EFFICACY OF ATOXIGENIC *Aspergillus flavus* (AFLASAFE KE01) ON THE POPULATION OF AFLATOXIGENIC FUNGI AND AFLATOXIN CONTAMINATION OF MAIZE (*Zea mays* L.) IN ALTERNATE SEASONS IN LOWER EASTERN KENYA

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DECLARATION AND RECOMMENDATION

I declare that this research thesis is my original work and has not been submitted wholly or in part for any award in any institution.

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Approval

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DEDICATION

I would like to dedicate this thesis to the most important person my husband Hezekiah Korir: You have given me lots of patience, love, support and statistical advice. This thesis is also dedicated to my parents, siblings and my daughter Chepkemoi for enduring my absence during my study.

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ABSTRACT

Aflasafe KE01 is a biological control product comprising of four atoxigenic strains of *Aspergillus flavus* which are native in Kenya. This study determined the efficacy of Aflasafe KE01 on the population of aflatoxin producing fungi and aflatoxin contamination of maize in alternate seasons. Field efficacy trials were carried out in aflatoxin hot spots in Kathiani and Wote sub Counties in lower Eastern Kenya. Twenty four maize fields were selected at random in each Sub County where 12 were treated with Aflasafe KE01 while 12 were not treated. Aflasafe KE01 was broadcast in the maize fields 2-3 weeks before tussling of maize at a rate of 10kg/ha. Soil samples were collected from each field prior to application of the product and one year later; while maize samples were collected at harvest in both seasons. *Aspergillus* section *Flavi* were isolated from soil and maize grains on modified Dicloran Rose Bengal agar and identified on 5/2 agar. Nitrate non-utilization $(Nit)^-$ mutants were developed for all *A. flavus* L strain isolates and tested for their genetic relatedness to the four Aflasafe KE01 strains. Levels of aflatoxin in maize grains were determined by enzyme-linked immunosorbent assay (ELISA) based Accuscan Pro-reader method. *Aspergillus flavus* L strain, *A. flavus* S strain and *A. parasiticus* were commonly isolated from the soil and maize grains while *A. tamarii* was only isolated from soil samples in in low proportions. A large proportion of *A. flavus* L strain isolates from soil (63.4%) and maize (90.2%) sampled from treated fields belonged to Aflasafe KE01 genetic groups implying displacement of aflatoxigenic fungi by the atoxigenic isolates in both soil and maize. In addition, Aflasafe KE01 strains survived between cropping seasons, implying that alternate season application of Aflasafe KE01 can be exploited. Application of Aflasafe KE01 resulted in up to 85% reduction in aflatoxin level. The study demonstrates the high efficacy of Aflasafe KE01 in aflatoxin reduction in hot spot areas of varying agro-climates.

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LIST OF ABBREVIATIONS AND ACRONYMS

AEZ	Agro-Ecological Zones
CAST	Council for Agricultural Science and Technology
CDC	Centers for Disease Control
CFR	Case Fatality Rate
CFU	Colony Forming Units
CIMMYT	Centro Internacional de Mejoramiento de Maiz y Trigo (International Maize and Wheat Improvement Center)
CU	Clean Up
DNA	Deoxy Nucleic Acid
EPZ	Export Processing Zone
FAO	Food and Agriculture Organization
IITA	International Institute of Tropical Agriculture
KALRO	Kenya Agricultural and Livestock Research Organization
MT	Metric Tones
PACA	Partnership for Aflatoxin Control in Africa
ppb	parts per billion
rpm	revolutions per minute
SSA	Sub-Saharan Africa
VCG	Vegetative Compatibility Groups

CHAPTER ONE
INTRODUCTION

1.1 Background information

Maize (*Zea mays* L.) is a primary staple crop that plays an important role in the livelihood of Kenyan people (Wekesa *et al*., 2003; Schroeder *et al*., 2013) with 90% of the rural households growing maize (FAO, 2011). It constitutes an important source of carbohydrates, protein, vitamin B, and minerals. As an energy source, it compares favourably with other cereals, root and tuber crops, and it is similar in energy content to dried legumes (Gitu, 2006). Contamination of maize by aflatoxins has become a major challenge to maize production and thus a threat to food security (Hell *et al*., 2008).

Aflatoxins are secondary metabolites produced by *Aspergillus* species such as *Aspergillus nomius, A. pseudotamarii, A. minisclerotigenes* and *A. bombycis* (Cotty *et al*., 1994) with the main producers being *A. flavus* and *A. parasiticus.* Aflatoxin contaminates many crops including groundnuts, cotton seed, rice and spices, among others (Bennett and Klich, 2003). Aflatoxin contamination of maize is associated with several species of *Aspergillus* section *Flavi.* These fungi vary widely in their ability to infect and decay crops and produce aflatoxin (Cotty, 1989). Thus, the potential of these fungi to contaminate crops with aflatoxin also varies. The most common aflatoxin, aflatoxin B1, is a toxic fungal metabolite associated with liver cancer, immune suppression (Turner *et al*., 2003; Shephard, 2008) and reduced growth in children (Gong *et al*., 2008).

Aflatoxin-producing members of *Aspergillus* section *Flavi* also differ in morphology, physiology and ecology (Cotty, 1989; Cotty *et al*., 1994). *Aspergillus flavus* forms sclerotia which allow it to survive saprophytically in the soil, groundnut grains, maize residues and cobs for extended periods of time (Wagacha and Muthomi, 2008). The inoculum in soil and crop debris acts as the primary source of inoculum that infects maturing maize crop (Atehnkeng *et al*., 2008a). Infected crops periodically replenish soil populations during drought seasons. Members of *Aspergillus* section *Flavi* are one of the most abundant and widely distributed soil-borne molds and can be found anywhere on earth (Yu *et al*., 2005).

A highly promising method for aflatoxin management has been the use of atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers (Dorner, 2004; Cotty *et al*., 2007). Identification of atoxigenic isolates of *A. flavus* native to Sub Sahara Africa might provide an environmentally sound, ecologically adapted, native, biological resource useful in tackling aflatoxin contamination.

Atoxigenic isolates of *A. flavus* used in biological control have been selected from highly toxic fungal communities in aflatoxin-contaminated maize produced in countries that had been associated with lethal aflatoxicosis (Atehnkeng *et al*., 2008a; Probst *et al*., 2011). Atoxigenic isolates of *A. flavus* can prevent aflatoxin levels in crops through direct interference with aflatoxin production during co-infection and modification of *A. flavus* population composition by competitive exclusion (Dorner, 2004). Competitive exclusion is dependent on isolate reproduction and competition in the local environment (Cotty *et al*., 2008). Isolates of *A. flavus* that produce the most conidia dominate *A. flavus* communities and have proportional influences on contamination regardless of ability to compete during host tissue invasion (Mehl and Cotty, 2010).

Interest in use of atoxigenic strains to manage aflatoxin contamination in crops has led to formulation of commercial biopesticides such as Aflaguard™ (USA) and Aflasafe ™ in Nigeria. In Kenya, a biopesticide which comprises of four atoxigenic isolates of *A. flavus* L strains native to Kenyan soil known as Aflasafe KE01 is currently under testing in farmers' fields. The atoxigenic strains are allowed to internally colonize sorghum grains (used as a carrier) for a brief period and dried before broadcasting it in the field.

1.2 Statement of the problem

In developing countries, many individuals are not only food insecure, but are chronically exposed to high levels of mycotoxins in their diet. Food safety results when microbial contaminants and chemical toxicants are present below tolerance levels in foods. Aflatoxin, a dangerous fungal contaminant, compromises food security in the most vulnerable groups of people in Africa. In Kenya there has been recurrent outbreak of aflatoxicosis and predominance of aflatoxin producing fungi in the country that has led to lethal aflatoxicosis. Aflatoxin B1, classified as a Group 1a carcinogen by the International Agency for Research on Cancer causes death when consumed in large quantities. Aflatoxin causes growth retardation in children and immunosuppression. Aflatoxin-producing fungi also cause direct economic losses by spoiling grain and lower their market value. Animals fed on aflatoxin-contaminated grain have lower productivity and slower growth.

When maize grains are contaminated, they are not safe for human consumption and Kenya has reported cases where tons of maize has been condemned by the government to avoid aflatoxicosis. Contamination of maize with aflatoxin leads to serious food insecurity in the country where maize is the staple food crop. Kenya has repeatedly experienced epidemics of acute aflatoxin poisoning that has resulted in deaths of several hundred Kenyans over the past three decades.

There are no effective fungicides for the management of aflatoxin producing fungi in food crops. Use of several fungicides on maize grains meant for human consumption has been banned in Europe and United States due to toxic effect of fungicides residues in grains. Dietary exposure of human population to fungicides residues found on food consist a potential threat to human health.

1.3 Objectives

1.3.1 Broad objective

To reduce aflatoxin contamination in maize through use of atoxigenic *Aspergillus flavus* (Aflasafe KE01)

1.3.2 Specific objectives

The objectives of this study were:

i. To determine the extent of displacement of aflatoxigenic species in soil and maize grains by atoxigenic strains of *Aspergillus flavus* (Aflasafe KE01).
ii. To determine the survival of atoxigenic *A. flavus* (Aflasafe KE01) strains in soil in alternate seasons.
iii. To determine the effectiveness of field application of atoxigenic *A. flavus* (Aflasafe KE01) in reducing aflatoxin contamination in maize at harvest.

1.4 Hypotheses

i. Atoxigenic *Aspergillus flavus* (Aflasafe KE01) strains do not displace aflatoxigenic species in soil and maize grains.
ii. Atoxigenic *Aspergillus flavus* (Aflasafe KE01) strains do not survive in soil between cropping seasons.
iii. Atoxigenic *Aspergillus flavus* (Aflasafe KE01) strains are not effective in reducing aflatoxin contamination of maize under field conditions.

1.5 Justification

Aflatoxin contamination of maize is a health and food security concern to small scale farmers who depend on maize as the staple food crop. Due to recurrent aflatoxicosis in Kenya, there is need for tools to manage contamination of locally produced maize. Different approaches have been advocated for the management of aflatoxin level in cereals which include proper crop rotation, appropriate fertilization, management of crop residues, harvesting of maize when dry, proper storage after harvest, breeding for resistant maize varieties (Hell *et al*., 2008) and use of biocontrol agents (Cotty *et al*., 2007).

Use of native atoxigenic strains in reduction of aflatoxin contamination has been shown to be effective in other parts of the world (Abbas *et al*., 2006; Atehnkeng *et al*., 2014). Management of aflatoxin contamination of food is required to capture optimal markets for maize, maintain food security and to improve health and wellbeing of people. Aflatoxin menace if left unmanaged will lead to serious food insecurity in the country where maize is the staple food crop and future outbreaks that will take lives of more Kenyans.

Atoxigenic strains of *A. flavus* native to Kenya are adapted to local cropping systems and can successfully compete for local resources (Mehl *et al*., 2012; Probst *et al*., 2011). These atoxigenic isolates are native, environmentally sound and biological resource useful in mitigating aflatoxin contamination of maize produced in Kenya. Indeed, atoxigenic isolate formulations have long-term stability under non-refrigerated conditions, a trait useful in regions where refrigeration is unavailable or expensive. In addition to reducing aflatoxin contamination in maize, the atoxigenic strains can survive for many years in soil and can spread from treated fields to neighbouring fields. This is a biocontrol benefit not shared with chemical pesticide applications.

1.6 Scope and limitation

The study was carried out in Kathiani and Wote Sub Counties in lower Eastern Kenya (aflatoxin hotspot areas) during the short rain of 2013 and 2014 cropping seasons. The study was aimed at testing the efficacy of a biocontrol product (Aflasafe KE01) in reducing aflatoxin contamination of maize. The study took duration of two years and involved both field and laboratory work. Unreliability of rainfall during long rains season in the study areas limited the study to the short rain seasons only. The efficacy of the biocontrol product in storage was not investigated due to time limitation.

CHAPTER TWO
LITERATURE REVIEW

2.1 Maize production and consumption in Kenya

Kenya has 1.6 million hectares of maize and there is limited scope for further expansion since most of the arable land in Kenya is already under cultivation (Kibaara *et al*., 2005). Maize is produced mainly in Rift Valley and some parts of Central, Western and Eastern regions. The chief growing areas are Trans Nzoia, Nakuru, Bungoma, and Uasin Gishu Sub Counties. In South Nyanza, other parts of the Rift Valley and Western Province, maize is grown alongside other subsistence crops like beans, potatoes and bananas. The Kenya Agricultural and Livestock Research Organization (KALRO) has developed an open pollinated maize composite (Katumani variety) adapted to the drier conditions and is grown in Machakos, Kitui, Tana River and Isiolo Counties (Wekesa *et al*., 2003).

The small- and medium-scale sector produces about 75% of the nation's maize crop, while the large-scale sector (farms over 25 acres) produce the other 25% (Kirimi *et al*., 2011). Maize is estimated to account for more than 20% of total agricultural production, and 25% of agricultural employment (FAO, 2011). Maize contributes about 68% of daily per capita cereal consumption, 35% of total dietary energy consumption and 32% of total protein consumption (FAO, 2010). Thus, Kenya's national food security is strongly linked to production of adequate quantities of maize to meet an increasing domestic demand (Odendo *et al*., 2002). Smallholder farmers grow maize either as a sole crop or as an intercrop, but intercropping is more prevalent for the vast majority of small farmers (Muthamia *et al*., 2001). So, in Kenya's smallholder sector, maize is commonly intercropped with a number of short-term crops such as groundnut (*Arachis hypogea*), potatoes (*Solanum tuberosum*) and mainly beans (*Phaseolus vulgaris*) or cowpeas (*Vigna unguiculata*) (Hassan *et al*., 1998).

The maize grains are ground to produce maize flour for stable maize meal (*ugali)*. It is also consumed as a food grain, fresh, boiled or mixed with other foods like grain legumes (Walingo, 2011). The stalks, leaves, and other remains from the maize cobs are used to feed livestock especially dairy cattle. The stalks and cobs are used to provide domestic fuel particularly in the rural areas and as organic manure (FAO, 2011). The grains are used in manufacture of corn oil and animal feeds hence it is a vital raw material for industrialization (IITA, 2009).

Maize production is constrained by many factors including, declining soil fertility (Mutegi *et al.*, 2012), high incidences of diseases, erratic climatic conditions and high cost of key inputs such as seeds, pesticides and fertilizer for resource-poor farmers (Ouma and De Groote 2011). Other constraints include weed infestation and postharvest losses due to grain damage by storage pests (Sjogren *et al*., 2010; Tefera *et al*., 2011).

2.2 Occurrence and impact of aflatoxin contamination of maize in Kenya

In 1981, Kenya experienced its first recorded outbreak of aflatoxicosis. A total of 20 patient cases of acute hepatic failure were reported, 12 cases occurred in persons who subsequently died after eating maize which contained as much as 12 000 ppb of aflatoxin B1. Liver tissue at necropsy contained up to 89 ppb of this mycotoxin (Ngindu *et al*., 1982). In 2004 an outbreak of acute aflatoxicosis in Kenya which was one of the most severe episodes of human aflatoxin poisoning in history also occurred (Probst *et al.*, 2007). A total of 317 cases were reported by 20 July 2004, with a case fatality rate of 39% (Azziz-Baumgartner *et al*., 2005). This epidemic resulted from ingestion of aflatoxin contaminated maize (Lewis *et al*., 2005). Maize samples from the affected area had concentrations of aflatoxin B1 as high as 4,400 ppb much higher than 10 ppb limit for maize suggested by Kenyan authorities the same as that allowed by the World Food Programme (Onsongo, 2004; Collins *et al*., 2010).

