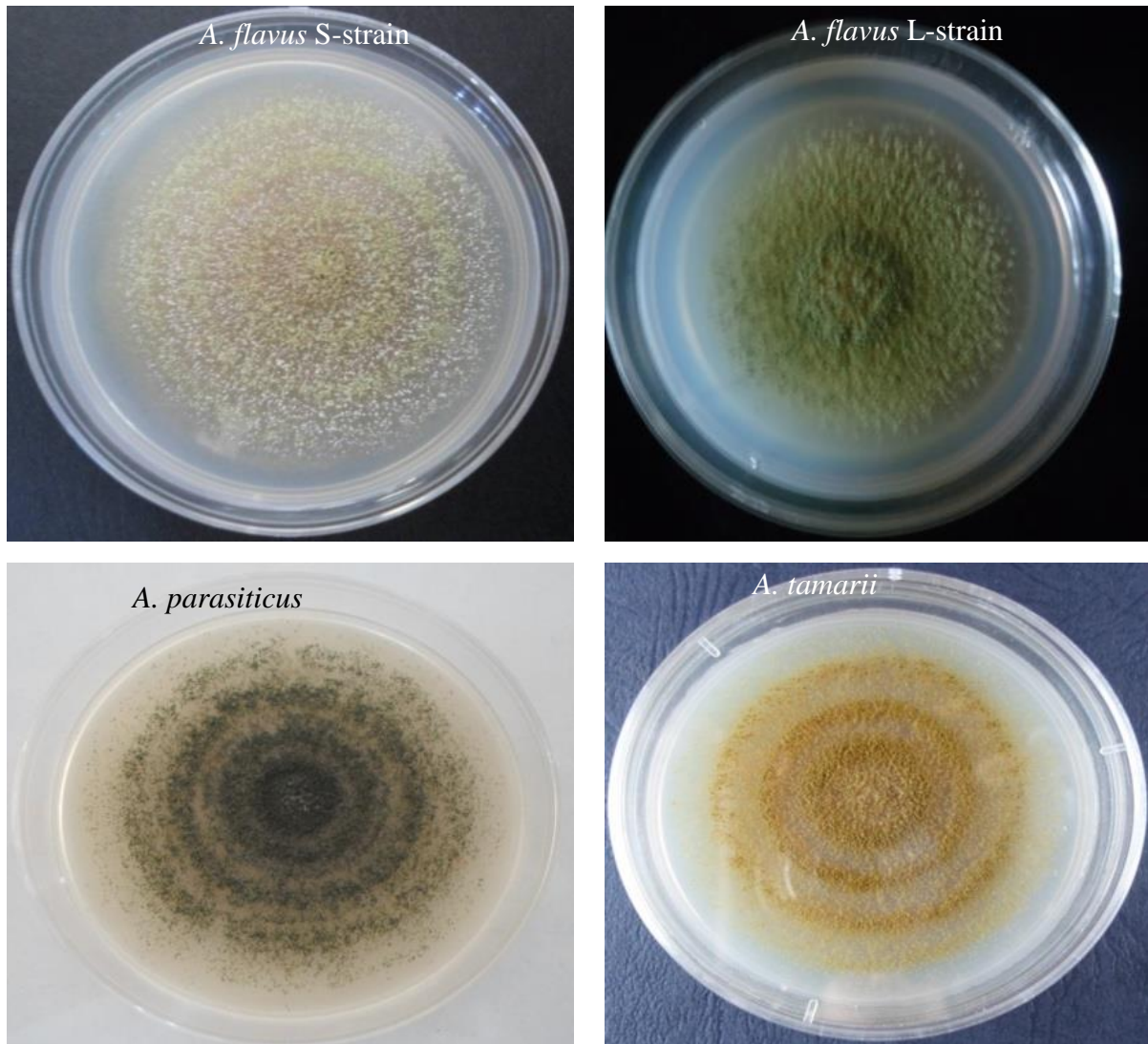


Fig. 4: *Aspergillus* section *Flavi* species isolated from soil and maize samples in lower Eastern Kenya

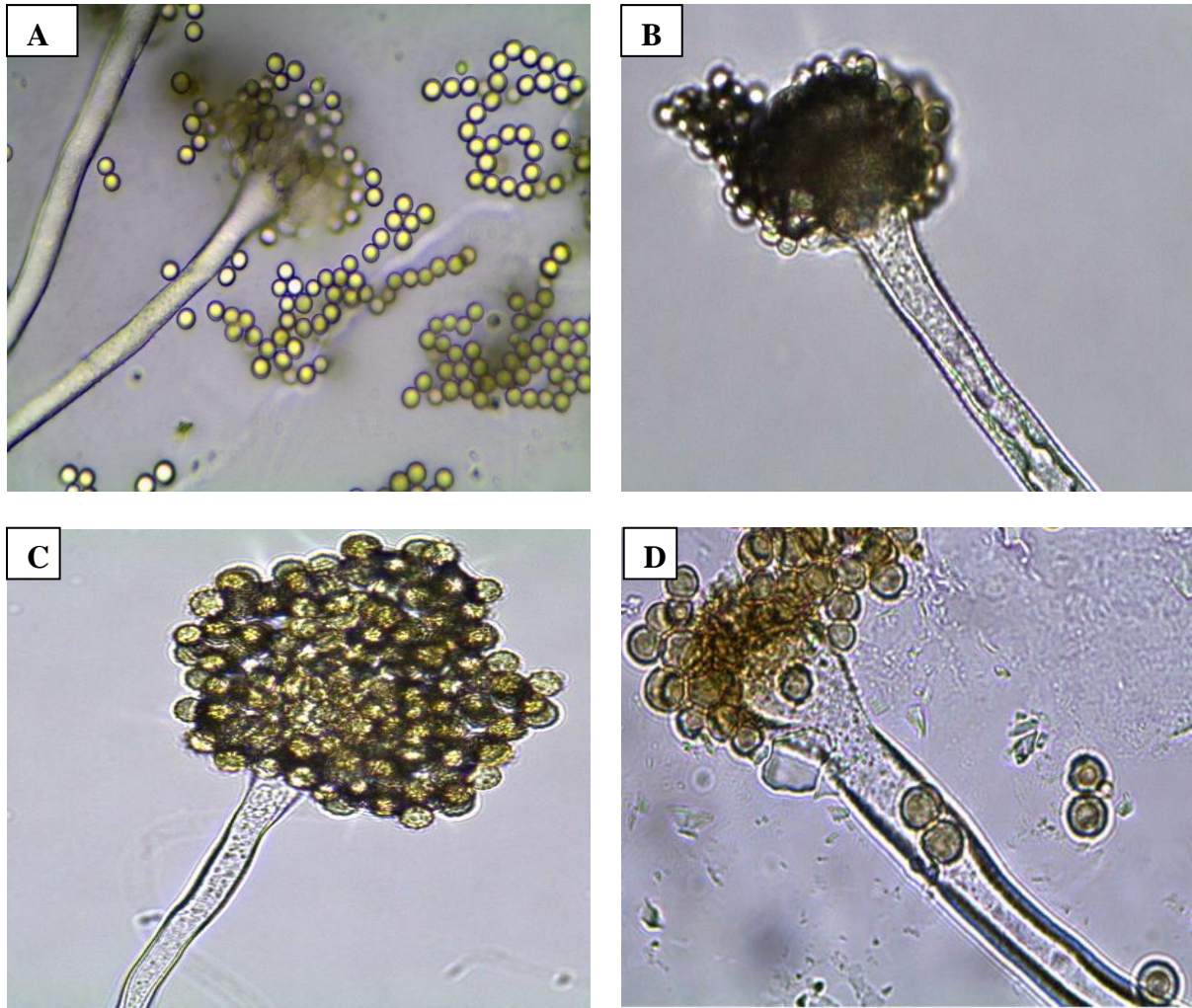


Fig. 5: Conidial heads of *Aspergillus* section *Flavi* from random maize and soil samples from lower Eastern Kenya.

A. flavus L-strain (A); *A. flavus* S-strain (B); *A. parasiticus* (C); *A. tamarii* (D).

4.3 Population of *Aspergillus* section *Flavi* in soils

Four morphotypes of *Aspergillus* section *Flavi* were commonly isolated from soil samples including: *Aspergillus flavus* S-strain, *A. flavus* L-strain, *A. parasiticus* and *A. tamarii*. The proportion of *A. flavus* S-strain in baseline soil samples was significantly higher ($P \leq 0.05$) with isolation frequency of between 34 and 69%, followed by *A. flavus* L-strain with 21 to 38.5%

isolation frequency. *Aspergillus tamaris* was isolated in the lowest frequency (3.2%). *Aspergillus flavus* S-strain was predominant in soil from Wote and Kaiti districts. The incidence of *A. parasiticus* was low in soil from all other districts except Kathiani district which had up to 38.5% isolation frequency. The population of *Aspergillus* section *Flavi* significantly ($P \leq 0.05$) varied among the four districts (Table 4). There was a significant difference in the overall population of *Aspergillus* section *Flavi* between Wote and Kaiti. The highest population was in soil samples from Wote district (Mean = 721CFU /g) while the lowest population was in samples from Kathiani district (Mean= 370CFU/g) as indicated in Fig. 6.

Table 4: Proportion (%) of *Aspergillus* section *Flavi* in soil sampled from four districts in lower Eastern Kenya

| Strain/Species | Wote | Kaiti | Nzambani | Kathiani | Mean |
|---------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------------|--------------------------|
| <i>A. flavus</i> L-strain | 21.1 _b ^b | 21.1 _b ^b | 38.5 _a ^b | 24.0 _b ^b | 26.2 ^b ± 4.2 |
| <i>A. flavus</i> S-strain | 69.1 _a ^a | 67.4 _{ab} ^a | 56.9 _b ^a | 34.4 _c ^a | 56.9 ^a ± 8.0 |
| <i>A. parasiticus</i> | 5.9 _{bc} ^c | 9.0 _b ^c | 1.0 _c ^c | 38.5 _a ^a | 13.6 ^{bc} ± 8.5 |
| <i>A. tamaris</i> | 3.8 _a ^c | 2.4 _a ^c | 3.5 _a ^c | 3.1 _a ^c | 3.2 ^c ± 0.3 |
| LSD ($p \leq 0.05$) | 8.9 | 9.0 | 9.6 | 9.3 | 19.1 |
| CV (%) | 62.3 | 62.8 | 67.4 | 64.9 | 49.5 |

Values accompanied by similar superscript letters within the columns are not significantly different ($P \leq 0.05$); Values accompanied by similar subscript letters within the rows are not significantly different ($P \leq 0.05$); LSD - Least significant difference CV - Coefficient of Variation