Case fatality ratio (CFR) was significantly higher in Makueni County than in Kitui County (CDC, 2004). Since 2004, outbreaks among subsistence farmers have recurred annually in Eastern Province (KEPHIS, 2006). In 2005, another aflatoxicosis outbreak in Makueni and Kitui Counties affected 75 people, resulting in 32 deaths (Julia, 2005). In 2006, 78 cases of aflatoxicosis were reported in Machakos, Makueni and Kitui, 28 of which resulted in deaths (Nyaga, 2010). In 2007, 21 out of 84 cases in Eastern province were fatal, 16 of these cases occurred in Igembe in Meru County. In 2008 nine cases were documented from Kibwezi, Kajiado, Mutomo and Makueni County, four of which were deaths (Muthomi *et al*., 2009; Wagacha and Muthomi, 2008). During the outbreak that occurred in 2010, the levels of aflatoxin-B1 in serum (4400 ppb) found in Kenya were among the highest ever recorded in the world (Obura, 2013).

2.3 The Genus *Aspergillus*

Moulds that produce a characteristic asexual spore head that looks like an aspergillum are placed together in the genus *Aspergillus.* There are approximately 250 named species of *Aspergillus* (Geiser, 2008). Furthermore, this common mould is involved in many industrial processes including enzymes (amylases), commodity chemicals (citric acid) and food stuffs like soy sauce produced by *A. oryzae* (Machida and Gomi, 2010).

Aspergillus (Emericella) *nidulans* has been used to elucidate the parasexual cycle, as well as to understand basic concepts in regulation of metabolic pathways, the cell cycle, intron splicing and hyphal polarity (Bentley and Bennett, 2008). Several species of *Aspergillus* contaminate grains and other foods with toxic metabolites that are a threat to the health of humans and other animals (Wild and Gong, 2010).

2.3.1 *Aspergillus* section *Flavi* species

Several species have been assigned to *Aspergillus* section *Flavi* mainly based on morphological parameters, including colony diameter, colour and texture, size and texture of conidia and conidiophore structure. They include, *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. parvisclerotigenus*, *A. oryzae*, *A. bombycis* and *A. tamarii* (Klich, 2002). Several species in this section are important mycotoxin producers including aflatoxins, cyclopiazonic acid, ochratoxins and kojic acid (Varga *et al*., 2011). Aflatoxins are mainly produced by *A. flavus* and *A. parasiticus*, which coexist with and grow on almost any crop or food (Varga *et al*., 2011). The species have received major considerations due to their ability to produce potent carcinogenic aflatoxins (Klich, 2007; Cotty *et al*., 2008) on important food crops prior to harvest, during harvest and storage (Yu *et al*., 2004). Typical *A. flavus* isolates produce aflatoxin B1 which is the most toxic of the many naturally occurring secondary metabolites produced by fungi (Varga *et al*., 2011). Another group of *A. flavus*-related isolates producing both B- and G-type aflatoxins has also been described as *A. minisclerotigenes* (Varga *et al*., 2011).

Other members of section *Flavi* known to produce aflatoxin are *A. pseudotamarii* and *A. bombycis* (Peterson *et al*., 2001). *Aspergillus pseudocaelatus* produces aflatoxins B and G, cyclopiazonic acid and kojic acid, while *A. pseudonomius* produces aflatoxin B1, chrysogine and kojic acid (Varga *et al*., 2011). *Aspergillus pseudotamarii* produces aflatoxin B1 while *A. nomius* and *A. bombycis* produce both B and G aflatoxins (Peterson *et al*., 2001).

Aspergillus flavus can be divided into S and L strains on the basis of sclerotia morphology (Mellon and Cotty, 2004). The S strain produces many small sclerotia (<400 µm in diameter), relatively few conidia and consistently high levels of aflatoxin (Probst *et al*., 2010). The L strain produces fewer, larger sclerotia (>400 µm in diameter), more conidia and, on average, less aflatoxin than the S strain. A significant percentage of L strain isolates produce no aflatoxin and are called atoxigenic (Dorner, 2004). Toxigenic and atoxigenic isolates of *A. flavus* can be subdivided genetically by their vegetative incompatibility (Bayman and Cotty, 1991).

Aspergillus flavus L strain has atoxigenic (non-aflatoxin producers) and toxigenic isolates which produce B aflatoxins (Cotty, 1997; Atehnkeng *et al*., 2008a). Atoxigenic isolates do not produce aflatoxin due to 0.8-1.5 kb deletion at five prime end of aflatoxin biosynthesis gene cluster (Ehrlich *et al*., 2004). The fungal lineage with S strain morphotype within section *Flavi* found in Kenya produce copious amount of aflatoxin B1 in contaminated maize (Probst *et al*., 2007). *Aspergillus parasiticus* and S_{BG} strain found in West Africa, produces both B and G aflatoxins while *A. tamarii* which occurs in sub-saharan Africa is regarded as non-aflatoxin producing species (Atehnkeng *et al*., 2008a; Ezekiel *et al*., 2014).

2.3.2 Biology and life cycle of *Aspergillus* section *Flavi*

Aspergillus section *Flavi* historically includes species with usually biseriate conidial heads in shades of yellow-green to brown and dark sclerotia (Varga *et al*., 2011). It comprises a closely related group of fungi that are found throughout the world and are present in soil and air (Klich, 2002; Abbas *et al*., 2005; Yu *et al*., 2005). *A. flavus* is a saprophytic fungus that is capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, hay, cotton, compost piles, stored grains, dead insects and animal carcasses (Hedayati *et al*., 2007; Accinelli *et al*., 2008).

The life cycle of the species in section *Flavi* can be divided into two phases: (1) colonization of plant debris in soil and (2) invasion of seeds and grain in actively growing crop plants (Horn, 2007). Soil serves as a reservoir for primary inoculum of *A. flavus* and *A. parasiticus* (Abbas *et al*., 2009; Probst *et al*., 2009). The fungus exists in form of mycelium which rapidly grows during its growth cycle. When the conditions are unfavourable for growth (dessication and poor nutrition) the mycelium congregates to form resistant structures called sclerotia (Yu *et al*., 2005). Under favourable conditions the sclerotia either germinate to produce additional hyphae or conidia (asexual spores), which can be further dispersed in the soil and air (Bennett *et al*., 1986; Cotty, 1988).

Sclerotia and conidia produced by *A. flavus* growing on crop debris and in the soil serve as primary inoculum for plants in the next cropping season. Later in the growing season, conidia produced on crop debris or on infected plants provide high levels of secondary inoculum when environmental conditions are conducive for disease development (Scheidegger and Payne, 2003).

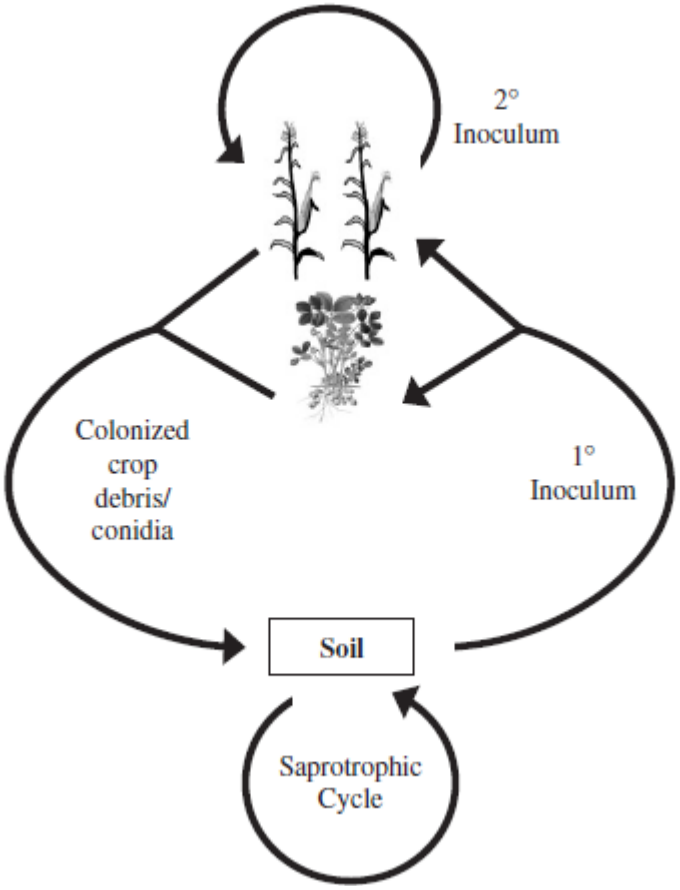

Figure 1: Life cycle of *A. flavus* and *A. parasiticus* in agricultural ecosystems.

Source: Horn, 2007. 1°- primary inoculum, 2°- secondary inoculum.

2.3.3 Vegetative Compatibility Groups

Phenotypic characteristics, including sclerotial morphology and ability to produce aflatoxins, are conserved within a VCG (Pildain *et al*., 2004).

Strains that are vegetatively compatibility with one another are frequently described as members of the same vegetative compatibility group (Cotty *et al*., 1994).

Vegetative compatibility is the ability of individual fungal strains to undergo mutual hyphal fusion to form a viable heterokaryon, previously defined as a fused cell containing nuclei of both parental strains in a common cytoplasm (Leslie, 1993). Vegetative compatibility isolates share a common gene pool, are placed in the same VCG, and are potentially able to exchange genetic material through the parasexual cycle (Glass *et al*., 2000). In contrast to compatibility isolates, hyphal contact between incompatibility isolates does not form stable heterokaryons (Hell *et al*., 2010).

2.4 Aflatoxin types and their biosynthesis

Aflatoxins are a group of structurally related toxic secondary metabolites produced mainly by certain strains of *A. flavus* and *A. parasiticus*. *Aspergillus flavus* in particular is a common contaminate in agricultural commodities, notably maize, peanuts, tree nuts, cottonseed, and spices (Bhatnagar *et al*., 2001; Bennett and Klich, 2003). *Aspergillus bombysis*, *A. ochraceoroseus*, *A. nomius*, *A. minisclerotigenes*, *A. pseudotamarii* and the strain S_{BG} are also aflatoxin-producing species but occur less frequently (Klich *et al*., 2000; Peterson *et al*., 2001; Pildain *et al*., 2008). When the spores of these species find a suitable nutrient source and favourable environmental conditions they rapidly colonize, establish and produce aflatoxins (Payne, 1992) on susceptible crops.

The four major aflatoxins are called B1, B2, G1, and G2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography (Bennett and Klich, 2003). Aflatoxins were first identified in the early 1960s and compose a family of toxic compounds (Wild and Turner, 2002). Aflatoxin B1 is predominant and the most toxic and potent hepato-carcinogenic natural compound ever characterized (Bhatnagar *et al*., 2001). The conditions favouring formation of the aflatoxins have been described, as their metabolism, toxicity, DNA adduct formation, mutagenic and carcinogenic activity (Eaton and Groopman, 1994). The immunosuppressive properties of aflatoxin B1, particularly on cell-mediated immunity, have been demonstrated in various animal models (Pestka and Bondy, 1994). A major metabolite of aflatoxin B1 is aflatoxin M1 which is usually excreted in the milk and urine of dairy cattle and other mammalian species that have consumed aflatoxin contaminated food or feed (Gourama and Bullerman, 1995).

Aflatoxins are polyketides with characteristic dihydro- (B1 and G1) or tetrahydro- (B2 and G2) bisfuran rings (Minto and Townsend, 1997). The production of aflatoxin involves a complex biosynthetic pathway consisting of at least 25 genes (Bhatnagar *et al*., 2003). All of the identified genes related to the biosynthesis are located within a 75kb DNA region in both *A. parasiticus* and *A. flavus*, and their relative positions in the cluster of both fungal species are similar (Yu *et al*., 2000; Ehrlich *et al*., 2005). Most of genes within the biosynthetic pathway are regulated by a single Zn_2Cys_6-type transcription factor, *aflR*, which is encoded by one of the genes in the cluster (Yu *et al*., 1997). The gene *aflJ*, adjacent to the *aflR*, is also involved in the regulation of transcription (Chang, 2003). Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path: acetate → polyketide → anthraquinones→ xanthones → aflatoxins (Bhatnagar *et al*., 2003; Yu *et al*., 2004).

2.5 Effect of aflatoxin on human and livestock health

Aflatoxin contamination of food crops is a common occurrence in both developing and developed countries (Yin *et al*., 2008; Zain, 2011). Aflatoxin contamination results in reduced crop value and diminished health of humans and domestic animals that consume the contaminated crops (Wu and Khlangwiset, 2010). Intake of low, daily doses of the toxins over long periods may result in chronic aflatoxicosis expressed as impaired food conversion and stunting in children (Abbas, 2005; Gong *et al*., 2008), cancer and reduced life expectancy (Cardwell and Henry, 2004; Farombi, 2006). Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and efficiency, anaemia, and jaundice (CAST, 2003). Contamination may limit the economic viability of agriculture in some regions and, in others reduce the acreage on which susceptible crops such as maize and groundnuts may be grown (Wu, 2004). The direct economic impact of aflatoxin contamination in crops results mainly from a reduction in marketable volume, loss in value in the national markets, inadmissibility or rejection of products by the international market, and losses incurred from livestock disease, consequential morbidity and mortality (PACA, 2012).

2.6 Aflatoxin management strategies

Several methods that have been used to manage aflatoxin contamination include chemical, cultural and biological. Cultural methods used to prevent aflatoxin contamination include, field management such as irrigation, timely planting, fertilizer application, weed control, insect control, management of crop residues, crop rotation, planting resistant cultivars, timely harvest and avoiding drought and nutritional stress (Bruns, 2003; Hell *et al*., 2008). Removal and destruction of debris of the previous harvest and cleaning stores before loading new produce would help reduced aflatoxin levels (Hell *et al*., 2000) Drying of grains to safe moisture levels of 10-13% before storage prevents aflatoxin contamination (Hell *et al*., 2008). Reduction of aflatoxin levels can be achieved through storage of grains in traditional granaries which are usually raised structures that are well ventilated and promotes drying of grains (Hell *et al*., 2000). Traditional processing methods such as dehulling, soaking and cooking maize have been reported to reduce the levels of aflatoxins by 46.6%, 28-72% and 80-93% in maize containing 10.7-270 ng/g of aflatoxin levels in Kenya (Mutungi *et al*., 2008). Exposure to acute aflatoxin levels is minimized during food processing and reparation (Kang'ethe, 2011). Nonetheless, these practices have been shown to produce inconsistent results and cannot be relied on to control aflatoxin contamination levels low during high-stress seasons (Payne, 1998; Klich, 2007).

Chemical methods include use of fungicides to prevent fungal growth on crops while pesticides have been used as storage protectant (Hell *et al*., 2010). Fumigation of seeds with chemicals like ethylene oxide and methyl bromide were found to be effective in reducing incidence of aflatoxigenic fungi in stored peanuts (Bankole *et al*., 1996). The biocontrol strategy refers to field application of atoxigenic strains of *A. flavus* that can competitively exclude toxigenic strains from colonizing crops and thereby reduce aflatoxin concentration (Abbas *et al*., 2006; Cotty *et al*., 2007). The competitiveness of applied isolate in the process of colonizing and becoming established in crops is important to the success of the use of atoxigenic isolates for biocontrol (Dorner, 2004). For competitive exclusion to be effective, atoxigenic strains must be predominant in agricultural environments where susceptible crops are to be infected by the toxigenic strains (Dorner, 2004). Dorner (2009) achieved successful aflatoxin reduction in maize through use of atoxigenic *A. flavus* strains in pre-harvest field conditions. Greatest successes to date in biological control of aflatoxin contamination in both pre- and post-harvest crops have been achieved through use of atoxigenic fungi to competitively displace toxigenic fungi (Cotty *et al*., 2007; Abbas *et al*., 2011).