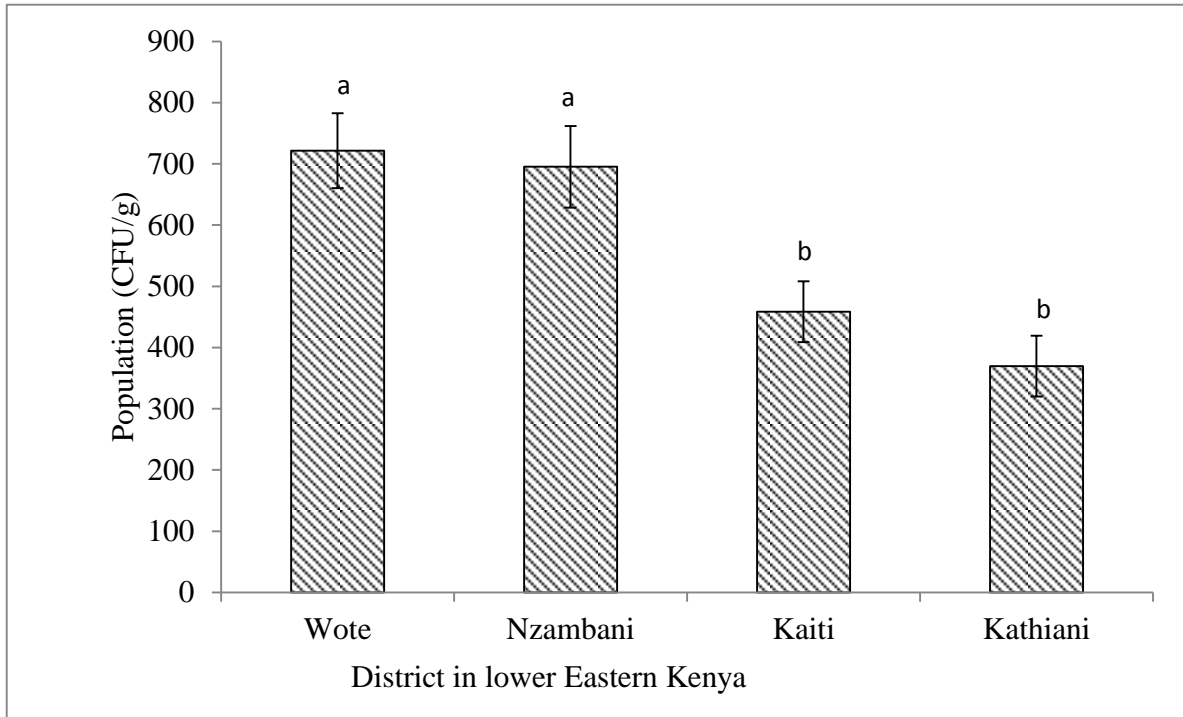


Fig. 6: Population (CFU/g) of *Aspergillus* section *Flavi* in soil sampled from four districts in lower Eastern Kenya

Bars with similar letter are not significantly different ($P \leq 0.05$); Error bars represent standard error of the mean

4.4 Correlation between the population of *Aspergillus* section *Flavi* in soil and maize production practices in lower Eastern Kenya

The population of *Aspergillus* section *Flavi* in soil had a significantly positive relationship with the use of local variety, oxen ploughing and the use of manure as a soil amendment (Table 5). However intercropping pigeon peas with maize and storing in granaries had a negative correlation with the population of *Aspergillus* section *Flavi*.

Table 5: Correlation among the population of *Aspergillus* section *Flavi* in soil and maize production practices in lower Eastern Kenya

| | Population (CFU/g) | Maize Variety (Local) | Intercropping (Pigeon peas) | Ploughing (Oxen) | Ammendment (Manure) | Storage (Granary) |
|--------------------------------|-----------------------|-----------------------------|--------------------------------|---------------------|------------------------|----------------------|
| Population (CFU/g) | - | | | | | |
| Maize variety (Local) | +0.47* | - | | | | |
| Intercropping (Pigeon peas) | -0.49* | +0.43* | - | | | |
| Ploughing (Oxen) | +0.77** | +0.92** | +0.13 ^{ns} | - | | |
| Amendment (Manure) | +0.78** | +0.91** | +0.03 ^{ns} | +0.99** | - | |
| Storage (Granary) | -0.16 ^{ns} | +0.57* | +0.34 ^{ns} | +0.37 ^{ns} | +0.40* | - |

** Correlation coefficient significant at $p \leq 0.01$; * correlation coefficient significant at $p \leq 0.05$; ns- not significant; correlation coefficient significant at $p \leq 0.05$

4.5 Efficacy of Aflasafe KE01 in reducing toxigenic *Aspergillus* and aflatoxin in treated maize fields

4.5.1 Sporulation of atoxigenic *A. flavus* L-strain (Aflasafe KE01) in treated maize fields

Sporulation of atoxigenic *A. flavus* L-strain (Aflasafe KE01) was seen in treated maize fields two weeks after inoculation (Table 6). Aflasafe KE01 sporulation was seen in 96% percent of the farms inoculated. However there were significant differences ($P \leq 0.05$) in sporulation of Aflasafe KE01 among the districts and farms. The highest sporulation was observed in Nzambani ranging from 50.9% to 100% while sporulation was lowest (49.3%) in Kaiti district.

Table 6: Sporulation (%) of Aflasafe KE01 in farmers' maize fields in four districts in lower Eastern Kenya

| Farm Number | Wote | Kaiti | Nzambani | Kathiani |
|-----------------------|----------|---------|----------|-----------|
| 1 | 54.9 cd | 1.0 de | 97.1 ab | 84.2 a |
| 2 | 52.1 cd | 24.6 d | 82.9 b | 43.5 d |
| 3 | 57.5 bcd | 0.0 e | 98.5 ab | 64.7 abcd |
| 4 | 54.3 cd | 99.1 a | 93.4 ab | 78.2 a |
| 5 | 50.0 cd | 74.2 b | 88.1 ab | 42.8 d |
| 6 | 71.0 b | 0.0 e | 100 a | 53.2 bcd |
| 7 | 55.2 cd | 80.1 ab | 50.9 c | 70.4 ab |
| 8 | 54.9 cd | 51.6 c | 64.4 c | 67.0 abc |
| 9 | 100.0 a | 69.0 c | 56.6 c | 43.9 cd |
| 10 | 49.6 cd | 63.2 bc | 61.6 c | 50.6 bcd |
| 11 | 62.1 bc | 68.0 bc | 56.4 c | 79.2 a |
| 12 | 46.4 d | 60.4 bc | 63.9 c | 82.4 a |
| Mean | 59.0 AB | 49.3 B | 76.2 A | 63.3 AB |
| LSD ($P \leq 0.05$) | 18.4 | 28.9 | 16.4 | 23.2 |
| CV (%) | 36.0 | 29.9 | 15.0 | 25.0 |

Values accompanied by similar lowercase letters within the same column are not significantly different at $P \leq 0.05$

Values accompanied by similar uppercase letters within the same row are not significantly different at $P \leq 0.05$

LSD - Least significant difference

CV - Coefficient of Variation

4.5.2 *Aspergillus* section *Flavi* isolated from maize samples

Aspergillus flavus L-strain (60%), *A. flavus* S-strain (38%), *A. parasiticus* (1.9%) and *A. tamarii* (0.1%) were the commonly isolated members of *Aspergillus* section *Flavi* from the maize samples. The population of *A. flavus* L-strain was significantly higher ($P \leq 0.05$) in maize grains from treated fields compared to the other members of *Aspergillus* section *Flavi* (Table 7). However, *A. flavus* S-strain was the most predominant in untreated fields. The proportion of *A. flavus* L-strain was higher ($P \leq 0.05$) in treated fields (84%) compared to *A. flavus* S-strain

(14.2%) and *A. parasiticus* (1.8%). However, in the untreated fields the overall proportion of *A. flavus* S-strain (61.8%) was higher compared to *A. flavus* L-strain (35.9%), *A. parasiticus* (2.1%) and *A. tamaraii* (0.2%). The highest proportion of *A. flavus* S-strain was in samples from untreated fields in Wote district (79%).