In many field experiments, particularly with peanut and cotton, significant reductions in aflatoxin contamination in the range of 70%-90% have been observed consistently by the use of atoxigenic *Aspergillus* strains (Dorner, 2004; Pitt and Hocking, 2006; Dorner *et al*., 2010).

2.7 Use of atoxigenic strains of *Aspergillus flavus* to manage aflatoxin production

Atoxigenic *A. flavus* strains have been found in Sub-Saharan Africa, which show promise for controlling aflatoxin in African crops (Bandyopadhyay *et al*., 2005; Atehnkeng *et al*., 2008b). In field trials involving inoculation of maize with toxigenic versus atoxigenic isolates of *A. flavus*, naturally occurring atoxigenic isolates found in Nigerian soils showed a 70.1–99.9% reduction in aflatoxin levels, compared to the toxigenic isolates (Atehnkeng *et al*., 2008b). In Australia, Pitt and Hocking (2006) reported 95% reduction of aflatoxin in peanuts with application of atoxigenic strains.

Two products of atoxigenic strains have been registered as biopesticides to control aflatoxin contamination in cotton and peanuts in several states of USA (Dorner, 2004). A biocontrol product, AF36 was first developed by Cotty and is used in cotton, while AflaGuard™, a commercially available product for aflatoxin biocontrol in the United States, is applied primarily to groundnut fields (Dorner and Lamb, 2006). This technology has been embraced in Africa with Nigeria taking the lead with the formulation of a biopestide,

Aflasafe ™ that has been used in several countries with reportedly lowering the level of aflatoxins in treated maize fields (Bandyopadhyay *et al*., 2013). Atehnkeng *et al*. (2014) reported a change in the densities of *A. flavus* L strain isolates in the soil inoculated with the biopesticide where there was an increase from 62.7% prior to inoculation to 99.1% at harvest and a significant reduction in the population of the toxigenic strains.

In Kenya, IITA has identified four atoxigenic strains of *A. flavus* in locally grown maize, which are now being used to make a Kenya-specific product called Aflasafe KE01. Aflasafe KE01 consists of four atoxigenic isolates that belong to vegetative compatibility groups (VCG) that do not have toxigenic members, which ensures that atoxigenic and toxigenic isolates within a VCG do not exchange genetic material and generate progenies that produce aflatoxins (Cotty, 2006; Ehrlich *et al*., 2007). This atoxigenic VCGs do not to make aflatoxins due to defects in the aflatoxin biosynthesis gene cluster (Donner *et al*., 2010).

Probst *et al*. (2011) carried out a study to determine whether atoxigenic isolates of *A. flavus* with potential value in biological control could be selected from highly toxic fungal communities in aflatoxin-contaminated maize produced in Kenya and were able to identify potential strains for biological control. The composition of Aflasafe varies across the continent, and for Kenya and Nigeria (Bandyopadhay *et al*., 2011) each product consists of a mixture of four atoxigenic strains native to, and targeted for the particular country.

2.8 Methods of aflatoxin quantification

Several methods including chromatographic, spectroscopic and immune-chemicals have been used for detecting and quantifying aflatoxins in food, feed, blood and urine (Wacoo *et al*., 2014). Chromatographic techniques are based on the physical interaction between a mobile phase and a stationary phase where the components to be separated are distributed between the two phases which is followed by fluorescence detections stage (Kokkonen *et al*., 2005; Cavaliere *et al*., 2006). The most commonly used chromatography techniques for analysis of aflatoxins are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC). These chromatographic techniques are very sensitive, they require trained skilled technician, cumbersome pretreatment of sample, and expensive apparatus/equipment (Sapsford *et al*., 2006).

Spectroscopic methods include mass spectrometry, infra-red and ion mobilization spectrometry. Mass spectrometry is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions (Wacoo *et al*., 2014).

An infrared spectrometer is used in the analysis of a compound where infrared radiations covering a range of different frequencies are passed through the sample and the radiant energy absorbed by each type of bonds in the molecules is measured. The Ion mobility spectrometry is a technique that is used in the characterization of chemicals on the basis of speed acquired by the gas-phase ions in an electric field (Sheibani *et al*., 2008). The advantages of this method are accuracy, sensitivity and rapid (Pearson *et al*., 2001).

Immunochemical techniques rely on the specificity of binding between antibodies and antigens. The formation of the antibody-antigen complexes can be quantified by following the change in the absorbance of photons of light energy spectrophotometrically (Anfossi *et al*., 2011). The major immunochemical methods used in aflatoxins analysis comprise radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoaffinity column assay (ICA), and immunosensors (Piemarini *et al*., 2007). Enzyme linked immunosorbent assay methods potentially have advantages over the other procedures because of their simplicity, sensitivity, low cost and the use of safe reagents (Chun *et al*., 2007; Ayejuyo *et al*., 2011). Accuscan pro Reveal Q+ is an ELISA based technique and has been used for testing mycotoxins. The method is easy to use, rapid, fully quantitative lateral flow test with microwell and immunoaffinity column that provides unparalled accuracy in only 9 minutes (Neogen Corporation, 2013).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Description of the study area

The study was conducted in two Sub Counties in lower Eastern Kenya namely Kathiani Sub County in Machakos County and Wote Sub County in Makueni County during the short rain seasons (Figure 2). Whereas the long rains (March to May) season accounts for 80% of the annual national maize output, the short rains season (October to December) is the major maize production season in the marginal drought-prone area where the aflatoxin outbreaks occur (Mutunga and Odour, 2002).

The two Sub Counties are aflatoxin hotspots (Muthomi *et al*., 2009; Probst *et al*., 2010) and they were also selected based on reliability of rainfall in an otherwise dry region. Lower Eastern Kenya generally lies in semi-arid climatic condition and experiences two rainy seasons namely the long rains (March to May) and short rains (October to December). The precipitation pattern is bimodal, with long rains falling between March and May and short rains from October to December (Moore, 1979). The dominant vegetation of Kathiani and Wote Sub Counties is dry bush with trees, and, in the higher areas, savanna with scattered trees (Ominde, 1998).

Kathiani Sub County (1°22’36.0” E, 37°18’20.0” S) lies at an altitude of between 1000 to 1600 m above sea level (asl) and the local area is characterized by a hilly terrain. The soils of Kathiani reflect the largely metamorphic parent material. The mean annual temperature varies from 15 to 25ºC and a total annual rainfall ranging between 400 mm and 800 mm (Appendix II). Wote Sub County (01°48’26.4” E, 37°31’57.4” S) lies at an elevation between 800–1700 m asl with an annual rainfall of between 300 and 600 mm and the mean annual temperature varies from 12 to 28ºC (Appendix II). The soils are sandy and have very high humus content (Ellenkamp, 2004). Climate data during the sampling period was accessed from the awhere weather platform (http://me.awhere.com Accessed on 19th June 2015)

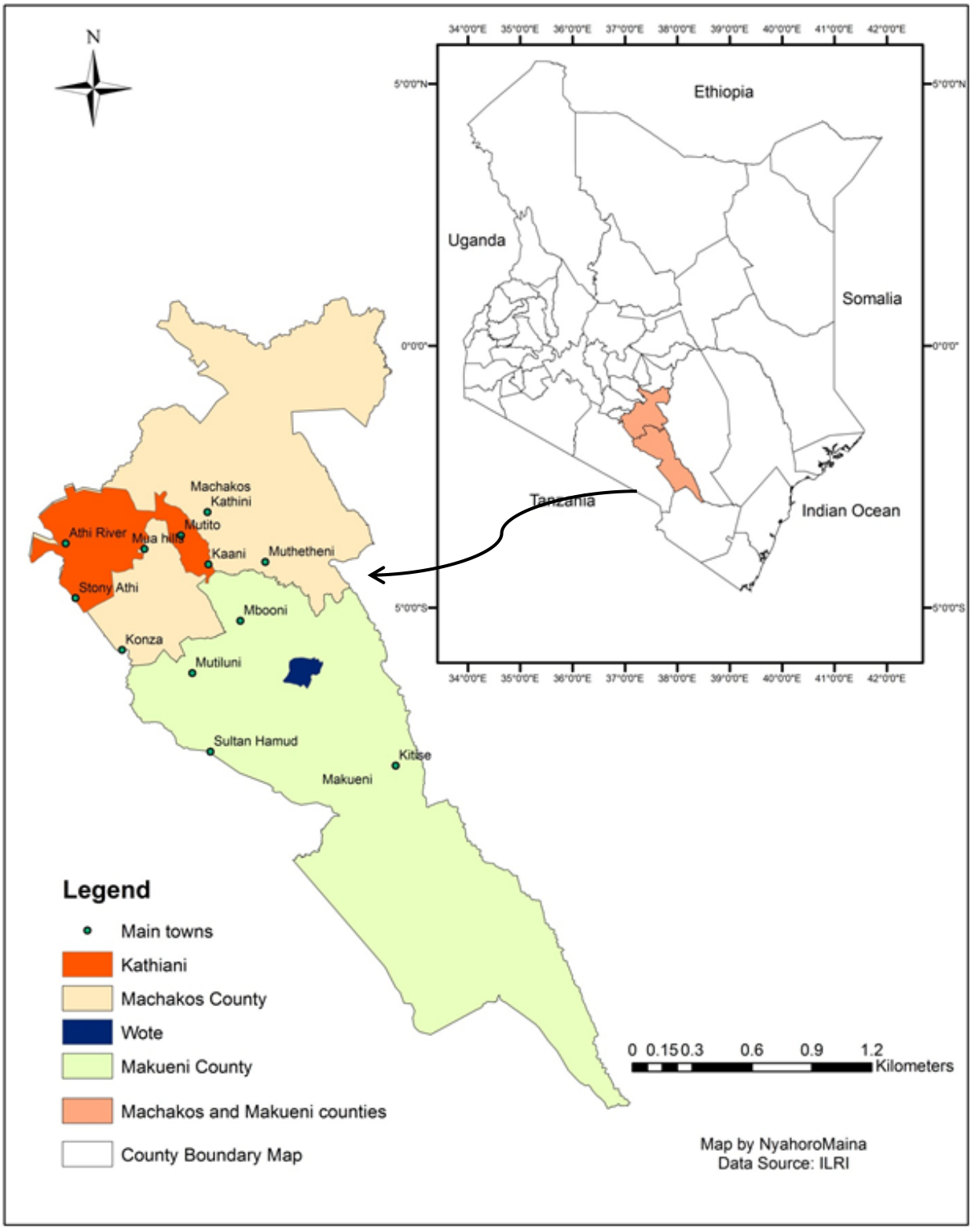

Figure 2: Map of Kenya showing Kathiani and Wote Sub Counties where the field trials were conducted.

3.2 Selection of farmers, application of atoxigenic *A. flavus* (Aflasafe KE01) and assessment of sporulation of atoxigenic *A. flavus* in maize fields

In each of the two Sub Counties, 24 farmers were randomly selected in consultation with agricultural extension officers. The farmers selected had a minimum of two acres under maize production and were willing to participate in the study. A maximum distance of 100 m was left between treated and untreated farms during selection to minimize variation between paired farms. In each Sub County twelve farms were treated with Aflasafe KE01 (This biocontrol product was obtained from IITA) while 12 farms were not treated. Aflasafe KE01 was applied through broadcasted by hand 2-3 weeks before tussling of maize at a recommended rate of 10 Kg/ha (IITA, 2009). Poultry were caged for 14 days after application of Aflasafe KE01 to prevent them from consuming the sorghum grains (used as a carrier). Assessment of sporulation of Aflasafe KE01 strains on the sorghum grains was done 10 days after application of the biopesticide. In each treated farm, four quadrants of $1m^2$ were randomly selected.

In each quadrant, the total number of sorghum grains and the total number of sporulated sorghum grains were recorded. The formation of yellowish green conidia that covers the surface of sorghum grains indicated that the atoxigenic *A. flavus* L strain had sporulated on the grains. The sporulation percentage was calculated as an average of the four quadrants.

3.3 Sampling of soil and maize from maize fields

A total of 96 soil samples were collected from maize fields 2-3 weeks before tussling of maize: Forty eight baseline soil samples (24 samples from each Sub County) were collected during the 2013/2014 cropping season. One year after application of Aflasafe KE01 (2014/2015 cropping season), 48 soil samples (24 samples from each Sub County: 12 from Aflasafe KE01 treated and 12 from untreated maize fields) were collected to determine the survival of Aflasafe KE01 strains in soil for the period of one year. Top soil (two cm) was sampled from each field when the maize was 2-3 weeks before tussling and prior to Aflasafe KE01 application. Sampling spoons and collectors hands were surface sterilized using 70% ethanol after sampling each field. The collectors were positioned at one end of the field; and selection of the first sampling location was done by walking 5 m from the edge of the field. The top two cm of soil was scooped and collected in a sampling bag. The next sampling spot was selected by walking 5 to 10 m in a diagonal transect depending on the size of the field.

Soil samples from all the collectors (from at least 15 different spots in the field) were mixed thoroughly to obtain a representative composite sample from which approximately 500 g was packed in Kraft bags and transported using a vehicle to the laboratory within 48 hours for analysis.

A total of 96 maize samples were collected at harvest: Forty eight maize samples were collected during the 2013/2014 cropping season (24 samples from each Sub County) and 48 maize samples collected during the 2014/2015 cropping season. 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. Collection of maize cobs within 5 m from field edge and contact with the soil was avoided during sampling. The samples from each farm were put in a zip lock bags, labeled and transported using a vehicle to the Regional Mycotoxin and Capacity Development Laboratory Katumani, Kenya within 48 hours.

3.4 Handling and processing of soil and maize samples

Soil samples were sun dried for 5-7 days and pound in a motor to fine particles before sieving through a 2mm mesh screen.

The samples were stored at room temperature (24 ± 2ºC) in Kraft bags before Microbial analysis. Maize samples were sun dried for 7 days, manually shelled by hand and oven dried at 45 °C for 48 hours in Kraft bags. Moisture content was determined by using a moisture meter (Foss Intratec 1241 analyzer moisture meter). Samples with moisture content above 13% were further oven dried. The maize grains were ground using a coffee bunn grinder (Bunn coffee mill, Bunn-o-matic Corporation, Springfield, Illinois, USA), thoroughly mixed and divided into two equal sub-samples for microbial and aflatoxin analysis. The sub-samples for microbial analysis were stored in zip-lock bags at 4°C while the sub-samples for aflatoxin analysis were stored at -20°C until analysis.

3.5 Preparation of culture media

Isolation, identification and characterization of members of *Aspergillus* section *Flavi* was done on Modified Rose Bengal agar and 5/2 medium while selection medium (SEL), Mit and Starch medium was used for *nit*$^{-}$ auxotrophs development, stabilization of mutant and complementation for vegetative compatibility analysis, respectively.

3.5.1 Modified Rose Bengal Agar

Isolation of *Aspergillus* section *Flavi* from maize and soil samples was done on Modified Rose Bengal agar. The medium contains dichloran which restricts the growth of *Aspergillus niger* and other fast growing fungi, it also contains rose Bengal, chloramphenicol and streptomycin which restricts bacterial growth (Garber *et al*., 2012).

One media bottle was prepared for every 500ml of medium. A stir bar and 10g (2%) of Bacto^{TM} agar was added to each bottle. The beaker was placed on a stir plate. A stir bar was added and for each liter of purified water, 3g sucrose, 3g $NaNO_3$, 0.75g KH_2PO_4, 0.25g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 10g NaCl, 1ml A and M micronutrients and 5ml rose Bengal stock solution was added. After all the ingredients dissolved, the final volume was brought to 1 liter and the pH adjusted to 6.5 (by adding KOH or HCL) while the solution is stirring. Thereafter, 500ml of the medium was measured and added to individual media bottles (with capacity of one litre). The bottles were loosely capped and placed on stir plate to disperse agar. The bottles were placed in microwave and heated for 15 minutes until agar melts. Then 5ml of chloramphenicol stock was added (2.5 ml/bottle). The bottles were removed, placed on a stir plate to mix for a few minutes and then placed in the autoclave basket. The media bottles were autoclaved for 20 minutes at 121ºC, at 15 MPa then removed and cooled to 55-60 ºC. In the biosafety cabinet, 5 ml dichloran stock solution/bottle and 2.5 ml streptomycin stock solution/bottle was added, placed on a stir plate at 70 ºC until pouring (Garber *et al*., 2012).

3.5.2 Preparation of 5-2 agar medium

The 5-2 agar medium is a chemically undefined, nutritionally minimal medium used for culture maintenance, spore production for inoculum and DNA isolation, and growing fungi prior to storage (Garber *et al*., 2012). One media bottle was prepared for every 500ml of medium. A stir bar and 10 g (2%) of Bacto^{TM} agar was added to each bottle. The beaker was placed on a stir plate. A stir bar was added and for each liter of media, 50 ml V-8^{TM} Juice and 950 ml of purified water will be added. The pH was adjusted to 6.0 by adding HCL or KOH buffer after all ingredients had dissolved. Thereafter, 500 ml of the medium was measured and added to individual media bottles. The bottles were loosely capped and placed on stir plate to disperse agar. The bottles were then removed, placed on a stir plate to mix for a few minutes and then placed in the autoclave basket. The media bottles were autoclaved for 20 minutes at 121 ºC after which they were removed and placed on a heated stir plate at 70 ºC until pouring (Garber *et al*., 2012).

3.5.3 Preparation of selection medium for auxotrophic mutant generation

This medium is used to select nitrate non-utilizing (*nit¯)* mutants (Garber *et al*., 2012). Chlorate in the medium is metabolized into toxic chlorite by fungi with fully functional nitrate. The medium was prepared following procedures described by Garber *et al*. (2012). One media bottle will be prepared for every 500 ml of medium.

A stir bar and 10g (2%) of Bacto™ agar was added to each bottle. The beaker was placed on a stir plate. A stir bar was added and for each liter of purified water, 30g sucrose, 3g $NaNO_3$, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 25g $KClO_3$, 10 ml rose Bengal stock solution was added. After all the ingredients dissolved, the final volume was brought to 1 litre and the pH adjusted to 7.0 while the solution was stirring. Thereafter, 500 ml of the medium was measured and added to individual media bottles. The bottles were loosely capped and placed on stir plate to disperse agar. The bottles were placed in microwave and heated on high for 15 minutes until agar melts. The bottles were removed, placed on a stir plate to mix for a few minutes and then placed in the autoclave basket. The media bottles were autoclaved for 20 minutes at 121ºC, then removed and placed on a stir plate at 70 ºC until pouring (Garber *et al*., 2012).

3.5.4 Mit agar (mutant culture and phenotyping medium)

This medium is used for purification and mutant characterization of nitrate auxotrophs generated from SEL medium. Once candidate mutants have been identified on SEL medium, they were transferred for three days on Mit medium for stabilization of mutants. One media bottle was prepared for every 500ml of medium. A stir bar and 10g (2%) of Bacto™ agar were added to each bottle. The beaker was placed on a stir plate. A stir bar was added and for each liter of purified water, 30g sucrose, 3g $NaNO_3$, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 15g $KClO_3$, 1 ml A and M micronutrients was added. After all the ingredients dissolved, the final volume was brought to 1 liter and the pH adjusted to 6.5 while the solution was stirring. Thereafter, 500 ml of the medium was measured and added to individual media bottles. The bottles were loosely capped and placed on stir plate to disperse agar. The bottles were placed in microwave and heated on high for 15 minutes until agar melts. The bottles were then removed, placed on a stir plate to mix for a few minutes and then placed in the autoclave basket. The media bottles were autoclaved for 20 minutes at 121ºC, then removed and placed on a stir plate at 70 ºC until pouring (Garber *et al*., 2012).

3.5.5 Preparation of starch agar

This is the medium on which complementation for vegetative compatibility analyses are performed. These complementation tests assess membership in specific vegetative compatibility groups (VCGs). One media bottle was prepared for every 500ml of medium. A stir bar, 10g (2%) of Bacto™ agar and 10g of soluble starch was added to each bottle. The beaker was placed on a stir plate.

A stir bar was added and for each liter of purified water, 36g dextrose, 3g $NaNO_3$, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, and 1ml A and M micronutrients were added. After all the ingredients dissolve, the final volume was brought to 1 liter and the pH adjusted to 6.0 while the solution was stirring. Thereafter, 500ml of the medium was measured and added to individual media bottles. The bottles were loosely capped and placed on stir plate to disperse agar. The bottles were placed in microwave and heated on high for 15 minutes until agar melts. The bottles were then removed, placed on a stir plate to mix for a few minutes and then placed in the autoclave basket. The media bottles were autoclaved for 20 minutes at 121ºC, placed on a stir plate at 70 ºC until pouring (Garber *et al*., 2012).

3.6 Determination of displacement of aflatoxigenic species in soil and maize grains by atoxigenic strains of *Aspergillus flavus* (Aflasafe KE01).

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

Isolation of *Aspergillus* section *Flavi* was done on Modified Rose Bengal agar following the method by Garber *et al*. (2012). One gram of each soil and ground maize samples was weighed and emptied into 10 ml of sterile distilled water in a 40 ml glass vial. The vials were placed on vortex mixer (Velp Scientifica, Europe) for three minutes at 1750 rpm. Using a pipettor and tip, 10, 20 and 40 µl aliquots (from 10^0 dilution) were dispensed into Modified Rose Bengal agar, three plates per sample which were labeled with date of inoculation, sample code and amount of inoculum inside a biological safety cabinet. The suspension was evenly distributed across the surface of Modified Rose Bengal agar plate using cell spreader starting from lowest to highest dilution. Once all isolation plates were inoculated, they were incubated for three days at 31ºC in the dark.

At the end of incubation period, colonies of *Aspergillus* section *Flavi* were counted; plates with 8-10 colonies were selected and the colonies with distinct greenish yellow colour were marked for transfer to 5/2 agar (50 ml of V8 juice and 10 g of agar in 1 L of distilled water) plates (Probst *et al*., 2007). The population (CFU/g of the substrate) of *Aspergillus flavus* L and S strains, *A. parasiticus* and *A. tamarii* were determined using the formula (Olsen *et al*., 1996):

$$\text{CFU/ml} = \frac{\text{Number of colonies x dilution factor}}{\text{Volume of culture plate}}$$

The colonies on Modified Rose Bengal agar plates were examined taking note of their colour and texture, size and number of sclerotia was also noted.

Using a permanent marker, each colony was circled on bottom of the plate and the total number of colonies written on the plate. For any plate with colonies exceeding 10, the colonies were not picked because of possibility of cross contamination and thus they were autoclaved and disposed. To pick up colonies, one conidiophore was lightly touched on one colony with the pointed tip of an applicator stick. The 5-2 agar plate was opened in the biological safety cabinet and the stick was stabbed into the center of the agar and this process was repeated for each colony. Using a permanent marker, each 5-2 agar plate was labeled with the correct sample identification and the date. The inoculated plates were placed in the incubator at 31ºC for five days (Garber *et al*., 2012).

3.6.2 Identification of *Aspergillus* section *Flavi*

After five days of incubation at 31ºC, identification of *Aspergillus* section *Flavi* isolates was done on 5/2 plates based on combination of macroscopic and microscopic characteristics exhibited by each species. Differentiation of members of *Aspergillus* section *Flavi* was mainly based on cultural and morphological parameters, including colony diameter, colour and texture, size and texture of conidia and conidiophore structure (Klich, 2002; Garber *et al*., 2012). Ridell slides were prepared for confirmation of morphological characteristics where *Aspergillus* colonies were sub-cultured on 5-2 agar and incubated for three days at 31ºC in the dark. Slide cultures of *Aspergillus* species and *A. flavus* morphotypes were made by placing 5-2 agar squares on a microscope slide raised with a V-shaped glass rod in a sterile glass petri dish plate covered with a sterile paper at bottom. Some of the *Aspergillus* mycelia and spores were transferred from the isolate to the four edges of the 5-2 agar block using a sterile toothpick.

A sterile cover slip was placed on the surface of the 5-2 agar block, the filter paper moistened with distilled water and the plate incubated for three days at 31ºC in the dark.

Slides for light microscopy were prepared by removing cover slips with grown colonies of *Aspergillus* section *Flavi* and placing them carefully on a microscope slide with a drop of water. The slide was viewed under a light microscope (Leica DM 500, Leica Microsystems, Wetzler, Germany) fitted with a camera (LEICA ICC 50, Leica Microsystems, Wetzler, Germany) and images were taken at ×1000 magnification. Members of *Aspergillus flavus* producing numerous small sclerotia and scanty conidia were identified as S- strains while those with few large sclerotia and numerous conidia were regarded as L-strain. Those with dark green conidia were regarded as *A. parasiticus* while those with brown conidia were regarded as *A. tamarii*.

3.6.3 Preservation of *A. flavus* isolates

Using a dispenser, two ml of distilled water was dispensed into four ml water vials, the vials were then placed inside a 500 ml beaker which was wrapped with aluminium foil and autoclaved at 121 ºC for 20 minutes at 15 mPa. Using sterile transfer tubes, three agar plugs of conidia were removed in a ring pattern from the youngest mature conidia (usually ½ to ¾ of the way from the centre to the edge of the 5/2 plate).

Pure cultures of twelve *A. flavus* L strain isolates from each sample were saved as agar plugs which were dispensed into four ml vials containing two ml sterile distilled water and the vials stored at ambient temperature (24 ºC) for vegetative compatibility analysis (Garber *et al*., 2012).

3.7 Determination of survival of atoxigenic *A. flavus* (Aflasafe KE01) strains in alternate seasons

3.7.1 Development of *A. flavus* L strain mutants

Nitrate non-utilization *nit*¯ mutants of each wild type isolate were generated on selective (SEL) medium supplemented with potassium chlorate. Using sterile transfer tubes, one agar plug was removed from the centre of SEL plate creating a well. Ten microlitres of the water containing the spore suspension was dispensed into centered well of a SEL media agar plate inside a biological safety cabinet, incubated at 31ºC in the dark for 7-14 days until spontaneous auxotrophic sector arose (Garber *et al*., 2012). Nitrate non-utilization (*Nit*$^{-}$) mutants are generated on chlorate containing medium to determine the VCG of the isolates (Beever and Parkes, 2007). The *Nit*$^{-}$ mutants are characterized by the type of nitrogen source utilized and classified as *cnx*$^{-}$*, or niaD*$^{-}$ mutants. When two mutant types from two different isolates are paired on starch medium, a zone of dense conidia formation indicates hyphal fusion and complementation. These reactions are considered evidence of belonging to the same VCG (Beever and Parkes, 2007).

3.7.2 Stabilization of *A. flavus* L strain mutants

Using a sterile transfer tube, agar plugs obtained from the tip of an auxotrophic sector of each isolate were transferred into a Mit agar plate by placing the plug carefully on the Mit medium inside a biological safety cabinet. The agar plugs were incubated at 31ºC (Memmert incubator, Germany) for three days to allow mutant stabilization (Garber *et al*., 2012).

Two milliliter of the 5-2 media in four ml vials were autoclaved at 121°C for 20 minutes and placed in slanting position to settle for two hours on flat surface after autoclaving.

When 5-2 medium was ready for transfers of auxotrophic sectors from *MIT* agar plates, it was placed inside biosafety cabinet together with *MIT* with auxotrophic sectors. Using a transfer tube, a plug was cut from the marked sector of the auxotroph (furthest tip of the auxotroph for each mutant). The plug of cut auxotrophic sector from *MIT* plate was transferred into the floor of the 5-2 agar four ml vial and incubated at 31°C for 5-7 days in dark. After sporulation in 5-2 slants occurred, one ml of sterile water was added into the four ml vials and the *nit*$^{-}$ mutants were ready to be used in vegetative compatibility analysis (Garber *et al*., 2012).

3.7.3 Complementation of *A. flavus* L strain mutants

Inside the biological safety cabinet, three wells were created at the centre of starch media plates using a sterile transfer tube in a triangular pattern with approximately one cm separation between the wells. The lid of each plate was labeled as T_1 at one well and T_2 at the second well and two lines were drawn on the sides of the plates spanning the side of the lid and the side of the plate to maintain the alignment of the lid with the plate. Ten microlitres of suspension containing tester pairs of vegetative compatibility groups were dispensed into each well labeled T_1 and T_2 (the basal wells). Tester pairs are mutants identified as *niaD*$^{-}$ (nitrate non-utilizing, nitrate reductase mutant) and *cnx*$^{-}$ (hypoxanthine and nitrate non-utilizing permease mutant).

Tester pairs were developed from *A. flavus* L strain isolates where each isolate was seeded (10 µl) into centered wells in hypoxanthine and nitrate (Czapek-Dox Broth) media agar plates to assess frequencies of *cnx*$^{-}$ and *niaD*$^{-}$ auxotrophs, respectively. Auxotrophs with mutation at the 5 *cnx*$^{-}$ genes coding for molybedenum-containing cofactor indispensable for nitrate reductase and xanthine dehydrogenase are unable to utilize hypoxanthine and *niaD*$^{-}$ auxotrophs cannot grow on Czapek-Dox Broth agar and have mutation in the structural gene of the nitrate reductase apoenzyme. Tester pairs of vegetative compatibility groups were found by pairing combinations of *niaD*$^{-}$ with *cnx*$^{-}$ mutants on starch agar. Ten microlitres of the *nit*$^{-}$ mutant isolate to be tested for vegetative compatibility were dispensed into the remaining well. The inoculated starch plates were incubated at 31ºC for 10 days. In vegetative compatibility analyses, isolates producing a stable heterokaryon were considered members of the same VCGs which descended from the same clonal lineage and are closely related (Garber *et al*., 2012).