Table 7: Proportion (%) of *Aspergillus* section *Flavi* in maize samples from Aflasafe KE01 treated and untreated fields in lower Eastern Kenya

| District | Treatment | Species/Strain | | | |
|----------|-----------|------------------------------|------------------------------|-----------------------|--------------------|
| | | <i>A. flavus</i> L-strain | <i>A. flavus</i> S-strain | <i>A. parasiticus</i> | <i>A. tamaraii</i> |
| Wote | Treated | 61.0 * | 38.2 | 0.8 ns | 0.0 |
| | Untreated | 22.7 | 76.5 * | 0.0 | 0.8 ns |
| Kaiti | Treated | 91.0 * | 2.8 | 6.2 ns | 0.0 |
| | Untreated | 58.3 | 41.7 ** | 0.0 | 0.0 |
| Nzambani | Treated | 86.8 * | 13.2 | 0.0 | 0.0 |
| | Untreated | 43.8 | 56.2 * | 0.0 | 0.0 |
| Kathiani | Treated | 97.2 ** | 2.8 | 0.0 | 0.0 |
| | Untreated | 18.8 | 72.9 ** | 8.3 ns | 0.0 |

Significance levels *($P \leq 0.05$) and ** ($P \leq 0.01$) are for testing for differences between the populations of *Aspergillus* section *Flavi* in treated and control plots within a district based on Student's t-test; ns = no significant difference

4.5.3 Aflatoxin levels in maize samples

High levels of total aflatoxin were recorded in maize samples from untreated fields (Table 8). Maize from untreated fields in Wote had the highest levels of aflatoxin (Mean = 230.8 ppb). Overall, aflatoxin levels in samples from Aflasafe KE01 treated fields were significantly ($P \leq 0.05$) lower (Mean = 44.8ppb) than in samples from untreated fields (Mean = 84.3ppb). A higher proportion of samples from treated fields across the four districts (77.1%) satisfied the set KEBS aflatoxin threshold compared to samples from untreated fields (60.4%) (Table 9).

4.5.4 Correlation among sporulation of Aflasafe KE01, population of *Aspergillus* section

***Flavi* and aflatoxin levels in maize**

There was a highly significant positive correlation ($P \leq 0.001$) between the populations of *Aspergillus flavus* S-strain and aflatoxin level; and there was a highly significant negative correlation in the population of *A. flavus* L-strain and aflatoxin level. There was also a negative correlation between the population of *A. flavus* L-strain and *A. flavus* S-strain. Negative correlation was observed between sporulation of Aflasafe KE01 and population of *A. flavus* S-strain and between sporulation of Aflasafe KE01 and aflatoxin levels in treated fields where aflatoxin levels reduced with increase in sporulation. However in treated fields there was a significant positive correlation between sporulation of Aflasafe KE01 and the population of *A. flavus* L-strain in maize grains from the same fields as indicated in Table 10.

Table 8: Total aflatoxin levels (ppb) in maize samples from Aflasafe KE01 treated and untreated fields in four districts of lower Eastern Kenya

| Treatment | Wote | Kaiti | Nzambani | Kathiani | Mean |
|---------------|---------------------------|--------------------------|------------------------|--------------------------|--------------------------|
| Treated | 167.5 ^a ± 63.0 | 3.0 ^b ± 1.0 | 2.0 ^b ± 0.4 | 6.8 ^b ± 4.4 | 44.8 ^b ± 18.4 |
| Control | 230.8 ^a ± 84.0 | 49.9 ^a ± 27.0 | 9.0 ^a ± 3.5 | 47.4 ^a ± 35.5 | 84.3 ^a ± 25.3 |
| Reduction (%) | 27.4 | 94.0 | 77.8 | 85.7 | 46.9 |
| T-value | 0.89 | 0.001 | 0.001 | 0.002 | |

Means of treated and control fields were transformed by standardizing the means of each field with the average of the control and treated fields. Comparison was done by student t-test ($P \leq 0.05$). Values accompanied by similar letters are not significantly different at $P \leq 0.05$.

Table 9: Proportion (%) of maize samples from four districts of lower Eastern Kenya that conformed to different aflatoxin standards

| Treatment | Aflatoxin threshold | District | | | | Mean |
|-----------|---------------------|----------|-------|----------|----------|---------------------------|
| | | Wote | Kaiti | Nzambani | Kathiani | |
| Treated | EC (4ppb) | 25.0 | 91.7 | 91.7 | 83.3 | 72.9 ^a ± 16.9 |
| Treated | KEBS (10ppb) | 33.3 | 91.7 | 100.0 | 83.3 | 77.1 ^a ± 15.0 |
| Treated | FDA (20ppb) | 33.3 | 100.0 | 100.0 | 83.3 | 79.2 ^a ± 15.8 |
| Treated | Above FDA | 66.7 | 0.0 | 0.0 | 16.7 | 20.8 ^c ± 15.8 |
| Untreated | EC (4ppb) | 25.0 | 58.3 | 50.0 | 50.0 | 45.8 ^{abc} ± 7.2 |
| Untreated | KEBS (10ppb) | 41.7 | 66.7 | 83.3 | 50.0 | 60.4 ^{ab} ± 9.2 |
| Untreated | FDA (20ppb) | 41.7 | 75.0 | 83.3 | 66.7 | 66.7 ^{ab} ± 9.0 |
| Untreated | Above FDA | 50.0 | 25.0 | 16.7 | 33.3 | 31.3 ^{bc} ± 7.1 |

Values accompanied by similar letters within the column are not significantly different ($P \leq 0.05$); Values accompanied by different lower case letters indicate significant difference at $P \leq 0.05$