3.8 Determination of effectiveness of atoxigenic *A. flavus* (Aflasafe KE01) in reducing aflatoxin levels in maize grains

Aflatoxin levels in maize samples was quantified using Enzyme Linked Immuno-Sorbent Assay Accuscan Pro reader based technique. Three sub-samples from each sample were analyzed. The maize sample in zip-lock bag was homogenized by shaking for one minute and five grams of the sample was weighed into 100 ml media bottle and mixed for three minutes with 25 ml of 65% ethanol using an orbital shaker (HS501 IKA-WERKE, Germany); the mixture was filtered through Whatmann 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 new reveal Q+ strip was placed into the clear sample cup and left for 6 minutes and the test strip read within one minute using the Accuscan Pro reader (Neogen Corporation, 2013). Aflatoxin levels were read in ppb with a lower limit of 2 ppb and a high limit of 150ppb. Samples with more than 150ppb were further diluted in 65% ethanol in the ratio of 1:9. For every 100µl of sample 900µl of 65% ethanol was added. Then sample was mixed with diluent as explained above.

3.9 Data analysis

Differences in all response variables between treated and untreated maize fields were tested by analysis of variance (ANOVA) using the PROC GLM procedure in SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Means of the response variables obtained from treated and untreated fields were separated using LSD at $\alpha = 0.05$ and paired t-test was done on data from treated fields between the seasons. Correlation between population of *A. flavus* L strain and aflatoxin levels in maize samples was also be determined using PROC CORR procedure in SAS (version 9.3, SAS Institute Inc., Cary, NC, USA).

CHAPTER FOUR

RESULTS

4.1 Efficacy of Aflasafe KE01 in displacement of toxigenic *Aspergillus* species in soil and maize samples

4.1.1 Sporulation of Aflasafe KE01 strains in maize fields in Kathiani and Wote Sub Counties

Sporulation of Aflasafe KE01 strains was significantly ($p \leq 0.05$) higher in 2014/2015 cropping season compared to the 2013/2014 season (Figure 3). During the 2013/2014 cropping season, sporulation was significantly ($p \leq 0.05$) higher in Kathiani Sub County (63%) compared to Wote Sub County (59%). However, during the 2014/2015 cropping season, sporulation was significantly ($p \leq 0.05$) higher in Wote (98%) than in Kathiani (90.3%) Sub County (Figure 3).

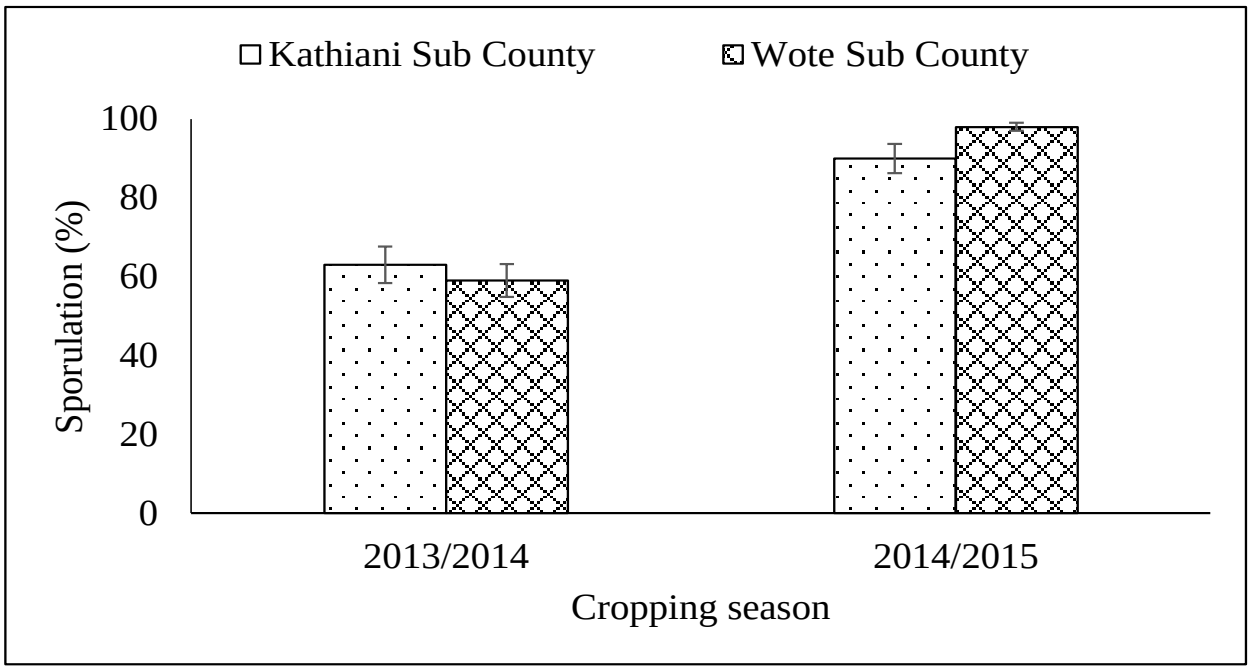

Figure 3: Sporulation (%) of Aflasafe KE01 strains in maize fields in Kathiani and Wote Sub Counties during the 2013/2014 and 2014/2015 cropping seasons.

Error bars represent standard error of the means.

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

The four commonly isolated members of *Aspergillus* section *Flavi* from soil samples were *Aspergillus flavus* L strain, *A. flavus* S strain, *A. parasiticus* and *A. tamarii*. However, in maize samples, only three members of *Aspergillus* section *Flavi* were isolated: *A. flavus* L strain, *A. flavus* S strain and *A. parasiticus* with the latter being isolated from maize sampled from untreated maize fields in Kathiani Sub County only.

The cultural and morphological characteristics of *Aspergillus* section *Flavi* isolated from soil and maize samples are shown in Plate 1 and Plate 2 *Aspergillus parasiticus* is characterized by dark-green conidial head with rough-walled. *Aspergillus flavus* has yellowish-green conidial head with smooth-walled spores while the spores of *Aspergillus tamarii* are brown and echinulated (Plate 2).

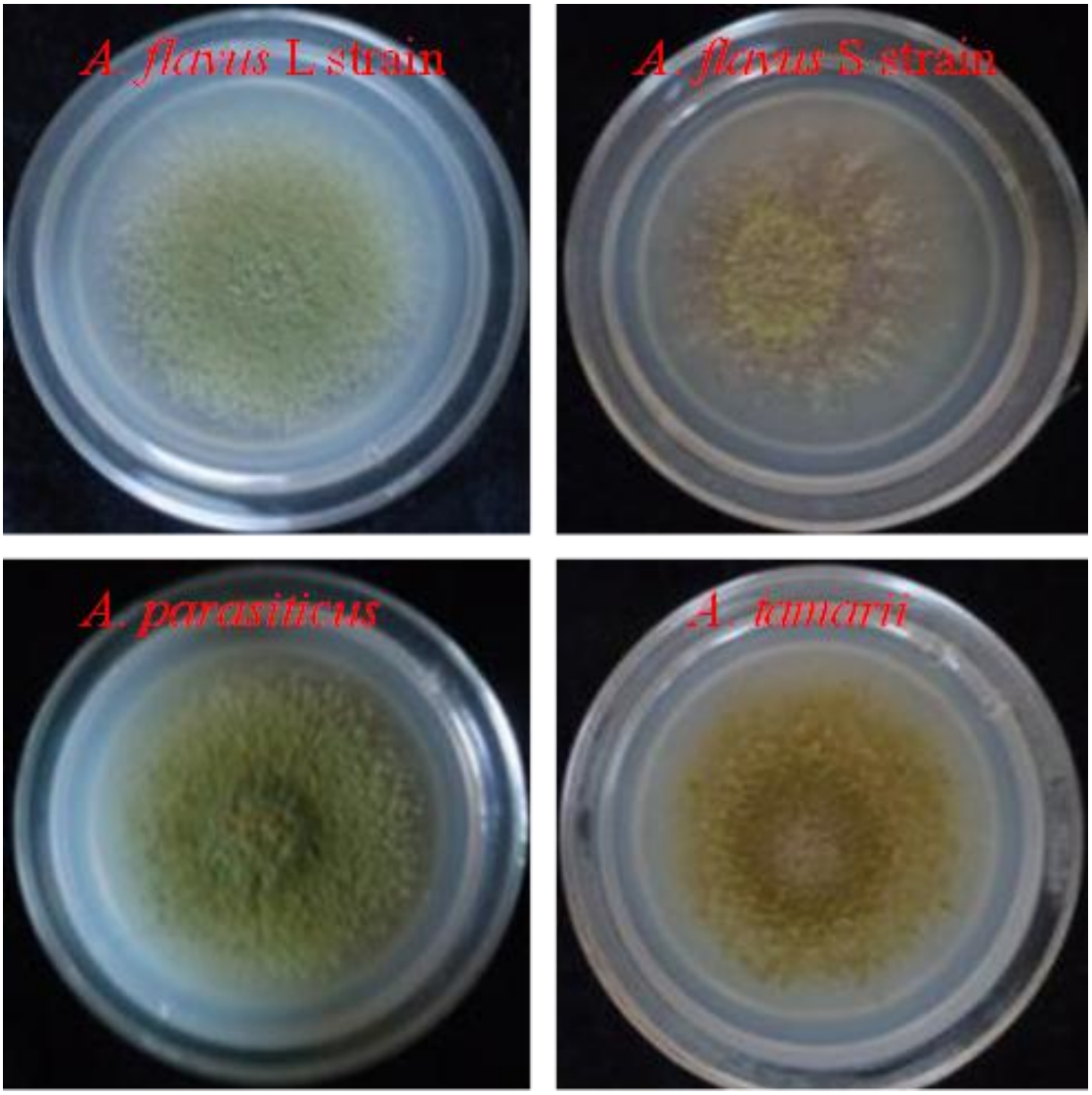

Plate 1: Cultures of *Aspergillus* section *Flavi* growing on 5/2 agar.

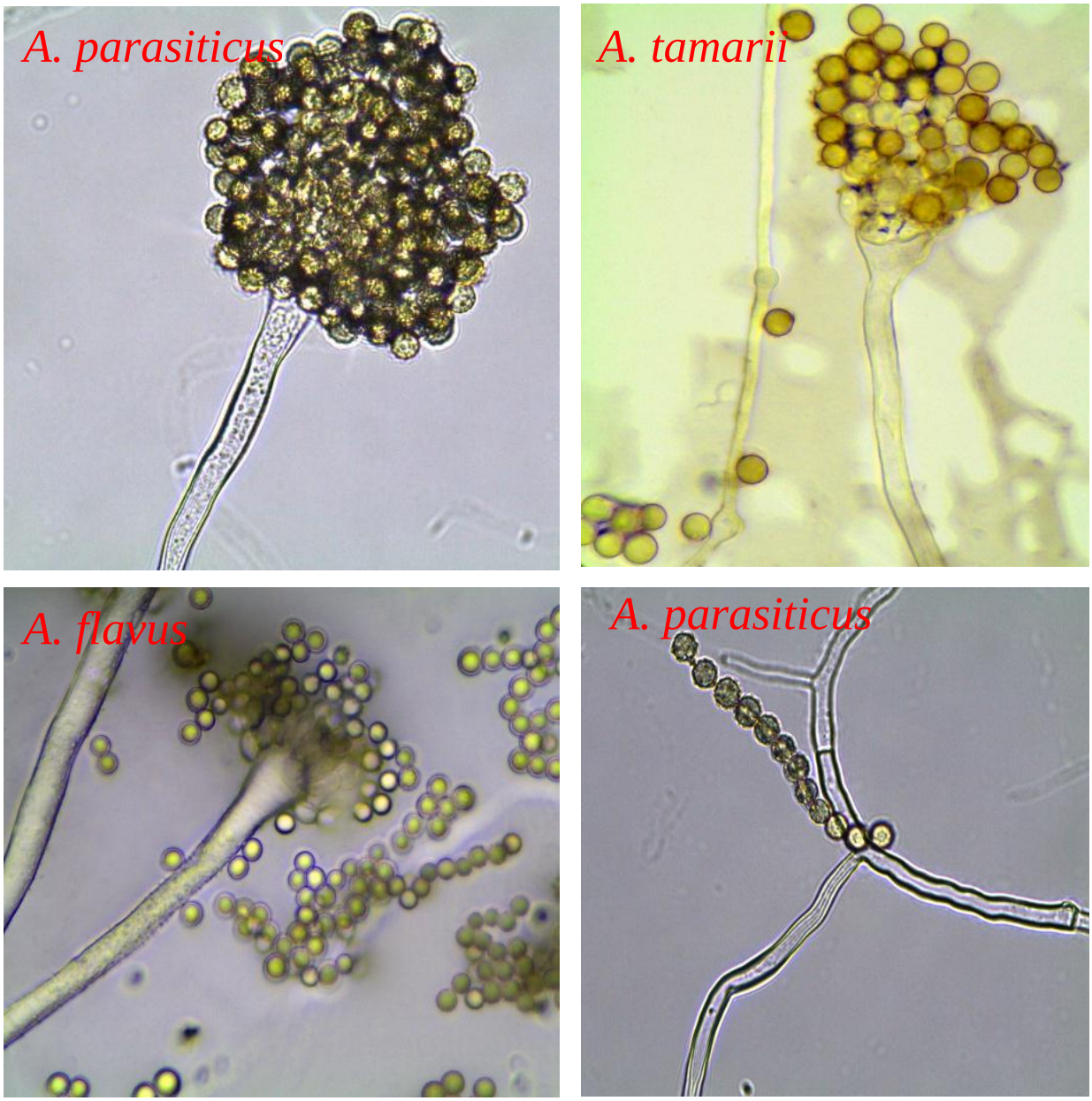

Plate 2: Conidial heads and spore characteristics of members of *Aspergillus* section *Flavi*.

4.1.3 Population (CFU/g) of *Aspergillus* section *Flavi* in soil and maize grains

There were significant ($P \leq 0.05$) differences between the population of *Aspergillus* section *Flavi* between Wote and Kathiani Sub County in both seasons. The population of *Aspergillus* section *Flavi* in soil was significantly ($P \leq 0.05$) higher in Wote Sub County compared to the population in Kathiani Sub County in both cropping seasons (Table 1).

Three morphotypes of *Aspergillus* section *Flavi* (*A. flavus* L strain, *A. flavus* S strain and *A. parasiticus*) were commonly isolated from maize grains in Kathiani and Wote Sub Counties. The population (CFU/g of maize) of *Aspergillus* section *Flavi* was significantly ($P \leq 0.05$) higher in 2014/2015 cropping season than in 2013/2014 cropping season.

In 2013/2014 cropping season, there was no significant difference in the population of *Aspergillus* section *Flavi* between Kathiani and Wote Sub Counties. However, in 2014/2015 cropping season, the population of *Aspergillus* section *Flavi* was significantly ($P \leq 0.05$) higher in Wote than in Kathiani Sub County. In Wote Sub County the population of *Aspergillus* section *Flavi* was significantly ($P \leq 0.05$) higher in 2014/2015 than in 2013/2014 cropping season (Table 1).

Table 1: Population (CFU/g) of *Aspergillus* section *Flavi* in soil and maize sampled during the 2013/2014 cropping season and one year after application of Aflasafe KE01

District	Treatment	*Aspergillus* section *Flavi* colony forming units (CFU)			
		2013/2014		2014/2015	
		Soil (prior to application)	Maize grain at harvest	Soil	Maize grain at harvest
Kathiani	Treated	365	17188	477	36500
	Untreated	464^{ns}	24650^{ns}	302^{ns}	34729^{ns}
	Mean	415	20919	390	35615
	LSD	81	578	97	604
	CV (%)	33	31	21	19
Wote	Treated	651	15000	651	59375
	Untreated	724^{ns}	41068^{ns}	480^{ns}	54684^{ns}
	Mean	688	28034	566	57030
	LSD	73	981	59	658
	CV (%)	17	29	24	26

ns = not significantly different ($p \leq 0.05$)

4.1.4 Shift in population of *Aspergillus* section *Flavi* in soil

The baseline (2013/14 cropping season), population of *A. flavus* S strain was significantly higher in soil sampled from Wote Sub County than in soil sampled from Kathiani Sub County (Table 2). The incidence of *A. parasiticus* was significantly higher in soil sampled from Kathiani than in soil sampled from Wote Sub County in 2013/14 cropping season.

The baseline proportion of the members of section *Flavi* was *A. flavus* S strain (37.5%), *A. flavus* L strain (23.9%), *A. parasiticus* (36.1%) and *A. tamarii* (2.5%) in Kathiani Sub County. In Wote Sub County, the baseline proportion of *A. flavus* S strain was 65.6% followed by *A. flavus* L strain (26.4%), *A. parasiticus* (6.4%) and *A. tamarii* with1.6% (Table 2).

Table 2: Baseline proportion (%) of *Aspergillus* section *Flavi* in soil samples in Kathiani and Wote Sub Counties in the 2013/2014 cropping season

Aspergillus section *Flavi* morphotype	Kathiani	Wote	Mean
A. flavus L strain	23.9^{a}_{b}	26.4^{a}_{b}	25.2_{b}
A. flavus S strain	37.5^{b}_{a}	65.6^{a}_{a}	51.5_{a}
A. parasiticus	36.1^{a}_{a}	6.4^{b}_{c}	21.3_{b}
A. tamarii	2.5^{a}_{c}	1.6^{a}_{c}	2.0_{c}
Mean	25.0	25.0	25.0
LSD ($p \leq 0.05$)	8.7	7.0	5.5
CV (%)	60.5	48.6	54.8

Means followed by the same superscript letters within a row are not significantly different ($p \leq 0.05$). Means followed by the same subscript letters within a column are not significantly different ($p \leq 0.05$)

In Kathiani Sub County, application of Aflasafe KE01 significantly increased the population of *A. flavus* L strain from 23% in 2013/2014 (baseline population) to 45% in 2014/2015 cropping season. However, the population of *A. flavus* S strain and *A. parasiticus* significantly decreased from 40% and 35% in 2013/2014 to 34% and 20% in 2014/2015 cropping season respectively due to application of Aflasafe KE01 (Figure 4).

There were significant differences between the Sub Counties and the treatments on the population of *Aspergillus* section *Flavi* during the 2014/2015 cropping season.

In Kathiani Sub County, the proportion of *A. flavus* L strain was significantly higher in soil sampled from treated fields than those sampled from untreated fields while *A. flavus* S strain was predominant in soil sampled from untreated fields. Application of Aflasafe KE01 significantly increased the proportion of *Aspergillus flavus* L strain in soil by 96.5% and decreased the proportion of *A. tamarii*, *A. parasiticus* and *A. flavus* S strain by 67.9%, 40.8% and 15.0%, respectively in Kathiani Sub County (Figure 4).

Application of Aflasafe KE01 did not significantly change the proportion of *Aspergillus* section *Flavi* in soil sampled from maize fields in Wote Sub County (Figure 4).

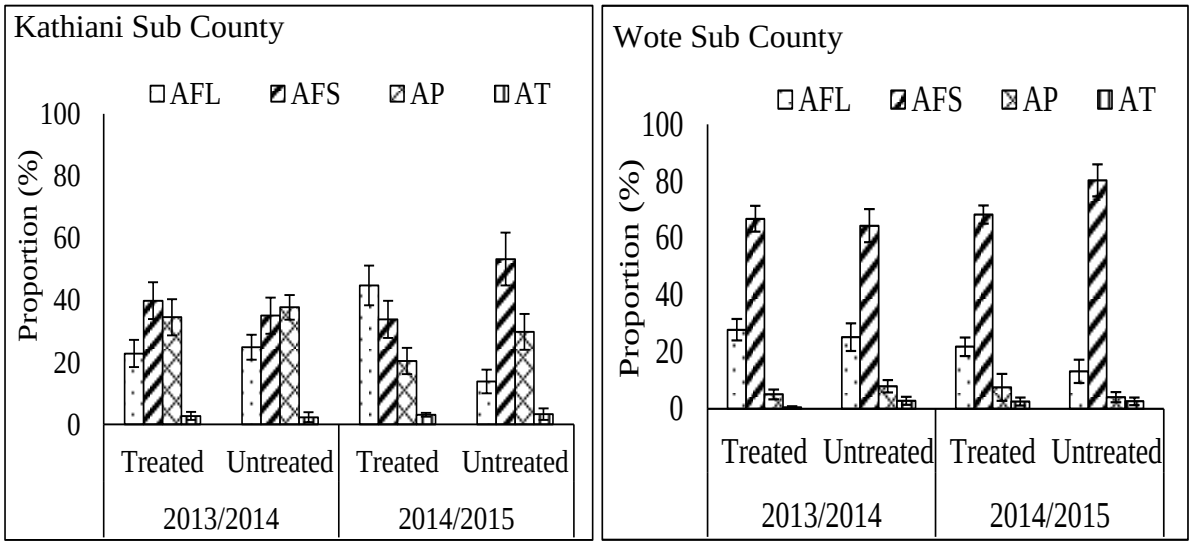

Figure 4: Proportion (%) of *Aspergillus* section *Flavi* in soil sampled from maize fields prior to and after application of Aflasafe KE01.

AFL - *Aspergillus flavus* L strain, AFS - *Aspergillus flavus* S strain, AP - *Aspergillus parasiticus*, AT - *Aspergillus tamarii.*
Error bars represent standard error of the means.

4.1.5 Population of *Aspergillus* section *Flavi* in maize grains

There was a significant effect of season on the population of the *A. flavus* L and *A. flavus* S strains. In 2013/2014 cropping season, the proportion of *A. flavus* L strain, *A. flavus* S strain and *A. parasiticus* was 47%, 50.3% and 2.6%, respectively. In 2014/2015 cropping season, a shift in population was observed, where the population of *A. flavus* L strain increased significantly from 47% to 63% while the proportion of *A. flavus* S strain decreased significantly from 50.3% to 35.5% (Table 3).

The predominant strain in maize sampled from treated fields was *A. flavus* L strain while *A. flavus* S strain was predominant in maize sampled from untreated fields in both cropping seasons. For example, in 2013/2014 cropping season, the proportion of *A. flavus* L strain was significantly ($P \leq 0.05$) higher (74.3%) in maize sampled from treated fields than in those from untreated fields (19.7%). The proportion of *A. flavus* S strain was significantly ($P \leq 0.05$) higher in maize sampled from untreated fields (75%) than those from treated (25.7%) maize fields (Table 3). During the 2013/2014 cropping season, application of Aflasafe KE01 significantly ($P \leq 0.05$) increased the proportion of *A. flavus* L strain in maize grains from 17% to 85% while the proportion of *A. flavus* S strain significantly ($P \leq 0.05$) decreased from 73% to 15% in Kathiani Sub County.

In Wote Sub County there was also a significant ($P \leq 0.05$) increase in the proportion of *A. flavus* L strain from 23% to 63% and a significant decrease in the proportion of *A. flavus* S strain from 77% to 37% in 2013/2014 cropping season. A similar trend was observed in 2014/2015 cropping season where there was a significant ($P \leq 0.05$) increase in the proportion of *A. flavus* L strain and a decrease in proportion of *A. flavus* S strain following application of Aflasafe KE01 in both Sub Counties (Figure 5).

In Wote Sub County, application of Aflasafe KE01 in alternate seasons led to a significant ($P \leq 0.05$) increase in the proportion of *A. flavus* L strain from 63.2% in 2013/2014 to 86.9% in 2014/2015 cropping season. *Aspergillus flavus* S strain significantly ($P \leq 0.05$) decreased from 36.7% in 2013/2014 to 13.1% in 2014/2015 in Wote Sub County. In Kathiani Sub County, application of Aflasafe KE01 in alternate season increased the proportion of *A. flavus* L strain from 73.4% in 2013/2014 to 85.5% in 2014/2015 cropping season. The proportion of *A. flavus* S strain decreased from 25.7% in 2013/2014 to 14.2% in 2014/2015 in Kathiani Sub County (Figure 5).

Table 3: Proportion of *Aspergillus* section *Flavi* in maize sampled from Aflasafe KE01 treated and untreated fields in 2013/2014 and 2014/2015 cropping seasons

Season	Treatment	AFL	AFS	AP
2013/2014	Treated	74.3*	25.7*	0ns
	Untreated	19.7	75.0	5.3
	Mean	47.0	50.3	2.6
	LSD	45.1	48.3	3.7
	CV (%)	37.8	35.5	49.2
2014/2015	Treated	85.5*	14.2*	0.3ns
	Untreated	39.5	56.8	3.7
	Mean	63.0	35.5	2.0
	LSD	39.0	38.4	3.6
	CV (%)	33.6	29.7	23.6

* Significance levels at ($P < 0.05$); ns = not significant.
Aspergillus flavus L strain, AFS - *Aspergillus flavus* S strain, AP - *Aspergillus parasiticus*

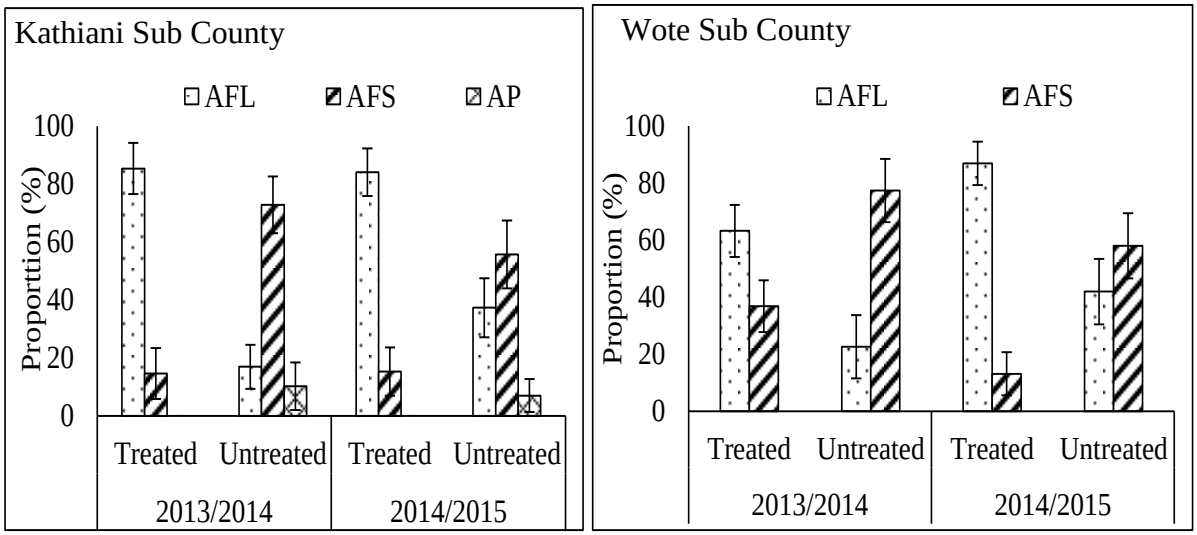

Figure 5: Proportion (%) of *Aspergillus* section *Flavi* in maize grains at harvest from Aflasafe KE01 treated and untreated maize fields.

AFL - *Aspergillus flavus* L strain, AFS - *Aspergillus flavus* S strain, AP - *Aspergillus parasiticus.*

4.2 Recovery of Aflasafe KE01 vegetative compatibility groups from soil and maize grains

Aflasafe KE01 VCGs were recovered from both soil and maize grains with a significantly ($p \leq 0.05$) higher recovery in samples from treated fields than from untreated maize fields. Formation of stable heterokaryon between tester pairs and unknown isolate confirmed vegetative compatibility. Negative vegetative compatibility analysis (VCA) reaction showing formation of a stable heterokaryon only between tester pairs (A), positive VCA reaction of a tester of a VCG with *nit*$^{-}$ mutant of an isolate tested for vegetative incompatibility (B and C) and positive VCA reaction of the two VCGs with *nit*$^{-}$ mutant (D) (Plate 3). Recovery of VCGs was significantly higher in grains than in soil samples.

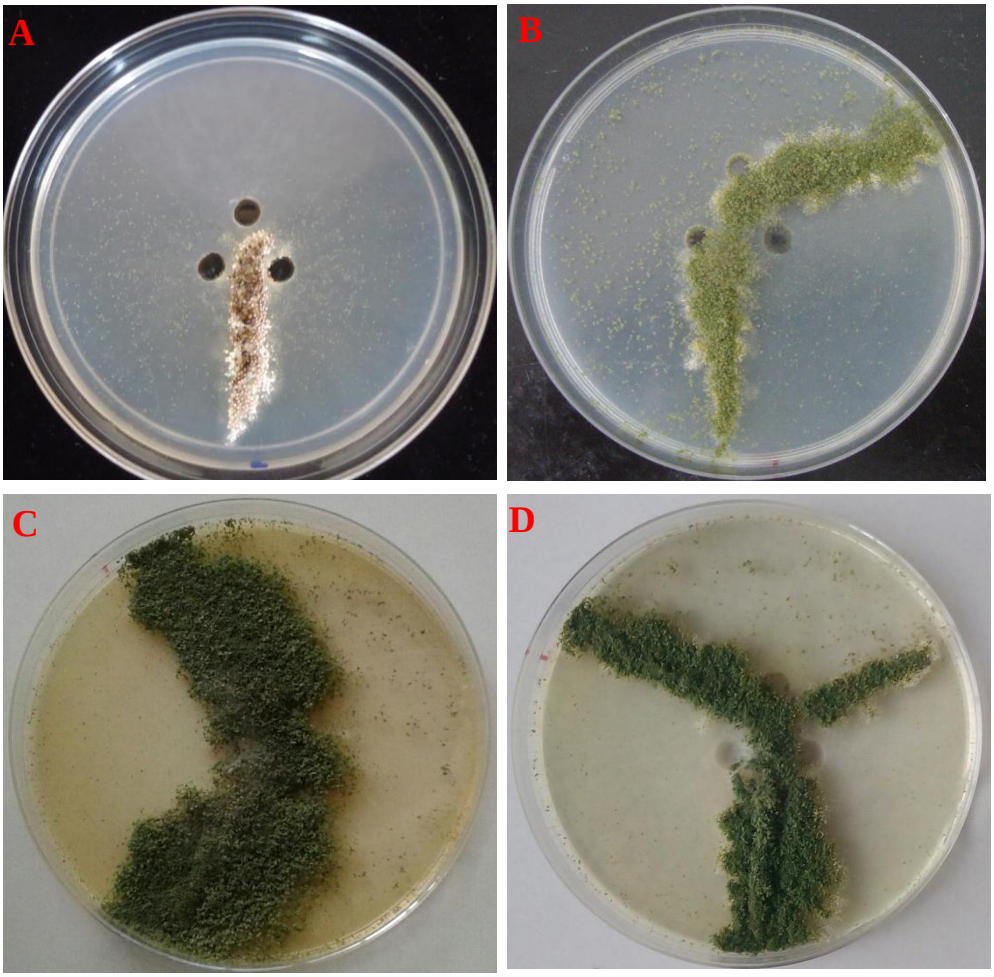

Plate 3: Vegetative compatibility reaction between tester pair and unknown isolate.