Table 10: Correlation among the population of *Aspergillus flavus* L-strain, *A. flavus* S-strain, aflatoxin levels and sporulation of Aflasafe KE01

| | <i>A. flavus</i> S-strain | <i>A. flavus</i> L-strain | Aflatoxin | Sporulation |
|---------------------------|---------------------------|---------------------------|-----------|-------------|
| <i>A. flavus</i> S-strain | 1.00 | | | |
| <i>A. flavus</i> L-strain | -0.97 ** | 1.00 | | |
| Aflatoxin | 0.41 * | -0.40 * | 1.00 | |
| Sporulation | -0.14 ns | 0.38 * | -0.13 ns | 1.00 |

** Correlation coefficient significant at $p \leq 0.01$; * correlation coefficient significant at $p \leq 0.05$; ns- not significant; correlation coefficient significant at $p \leq 0.05$

4.6 Aflatoxin producing potential of *Aspergillus* section *Flavi* isolates

Aflatoxin B1, B2, G1 and G2 were detected from *Aspergillus* section *Flavi* isolates using TLC method. *Aspergillus parasiticus* was found to produce all the four aflatoxin types: B1, B2, G1 and G2 (Table 11). However, production of aflatoxin G1 (Mean = 3084ppb) was more pronounced than B1 in isolates of *A. parasiticus*. Moreover, *A. flavus* S-strain was also found to produce aflatoxin B1, B2 and G1. *Aspergillus flavus* S-strain produced more aflatoxin B1 (Mean = 3964ppb) compared to the other morphotypes of *Aspergillus* section *Flavi*. However, there was no significant difference ($p \leq 0.05$) in production of aflatoxin B1 between *A. flavus* S-strain and *A. parasiticus*. Both *A. flavus* S-strain and *A. parasiticus* produced the four types of aflatoxin: B1, B2, G1 and G2. Although there was no significant difference in production of B1 and G1 aflatoxins in *A. parasiticus*, the level of aflatoxin B1 produced by *A. flavus* S-strain was significantly higher (Mean = 3964ppb) than aflatoxin B2 (Mean = 405ppb), G1 (Mean = 40ppb) and G2 (Mean = 9.2ppb). Almost all (99%) of *A. tamarii* isolates were atoxigenic although one isolate identified as *A. pseudotamarii* was found to produce B1 toxin.

Aflatoxin B1 was predominantly produced (range: 91 - 3344ppb) by *A. flavus* S-strain, *A. flavus* L-strain and *A. parasiticus* isolates from maize samples (Table 12). The potential of *Aspergillus* section *Flavi* to produce aflatoxin B1 was lower in fields that were treated with Aflasafe KE01 compared to untreated fields. Most (89.2%) of *Aspergillus flavus* L-strain from treated fields were atoxigenic while a few (8.8%) of the *A. flavus* S-strain in treated fields were atoxigenic. There was significantly ($p \leq 0.05$) high proportion (77.4%) of atoxigenic strains in Aflasafe KE01 treated maize fields whereas in untreated fields, there was high proportion (77.7%) of toxigenic strains (Fig. 7).

Table 11: Aflatoxin production (ppb) by *Aspergillus* section *Flavi* isolates from soil samples collected from lower Eastern Kenya

| Species | Type of aflatoxin | Aflatoxin level (ppb) | % of isolates |
|---------------------------|-------------------|-----------------------|---------------|
| <i>A. flavus</i> S-strain | B1 | 3964.0 ^a | 94.1 |
| | B2 | 405.0 ^b | 81.9 |
| | G1 | 40.0 ^c | 5.9 |
| | G2 | 9.2 ^c | 1.5 |
| | No toxin | - | 5.9 |
| <i>A. flavus</i> L-strain | B1 | 1396.1 ^a | 55.8 |
| | B2 | 105.9 ^b | 41.4 |
| | G1 | - | - |
| | G2 | - | - |
| | No toxin | - | 44.2 |
| <i>A. parasiticus</i> | B1 | 3426.0 ^a | 100.0 |
| | B2 | 372.1 ^b | 66.7 |
| | G1 | 3347.0 ^a | 100.0 |
| | G2 | 665.7 ^b | 91.7 |
| <i>A. tamarii</i> | B1 | 0.0 [*] | - |
| | B2 | - | - |
| | G1 | - | - |
| | G2 | - | - |

* one isolate of *A. pseudotamarii* produced 601 ppb of aflatoxin B1; Values accompanied by different lower case letters indicate significant difference at $P \leq 0.05$

Table 12: Aflatoxin production (ppb) by *Aspergillus* section *Flavi* isolates from maize fields

| Species/strain | Aflatoxin in treated fields | | | | Aflatoxin in untreated fields | | | |
|---------------------------|-----------------------------|-------|--------|-------|-------------------------------|----------|-----------|----------|
| | B1 | B2 | G1 | G2 | B1 | B2 | G1 | G2 |
| <i>A. flavus</i> L-strain | 91.0 | 20.3 | | | 385.0 * | 23.6 ns | | |
| <i>A. flavus</i> S-strain | 2814.0 | 268.5 | 27.2 | | 3844.0 * | 386.7 * | 18.0 ns | |
| <i>A. parasiticus</i> | 2857.0 | 222.6 | 2665.0 | 489.7 | 2925.0 ns | 133.7 ns | 3084.0 ns | 540.4 ns |
| <i>A. tamarii</i> | - | - | - | - | - | - | - | - |

Significance level $(P \leq 0.05)$ is for testing for differences in aflatoxin production of *Aspergillus* section *Flavi* isolates between treated and untreated plots based on Student's t-test; ns = not significant

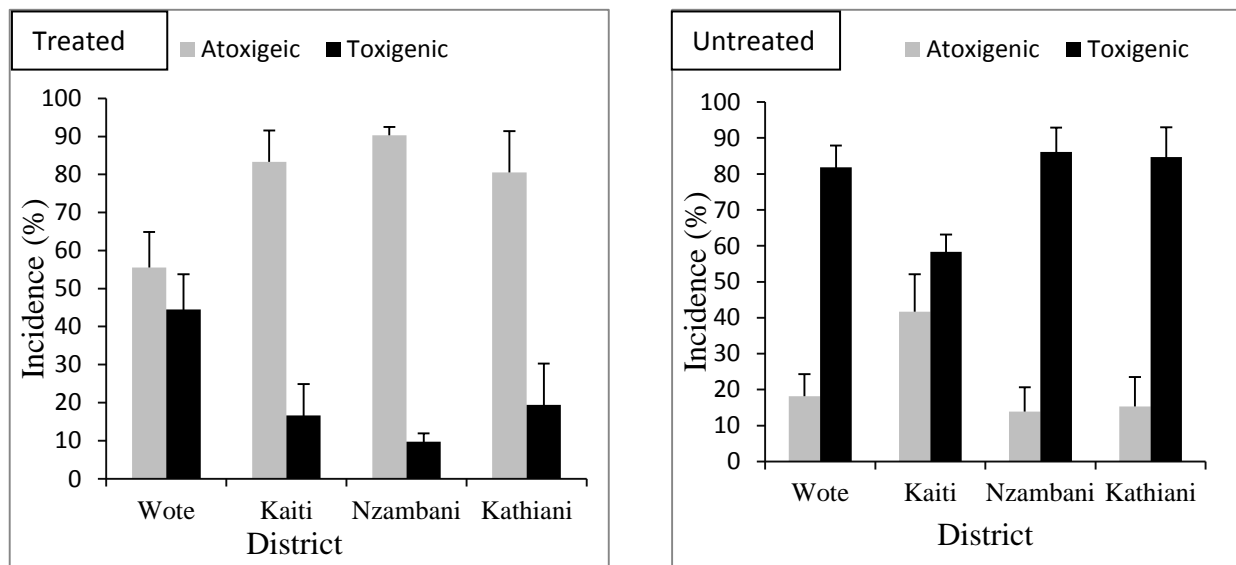


Fig 7: Incidence (%) of atoxigenic and toxigenic members of *Aspergillus* section *Flavi* isolates from Aflasafe KE01 treated and untreated fields

CHAPTER FIVE: DISCUSSION

5.1 Maize production practices and population of *Aspergillus* section *Flavi* in soil in Lower Eastern Kenya

Most (69.7%) of the farmers in lower Eastern Kenya planted the local variety of maize and the improved variety Duma. Maize was commonly intercropped with pigeon peas and common beans. The farmers were also found to majorly use manure for soil amendment after oxen ploughing; and after harvesting majority of the farmers stored their maize in granaries. The findings in this study corroborate earlier observations where farmers in lower Eastern Kenya were found to mainly plant the local variety known as *Kinyanya* and Duma due to ready availability, cheap price, early maturity, high flour yield and drought tolerance (Recha *et al.*, 2012) although new varieties with similar traits are currently available (Brooks *et al.*, 2009). Some areas in Africa like western Kenya (Wambugu *et al.*, 2012) and South Africa (Sibiya *et al.*; 2013) have also been observed to grow mostly their own local varieties compared to improved varieties. According to Recha *et al.* (2012), 95% of farmers in lower Eastern Kenya obtained the local varieties easily from informal systems through exchange with neighbours or from markets or recycling from their own farms (Muhammad *et al.*, 2003) to cut on costs due to economic constraints. The use of recycled seeds may favour persistence of *Aspergillus* species across seasons. Furthermore the local varieties are more susceptible to disease and pests compared to improved varieties (Sibiya *et al.*, 2013).

Intercropping cereal such as sorghum with maize has been reported to retain significantly high populations of *A. flavus* S-strain (Jaime- Garcia and Cotty, 2010). Intercropping maize with legumes such as pigeon peas and common beans replenish the soil with organic soil matter

(Recha *et al.*, 2012). Furthermore the legumes tend to cover the ground reducing moisture loss which favours growth of *Aspergillus* species. However a recent study by Mutiga *et al.* (2015) has indicated reduced aflatoxin level in maize that is intercropped than monoculture maize. Brooks *et al.* (2009) demonstrated the use of organic manure was more common in lower Eastern Kenya and other places in the world because it is cheap to acquire and eco-friendly (Patil *et al.*, 2013). Organic manure increases infiltration, water holding capacity, nutrients and microbial activity in the soil (Recha *et al.*, 2012). These conditions provide a favourable environment for growth of *Aspergillus* in the soil.

The use of hand hoes, ox-drawn plough and tractor drawn plough are commonly used in lower Eastern Kenya (Karuma *et al.*, 2014). Tilling of farms buries crop residues underneath the soil which may enable the *Aspergillus* species to overwinter in the soil across seasons. Furthermore oxen plough has been associated with increase in moisture content although there was no significant advantage over the other methods of tillage (Karuma *et al.*, 2014). Recent study by Atehnkeng *et al.* (2015) has pointed out that aflatoxin contamination of maize can occur at any stage from production, harvesting, postharvest handling, processing, and storage. According to Narrod *et al.* (2013) most farmers in Africa mainly store their maize in traditional granaries as has been seen in this study. However grains threshed when still damp and heaped up in granaries may increase susceptibility to microbial attack (Recha *et al.*, 2012). Furthermore, cultural practices, cultivars used and land management strategies may influence crop colonization by *Aspergillus* section *Flavi* and consequently increase the aflatoxin content of maize (Setamou *et al.*, 1997; Diedhiou *et al.*, 2011).

Aspergillus flavus S-strain was the most predominant species isolated from soil before the application of Aflasafe KE01. The findings of this study concur with the work done by Probst *et al.* (2009) where *A. flavus* S-strain was predominant in soil from lower Eastern Kenya. *Aspergillus flavus* S-strain was identified as the causal agent of outbreak of an acute aflatoxicosis in Kenya in 2004 (Probst *et al.*, 2007). Recent studies have shown that the *A. flavus* S-strain isolated in lower Eastern Kenya is phylogenetically similar to those from U.S and Asia (Probst *et al.*, 2012; 2014). On the contrary, regions neighbouring lower Eastern Kenya like Coast and Rift Valley have been shown to have low to no incidences of *A. flavus* S-strain with high level of *A. flavus* L-strain (Probst *et al.*, 2009). The Prevalence of *A. flavus* S-strain can be attributed to both environmental and survival structures. The high temperature experienced in lower Eastern Kenya may favour the growth and survival of *A. flavus* S-strain (Probst *et al.*, 2009, Jaime-Garcia and Cotty, 2010). The ability to produce large number of sclerotia (Jaime-Garcia and Cotty, 2004), which act as survival structures enables the *A. flavus* S-strain to persist across seasons. *Aspergilli* are known to reside in soil and decaying plant residues (Probst *et al.*, 2014). The high prevalence of *A. flavus* S-strain poses a potential risk of aflatoxin contamination in crops grown in lower Eastern Kenya since it is known to colonize crops like maize and produce copious amount of aflatoxin (Jaime-Garcia and Cotty, 2006; Cotty *et al.*, 2008; Probst *et al.*, 2010).