4.2.1 Recovery of Aflasafe KE01 vegetative compatibility groups from soil

Recovery of Aflasafe KE01 VCGs from soils was significantly higher ($p \leq 0.05$) in 2014/2015 (after application) than in 2013/2014 (prior to Aflasafe KE01 application) cropping season in Kathiani and Wote Sub Counties. In baseline soil samples, there was low recovery of VCGs in both Sub Counties. However, in both Sub Counties a significantly ($p \leq 0.05$) higher recovery of Aflasafe KE01 VCGs was recorded in treated fields than in untreated fields one year after application of the bio-product (Figure 6). In Kathiani Sub County, the proportion of Aflasafe KE01 VCGs increased from 45% in soil sampled from fields that were to be treated (2013/2014) to 55% one year after application of (2014/2015). Although the proportion of *A. flavus* L strain did not change after application of Aflasafe KE01 in Wote Sub County, the proportion of *A. flavus* L strain isolates that belonged to Aflasafe KE01 genetic group increased significantly from 32% (baseline) to 63.4% one year after application (Figure 6).

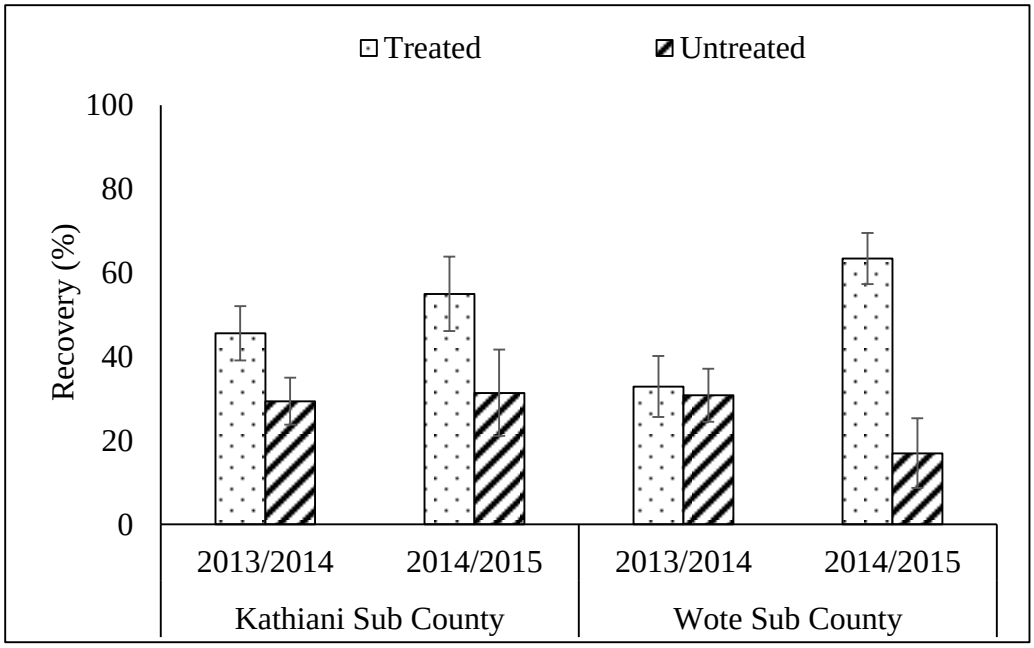

Figure 6: Recovery (%) of Aflasafe KE01 vegetative compatibility groups in soil before and one year after treatment of maize fields with Aflasafe KE01.

Error bars represent standard error of the means.

4.2.2 Recovery rate of Aflasafe KE01 individual VCGs from soil samples one year after application

The four VCGs that constitute Aflasafe KE01 were recovered from soil sampled from treated fields in the two Sub Counties in the following proportions: KN001 (16.7%), KN00A (16.5%), KN011 (14.1%) and KN012 (10.6%). However, there was no significant difference ($p \geq 0.05$) in recovery of individual VCGs between the two Sub Counties. In Kathiani Sub County, VCGs belonging to KN001 and KN00A were the most recovered from soil sampled from treated fields with rates of 17.2% and 16.3%, respectively. In Wote Sub County, VCGs belonging to KN011 (18.2%) and KN00A (17.2%) were recovered from soil sampled from treated maize fields in the highest rate (Figure 7).

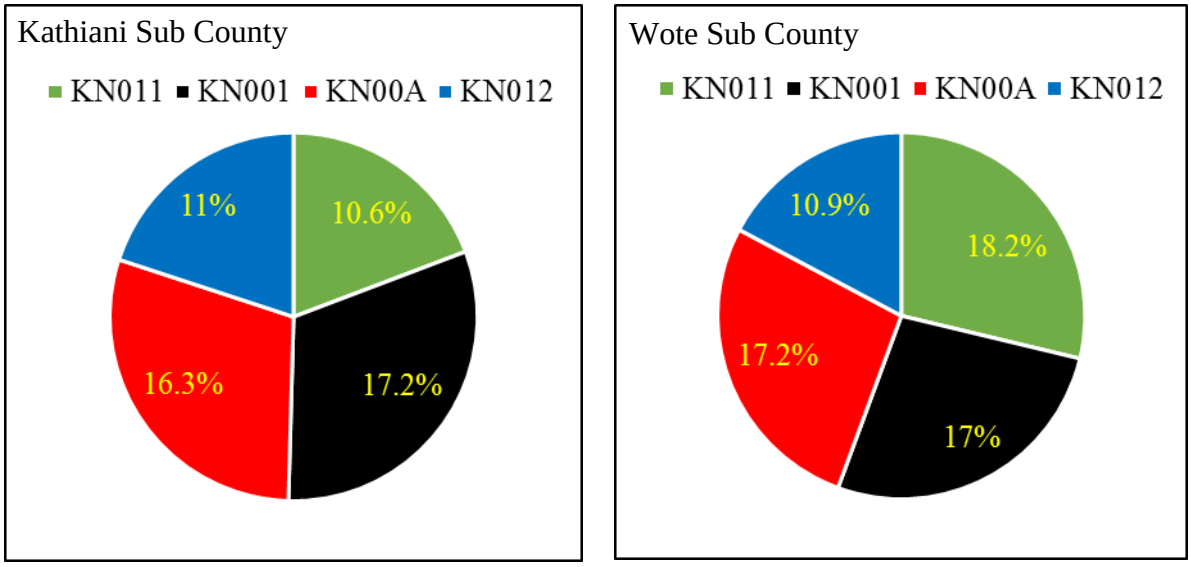

Figure 7: Proportion (%) of Aflasafe KE01 individual vegetative compatibility groups recovered from soil samples one year after application of the bio-control product.

4.2.3 Recovery of Aflasafe KE01 vegetative compatibility groups from maize grains

The recovery of Aflasafe KE01 VCGs from maize grains was significantly affected by the cropping season and treatments (Table 6). Recovery of Aflasafe KE01 VCGs from maize grains was significantly ($p \leq 0.05$) higher in 2014/2015 than in 2013/2014 cropping season. Recovery of Aflasafe KE01 VCGs was significantly ($p \leq 0.05$) higher in maize sampled from treated fields than those from untreated fields in the two seasons.

In 2013/2014 cropping season, maize sampled from treated fields had significantly higher recovery level of Aflasafe KE01 VCGS (58.5%) than maize sampled from untreated fields (12.4%). The same trend was observed in 2014/2015 where maize sampled from treated fields had significantly higher recovery level of Aflasafe KE01 VCGs (80.9%) than in untreated fields with recovery level of 47.9%. Application of Aflasafe KE01 in alternate seasons significantly ($p \leq 0.05$) increased recovery of Aflasafe KE01 VCGs in treated fields from 58.5% in 2013/2014 to 80.9% in 2014/2015 cropping season in the two Sub Counties (Table 4).

Table 4: Recovery of Aflasafe KE01 vegetative compatibility groups from maize grains sampled from Aflasafe KE01 treated and untreated fields in Kathiani and Wote Sub Counties

Treatment	2013/2014	2014/2015	Mean
Treated	$58.5^{b}{}_{a}$	$80.9^{a}{}_{a}$	69.7_{a}
Untreated	12.4_{b}	47.9_{b}	30.2_{b}
Mean	35.5^{b}	64.4^{a}	

Means followed by the same superscript letters within a row are not significantly different ($p \leq 0.05$). Means followed by the same subscript letters within a column are not significantly different ($p \leq 0.05$).

Recovery of Aflasafe KE01 VCGs was significantly ($p \leq 0.05$) higher in treated fields than in untreated fields in both Kathiani and Wote Sub Counties in the two cropping seasons. Subsequent application of Aflasafe KE01 led to a significant ($p \leq 0.05$) increase in recovery of Aflasafe KE01 VCGs. For example, in Kathiani Sub County there was an increase in recovery from 59% in 2013/2014 to 90.2% in 2014/2015 cropping season. The same trend was observed in Wote Sub County where there was an increase in recovery from 58.1% in 2013/2014 to 82% in 2014/2015 cropping season (Figure 8).

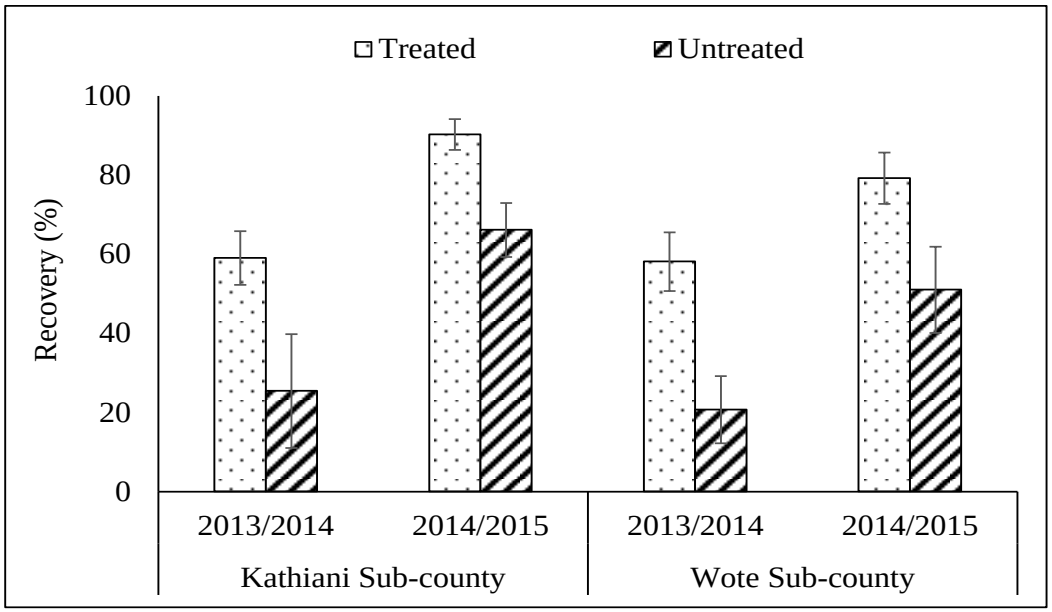

Figure 8: Recovery (%) of Aflasafe KE01 vegetative compatibility groups in maize grains sampled at harvest from Aflasafe KE01 treated and untreated fields.
Error bars represent standard error of the means

4.2.4 Recovery of Aflasafe KE01 individual VCGs from maize grains sampled from treated maize fields

The four Aflasafe VCGs were recovered in the following proportions from maize sampled from treated fields in the two Sub Counties: KN011 (29.0%), KN00A (14.3%), KN001 (10%), and KN012 (4.3%) during the 2013/2014 cropping season. In 2014/2015 cropping season, they were recovered as follows: KN00A (35.7%), KN011 (25.5%), KN001 (14.7%) and KN012 (10.3%). However, there was no significant difference ($p \leq 0.05$) in recovery of individual VCGs between the Sub Counties (Figure 9).

In 2013/2014 cropping season, the VCGs belonging to KN00A was the most recovered with rates of 30.7% and 27.3% in Kathiani and Wote Sub County, respectively while in 2014/2015 the VCGs belonging to KN001 was the most recovered with rates of 32.6% and 38.7% in Kathiani and Wote Sub County, respectively (Figure 9).

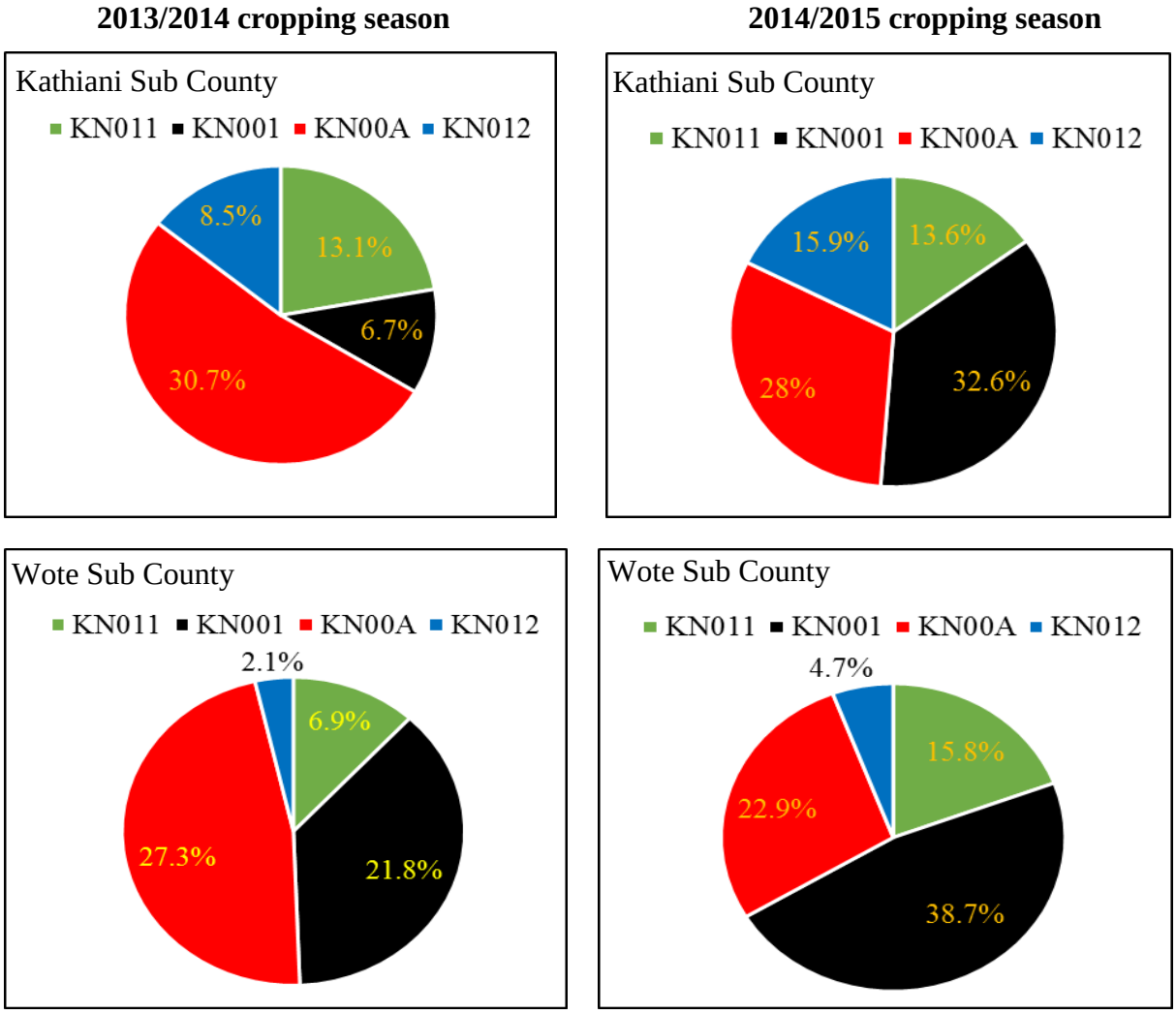

Figure 9: Proportion (%) of Aflasafe KE01 individual vegetative compatibility groups recovered from maize samples at harvest.