Soil from Wote district had the highest population of *Aspergillus* section *Flavi* while soil from Kathiani district had the lowest. Areas that have high humidity increases fungal growth and subsequent sporulation (Diedhiou *et al.*, 2011). Farmers in Wote district used manure and oxen ploughing which increase soil moisture content hence potentially favoring the growth of

Aspergillus section *Flavi* in soil unlike farmers in Kathiani district who used inorganic fertilizers and tractors for tillage.

5.2 Efficacy of atoxigenic *A. flavus* (Aflasafe KE01) in managing toxigenic *Aspergillus* and aflatoxin in maize fields

Sporulation of Aflasafe KE01 is important in providing the inoculum of the atoxigenic strains which will eventually colonize the soil and consequently the maize field. Sporulation of Aflasafe KE01 was highest in Nzambani district although it did not differ significantly from Wote and Kathiani districts. The study areas had a similar trend in humidity ranging between 45- 95%, temperature ranging between 17- 29° C and there was no rainfall between time of Aflasafe KE01 inoculation to monitoring. A similar product Aflaguard™ grows actively during periods of increased moisture (Environmental Protection Agency, 2004). Therefore the weather conditions could have accounted for low sporulation in some farms. Kaiti had the lowest proportion of Aflasafe KE01 sporulation because 25% of the inoculated farms did not have the sorghum grains; this could be attributed to free range poultry in the fields that ate the product after it was applied.

The population of *Aspergillus flavus* L-strain was significantly higher than other members of *Aspergillus* section *Flavi* in Aflasafe KE01 treated fields while *A. flavus* S-strain predominated in untreated fields. The high incidence of atoxigenic strains in treated fields could be attributed to the application of Aflasafe KE01. Similar findings have been reported in the USA where the application of atoxigenic strains led to shift of *Aspergillus* community in favor of *A. flavus* L-strain in cottonseed (Cotty , 1990; 1994; 2006; Cotty *et al.*, 2007) and in maize in Nigeria (Atehnkeng *et al.*, 2008b). A similar technology that uses atoxigenic *A. flavus* has been used in

the US to reduce population of toxigenic strains in peanut fields (Horn and Dorner, 2009). Introduction of atoxigenic strains results in exclusion of toxigenic strains by reshaping the fungal community (Grubisha and Cotty, 2015). The greater colonization ability of the atoxigenic strain gives it ecological competence (Abbas *et al.*, 2005) which enables it to competitively exclude the other members of *Aspergillus* section *Flavi*. Furthermore the founder effect plays a role in that the atoxigenic strain is introduced in the field with its food i.e. the sorghum carrier hence it is able to sporulate and outgrow the other species.

Aflasafe KE01 reduced aflatoxin levels in maize grains in treated fields by 47% and thus 77% of samples in treated fields met the KEBS threshold whereas in the untreated fields 60.4 % of the sample met the KEBS threshold. There was a positive correlation between the population of *A. flavus* S-strain and Aflatoxin level. A similar correlation was seen in Probst *et al.* (2007; 2012), Jaime-Garcia and Cotty (2006) and Mauro *et al.* (2015). Furthermore studies by Atehnkeng *et al.* (2008) showed that the atoxigenic strains can displace toxigenic strains both in harvested grain under controlled conditions and in developing maize kernels under conditions that favor high levels of aflatoxin contamination during crop production. The atoxigenic strain reduces aflatoxin levels by physically excluding the toxigenic strain and also by competing for nutrients required by toxigenic strains for aflatoxin biosynthesis (Bayman and Cotty, 1993). Biocontrol plays an important role in aflatoxin management by protecting the crop from harvest to consumption and not only does it protect the treated crop but also other rotated crops and season crops that missed a treatment (Ranajit and Cotty, 2013).

5.3 Aflatoxin production by *Aspergillus* section *Flavi* isolates from soil and maize

The *Aspergillus* section *Flavi* isolates were found to produce aflatoxin B1, B2, G1 and G2. These results are in line with Probst *et al.* (2009; 2014) and Mutegi *et al.* 2012 where isolates of *A. flavus* S- strain were found to produce high levels of aflatoxins. International Agency for Research on Cancer (IARC) has implicated aflatoxin B1 as a human carcinogen (IARC, 2002). Production of B aflatoxins only in most members of *A. flavus* is due to a 1.5 to 2.2 kb deletion in the aflatoxin biosynthesis gene cluster that results in loss of the gene *cypA*, required for G aflatoxin production (Ehrlich *et al.*, 2004). Furthermore, a study by Okoth *et al.* (2012) and Probst *et al.* (2014) projected the presence of S morphotypes isolated from Kenya producing both B and G aflatoxins which is in line with the findings of this study. In addition to this Cardwell and Cotty (2002), observed that S_{BG} producing strains were more prevalent in drier areas. Similar findings were reported by Atehnkeng *et al.* (2008a) where S-strain producing both B and G aflatoxin were detected.

S_{BG} isolates producing both B and G were also detected in soil isolates, they are phylogenetically divergent from but morphologically similar to the *A. flavus* S-strain that produces small sclerotia and large amounts of both B and G aflatoxins (Cotty and Cardwell, 1999). Low level of aflatoxin G was detected from the *A. flavus* S-strain compared to *A. parasiticus*. *Aspergillus flavus* S-strain producing B aflatoxin was the most predominant and was found to produce significantly high level of aflatoxin B1 compared to *A. flavus* L-strain but there was no significance difference with *A. parasiticus* in soil sample isolates. Furthermore the *A. parasiticus* was found to produce more G aflatoxin than *A. flavus* S-strain. This concurs with the study by Mutegi *et al.* (2012) were *A. flavus* S-strain was found to produce copious amounts of B1 aflatoxins in peanuts. A recent study

by Ezekiel *et al.* (2014a) demonstrated *A. parasiticus* produces more aflatoxin than *A. flavus*. Production of G aflatoxins in *A. parasiticus* could be attributed to the presence genes coding for biosynthesis of the toxin.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Farmers in lower Eastern Kenya mainly planted the local maize variety and intercropped it with pigeon peas, did tillage using oxen plough, applied manure to the fields and stored their maize in granaries. However, predominance of *A. flavus* S-strain was observed in this region. This shows that the farming practices predispose, increase proliferation and survival of *A. flavus* S-strain in the soil.

There was low incidence of toxigenic strains and aflatoxin level in fields that were treated with Aflasafe KE01 compared to untreated fields. This indicates that Aflasafe KE01 was efficacious in reducing the population of toxigenic strains and aflatoxin levels in maize in lower Eastern Kenya.

Aflatoxin B1, B2 G1 and G2 were detected from members of *Aspergillus* section *Flavi*. Aflatoxin B1 was commonly produced by *A. flavus* S-strain and *A. parasiticus*. Atoxigenic *A. flavus* L-strain were found coexisting with toxigenic strains in baseline soil. However, in Aflasafe KE01 treated fields the incidence of atoxigenic strains was significantly higher. This indicates that soil is a complex ecosystem and holds both toxigenic and atoxigenic strains but the application of Aflasafe KE01 shifts the population of *Aspergillus* section *Flavi* in favor of the atoxigenic strains.

6.2 Recommendations

Based on the findings of this study the following are recommended:

- i. Awareness creation should be done on farmers to enlighten them on intercropping with non-host crops, proper sanitation, proper management of crop residues and debris from the fields by doing intense tillage.
- ii. Further research should be carried out to assess susceptibility of different maize varieties to *Aspergillus* species and aflatoxin contamination.
- iii. Effect of soaking Aflasafe KE01 in water (hydropriming) before application of the product should be evaluated to assess whether it improves sporulation of Aflasafe KE01 on the sorghum grain.
- iv. Survival of AflasafeKE01 should be assessed in subsequent seasons.
- v. Multi-seasonal application of Aflasafe KE01 should be carried out to completely suppress toxigenic strains.

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APPENDICES

Appendix I: Preparation of stock solutions

A. Rose Bengal stock solution

Five hundred milligrams of rose Bengal (Sigma-Aldrich Company, St' Louis, USA) will be weighed and transferred into a 100 ml volumetric flask. Thirty milliliters of 100% ethanol will be added and final volume will be brought to 100ml by adding distilled water and stored at ambient temperature ($23 \pm 2^{\circ}\text{C}$).

B. Streptomycin stock solution

One gram of streptomycin sulphate (Duchefa Biochemie, Haarlem, Netherlands) will be weighed and transferred to a 100 ml volumetric flask. The final volume will be brought to 100 ml with sterile distilled water. The solution will be filter sterilized and aliquot to sterile 5 ml falcon tubes and stored in a freezer (-4°C).

C. Chloramphenicol stock solution

Five grams of chloramphenicol (Sigma-Aldrich, St. Louis, USA) will be weighed and transferred to a 500 ml volumetric flask and the volume brought to 500 ml with 100% ethanol and stored at room temperature ($23 \pm 2^{\circ}\text{C}$).

D. Dichloran

Two hundred and fifty milligram of dichloran (Sigma-Aldrich, St. Louis, USA) will be dissolved in 60 ml acetone and transferred to 250 ml volumetric flask and the volume of the solution increased to 250 ml with 100% ethanol and stored at room temperature ($23 \pm 2^{\circ}\text{C}$).

E. A & M micronutrients

Manganese sulphate (0.11 g) and 0.5 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ will be added to a beaker containing 800 ml of purified water and acidified to dissolve at pH of 2.0-2.5. Then, 17.6 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 0.3 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ will be added. Each of the above ingredients will be completely dissolved before adding the next. The solution will be transferred to 1 liter volumetric flask and the final volume brought to 1 liter with distilled water. Thereafter dispensed in aliquot of 5-10 ml in pre-sterilized screw cap tubes and stored in a freezer (-22 °C).

**Appendix II: Annual average temperature (°C), precipitation (mm) and relative humidity (%) in lower Eastern Kenya
in 2013 and 2014**

| District | Year | Minimum temperature (°C) | Maximum temperature (°C) | Precipitation (mm) | Minimum relative humidity (%) | Maximum relative humidity (%) |
|----------|------|--------------------------|--------------------------|--------------------|-------------------------------|-------------------------------|
| Kaiti | 2013 | 18.15 | 28.28 | 1.64 | 46.83 | 92.54 |
| Kathiani | 2013 | 17.22 | 27.43 | 2.16 | 46.92 | 93.14 |
| Nzambani | 2013 | 18.07 | 28.03 | 1.90 | 47.51 | 92.51 |
| Wote | 2013 | 17.33 | 27.66 | 1.64 | 45.83 | 93.02 |
| Kaiti | 2014 | 17.83 | 28.31 | 1.48 | 47.11 | 93.15 |
| Kathiani | 2014 | 16.73 | 27.49 | 1.43 | 45.64 | 92.99 |
| Nzambani | 2014 | 17.61 | 28.03 | 1.25 | 46.64 | 92.66 |
| Wote | 2014 | 16.98 | 27.7 | 1.48 | 46.17 | 93.57 |

Source: <http://me.awhere.com>

Appendix III: Questionnaire on maize production practices in lower Eastern Kenya

1. Name of enumerator: _____
2. Contact of enumerator: _____
3. Date: _____; Sample code: _____
4. Name of the farmer: _____; Phone No.: _____
5. District _____; Location: _____;
Sub-location: _____
6. GPS coordinates: longitude (E/W): _____; latitude
(S/N): _____; Altitude (m): _____
7. Current cropping system: Pure or Intercropped:
8. What is the crop (s) intercropped with maize (this season): _____
9. What is (are) the previous crop (s) grown on the same field?

10. What is the name of maize variety sampled: _____
11. What was the source of your maize seeds? (a) Own (b) Neighbor (c) Agro-shop
12. Did you use fertilizers? (Yes/No): _____
13. If Yes, give the name and quantity of the fertilizer used: _____
14. Did you use manure? (Yes/No) : _____

15. If yes, what type of manure did you use? _____
16. What is the tillage method used? _____
17. Where do you store maize after the harvest? _____
18. Observations or remarks: _____