4.3 Efficacy of Aflasafe KE01 in reducing aflatoxin levels in maize

4.3.1 Aflatoxin levels in maize grains at harvest

Maize grains sampled from treated and untreated fields during the two seasons were contaminated with aflatoxin (Table 5). However, samples from the treated fields had significantly lower aflatoxin levels compared to samples from untreated fields in both Sub Counties in the two cropping seasons. In 2013/2014 cropping season, the mean aflatoxin level in maize grains from treated fields was 87.1 ppb while that from untreated fields was 135.1 ppb.

In 2014/2015 cropping season the mean aflatoxin level in maize samples from Aflasafe KE01 treated maize fields was 36.9 ppb and that from untreated fields was 143.7 ppb. Application of Aflasafe KE01 in alternate seasons decreased aflatoxin level by 57.6% in treated fields in the two Sub Counties. Level of aflatoxin was significantly higher in Wote Sub County than in Kathiani Sub County in both seasons (Table 5).

In 2013/2014 cropping season, the aflatoxin levels in maize sampled from Wote Sub County was significantly higher compared to samples from Kathiani Sub County. In Kathiani Sub County, application of Aflasafe KE01 resulted in 85.7% and 78.4% reduction in aflatoxin level in 2013/2014 and 2014/2015 cropping seasons, respectively. In Wote Sub County, Aflasafe KE01 resulted in 27.4% and 51.4% reduction in the aflatoxin level in 2013/2014 and 2014/2015 cropping seasons, respectively (Table 5).

Table 5: Aflatoxin level (ppb) in maize sampled at harvest from Aflasafe KE01 treated and untreated fields in Kathiani and Wote Sub Counties in alternate seasons

Season	Treatment	Kathiani Sub County		Wote Sub County	
		Range	Mean	Range	Mean
2013/2014	Treated	<2.0-50.1	6.8[a]	<2.0-605.0	167.5[a]
	Untreated	<2.0-435.0	47.4[b]	<2.0-724.3	230.8[a]
% Reduction[a]			**85.1**		**27.4**
2014/2015	Treated	<2.0-376.0	49.6[a]	<2.0-190.8	24.2[a]
	Untreated	<2.0-2450.0	229.8[a]	<2.0-316.0	49.8[a]
% Reduction[a]			**78.4**		**51.4**

[a] Indicates reduction in levels of aflatoxin in maize grains sampled from Aflasafe KE01 treated and untreated fields

4.3.2 Correlation between population of *Aspergillus flavus* and aflatoxin levels in maize grains

According to Pearson's correlation analysis, there was a significant positive correlation ($r = 0.45$, $p < 0.0001$) between the population of *A. flavus* S strain and aflatoxin level (Table 6). However, there was a negative correlation between the population of *A. flavus* L strain and aflatoxin level ($r = -0.44$, $p < 0.0001$). A negative correlation was also observed between the population of *A. flavus* S strain and *A. flavus* L strain ($r = -0.95$, $p < 0.0001$).

Table 6: Correlation among the population of *A. flavus* L strain, *A. flavus* S strain, and aflatoxin level in maize grains sampled at harvest during the 2013/2014 and 2014/2015 cropping seasons

	2013/2014			2014/2015		
	AFL	AFS	Aflatoxin level	AFL	AFS	Aflatoxin level
AFL	1			1		
AFS	-0.94^{***}	1		-0.97^{***}	1	
Aflatoxin level	-0.36^{*}	0.39^{**}	1	-0.22^{ns}	0.23^{ns}	1

Correlation significance: *** significant at $p < 0.0001$, ** significant at $p < 0.01$, * significant at $p < 0.05$, ns = not significant
AFL - *A. flavus* L strain; AFS - *A. flavus* S strain.

CHAPTER FIVE

DISCUSSION

5.1 Efficacy of atoxigenic *Aspergillus flavus* (Aflasafe KE01) strains in displacement of toxigenic *Aspergillus* species in soil and maize

Aspergillus flavus S strain was the most abundant morphotype of *Aspergillus* section *Flavi* in soil prior to application of Aflasafe KE01 and in untreated fields. The predominance of *A. flavus* S strain in soil may be due to high temperature and low rainfall in Kathiani and Wote Sub Counties. Warm and dry weather conditions have been reported to favour infectivity of *A. flavus* S strain resulting in contamination of maize with aflatoxin prior to harvest (Bock *et al.*, 2004; Guo *et al.*, 2005). The predominance of *A. flavus* S strain in soil in this study was in agreement with reports by Probst *et al.* (2010) who stated that fungal communities in Kenya associated with severe maize contamination with aflatoxin and deaths in human were highly dominated by S strain of *A. flavus*.

Application of Aflasafe KE01 resulted in an increase in the proportion of *A. flavus* L strain and a decrease in the proportion of *A. flavus* S strain and *A. parasiticus* in soil sampled from Kathiani Sub County. These results were in agreement with those of Cotty *et al*. (2008) who reported that application of atoxigenic strain of *A. flavus* in soil shifts fungal community composition towards predominance of atoxigenic fungi. Applied atoxigenic strains of *A. flavus* displace aflatoxin producers from the crop environment thus reducing aflatoxin contamination (Cotty *et al*., 2007). However, in Wote Sub County, application of Aflasafe KE01 did not significantly change the proportion of *Aspergillus* section *Flavi* in soil samples which could be attributed to low amount of rainfall (237 mm) in the Sub County at the time of application of Aflasafe KE01 hence lower sporulation (Appendix II). High amount of soil moisture enhance rapid production of conidia by the applied atoxigenic strains of *A. flavus* (Aflasafe KE01) resulting to increase in their population while low soil moisture inhibit production of conidia (Cotty *et al*., 1994). High temperature (27.7 °C) and low rainfall (223 mm) experienced in the study areas during the two cropping seasons might have increased the population of atoxigenic fungi (Appendix II*)*. These findings are in agreement with those of Wilson and Payne (1994) who reported that climatic factors such as drought and high temperature accompanied by reduced rainfall increase the population of aflatoxin producing fungi and aflatoxin contamination in crops.

Soil sampled from maize fields in Wote Sub County had significantly higher proportion of *A. flavus* S strain even after application of Aflasafe KE01 implying the need for continuous application of the biocontrol product to reduce the inoculum of toxigenic fungi.

The predominance of *A. flavus* in the soil could be due to presence of crop debris which act as their reservoir. These findings are in line with those of Jaime-Garcia and Cotty (2004) who reported that crop residues support survival and propagation of *A. flavus* in soil over relatively long periods. The proportion of *A. flavus* L strain was significantly higher than that of *A. flavus* S strain in maize sampled from treated fields in both Kathiani and Wote Sub Counties. The population of *A. flavus* S strain was predominant in maize sampled from untreated fields, which could be attributed to the higher population of *A. flavus* S strain in the soil in both Sub Counties. Horn (2003) showed that the higher the inocula of *A. flavus* in a field, the higher the risk of maize contamination with aflatoxin. According to a study by Abbas *et al.* (2009), *A. flavus* not only colonizes living plant tissue, but also grows as a saprophyte on plant debris in the soil. These residues serve as a reservoir for the fungus and under favourable conditions; it will resume growth and release new conidia that can be transmitted by air or insects to serve as new inoculum on host plants or debris in the field. *Aspergillus flavus* typically lives as a saprophyte in the soil depending on organic matter to propagate and survive (Probst *et al*., 2014).

The proportion of *A. flavus* L strain was significantly higher in maize sampled from fields treated with Aflasafe KE01 compared to samples from untreated fields. Application of Aflasafe KE01 in alternate seasons significantly increased the population of *A. flavus* L strain and significantly decreased the population of S strain of *A. flavus* and *A. parasiticus* in maize grain samples in the alternate season. Atoxigenic strains of *A. flavus* competitively displace toxigenic strains from colonizing the crop thereby excluding them (Cotty *et al*., 2007). These results compare favourably with the findings by Cotty and Antilla (2003) who reported a shift in the proportion of atoxigenic strain from 2 to 80% of *A. flavus* community in maize or in soil as a result of application of atoxigenic strains. The findings from this study also concurred with those reported in the United States which demonstrated that application of atoxigenic isolates of *A. flavus* resulted in domination of fungal communities by the applied atoxigenic isolates and greatly reduced the toxigenic strains (Cotty, 2006; Cotty *et al*., 2007). Dorner and Horn (2007) and Atehnkeng *et al.* (2014) reported that a mixture of atoxigenic strains of *A. flavus* have a competitive advantage and are more aggressive than toxigenic strains in peanuts and maize. Atoxigenic strains of *A. flavus* have been deployed commercially in USA to reduce aflatoxin contamination of crops through competitive displacement of aflatoxin producers (Cotty *et al*., 2008; Dorner, 2003).

In this study, predominance of *A. flavus* L strain in maize samples from treated fields could be due to application of Aflasafe KE01 which increased the population of L strains of *A. flavus* that competitively excluded aflatoxigenic fungi.

5.2 Survival of Aflasafe KE01 strains in soil in alternate seasons

Aflasafe KE01 genetic groups occur naturally in Kenyan soils which explain recovery of these VCGs in samples collected from untreated fields. Results from this study showed that Aflasafe KE01 genetic groups in soil were carried over to alternate season. This implies that Aflasafe KE01 vegetative groups are stable and can survive in soil in several seasons benefiting subsequent and rotational crops even when there is no application of the product. During unfavourable conditions (dessication and poor nutrition), *A. flavus* survive in form of sclerotia in soil and conidia/mycelia in crop debris (Yu *et al*., 2005; Wagacha and Muthomi, 2008). Factors that affect survival of *A. flavus* in soil include precipitation, soil temperature, intervals of drought and soil biota (Wicklow *et al*., 1993). Bandyopadhyay and Cotty (2013) reported that a single application of atoxigenic strains may benefit not only the treated crop but also rotation crops and second season crops that miss a treatment. Atehnkeng *et al*. (2014) also reported that due to greater stability and resilience of multiple atoxigenic VCGs over years, small scale farmers do not have to periodically apply the biopesticide. In addition, the effect of biocontrol strains has been reported to be carried over in storage and transportation even when the conditions are conducive for growth of atoxigenic *A. flavus* (Bandyopadhyay and Cotty, 2013; Atehnkeng *et al*., 2014).

5.3 Aflatoxin levels in maize grains and its correlation to the population of *Aspergillus* section *Flavi*

Maize samples from treated fields had lower (85%) aflatoxin level compared to maize from untreated fields. These results are in line with those of Atehnkeng *et al.* (2014) who found that application of atoxigenic strains led to significant reduction in the aflatoxin contamination in grain due to competitive exclusion of toxigenic strains by the atoxigenic strains in the soil and grains. Application of atoxigenic *A. flavus* to soil have been reported to substantially reduce aflatoxin concentration in harvested maize grains and peanuts (Dorner, 2004; Abbas *et al*., 2006; Dorner and Horn 2007; Atehnkeng *et al.*, 2008b). Reduction of aflatoxin levels in crops by atoxigenic strains involves both competitive exclusion during host tissue invasion and disruption of aflatoxin biosynthesis (Mehl and Cotty, 2010; Mehl and Cotty, 2011). Application of Aflasafe KE01 significantly reduced the population of toxigenic strains that led to reduction in aflatoxin contamination of the maize grains in Kathiani and Wote Sub Counties.

Reductions in average aflatoxin-producing potential result in reduced contamination of crops (Bhatnagar *et al.*, 2001). Doster *et al.* (2014), stated that atoxigenic strains (in AF36) has potential for greatly reducing the aflatoxin-producing potential of *A. flavus* populations throughout the vast regions within which almond and pistachio are commercially cultivated in California.

Maize samples from treated fields had significantly higher proportion of *A. flavus* L strain, which may be as a result of successful displacement of aflatoxin-producing fungi leading to lower aflatoxin level. The higher aflatoxin level in maize grains from untreated fields in both Kathiani and Wote Sub Counties may be due to predominance of *A. flavus* S strain which produces copious amount of aflatoxins. *Aspergillus flavus* S strain has been reported to be important producers of aflatoxin in several areas worldwide (Jaime-Garcia and Cotty, 2006; Probst *et al*., 2007). Probst *et al.* (2011), showed that high levels of aflatoxin recorded in the affected areas of Lower Eastern Kenya was due to the dorminance of *A. flavus* S strain and scarcity of *A. flavus* L strain in maize associated with the 2004 epidemic in Kenya. A study by Mehl and Cotty (2010) demonstrated that isolates of *A. flavus* that produce the most conidia dominate *A. flavus* communities and have proportional influences on contamination. Jaime-Garcia and Cotty (2004) showed that *A. flavus* S strain isolates produce very high levels of aflatoxins and numerous small sclerotia, both factors that might facilitate long-term survival. Crops associated with communities having high average aflatoxin producing potential are much more likely to become contaminated than crops associated with communities with low average aflatoxin producing potential (Probst *et al*., 2007).

A negative correlation was also observed between the population of *A. flavus* S strain and *A. flavus* L strain. This implies that as the population of *A. flavus* L strain increases, the population of *A. flavus* S strain decreases. This may be due competitive exclusion of the toxigenic *A. flavus* S strain by the applied atoxigenic *A. flavus* L strains that constitutes Aflasafe KE01.

Aflatoxin level in maize samples from Kathiani and Wote Sub Counties was positively correlated to the population of *A. flavus* S strain and negatively correlated with the population of *A. flavus* L strain. A significant percentage of *A. flavus* S strain consistently produce high concentrations of aflatoxin while a large proportion of *A. flavus* L strain isolates produce no aflatoxin (Dorner, 2004; Probst *et al*., 2010). The positive correlation implies that as the population of *A. flavus* S strain increases, the levels of aflatoxin increases.

Negative correlation implies that aflatoxin level decreases as the population of *A. flavus* L strain increases. These results are in agreement with those of Probst *et al.* (2007) who reported that there was strong positive correlation between percentage of the S strain in the infecting *A. flavus* community and the aflatoxin content in maize samples from endemic areas in Kenya. In cottonseed, maize and peanuts, population densities of toxigenic *A. flavus* and *A. parasiticus* have been found to be linearly related to aflatoxin concentration (Dorner and Horn, 2007; Probst *et al*., 2011).

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the findings of this study, the following conclusions are drawn:

Atoxigenic *A. flavus* (Aflasafe KE01) strains were efficacious in displacing aflatoxigenic fungal species in soil and maize.

Atoxigenic *A. flavus* (Aflasafe KE01) strains survived between cropping seasons, implying that alternate season application of Aflasafe KE01 can be exploited

Aflatoxin levels in maize samples from Aflasafe KE01 treated fields were lower by up to 85% compared to samples from untreated fields. This implies that Aflasafe KE01 is effective in reducing aflatoxin contamination in maize and is therefore a promising biocontrol product in managing aflatoxin contamination.

6.2 Recommendations

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

i. There is need for awareness raising to enlighten maize farmers on proper agronomic and storage practices in order to mitigate the challenge of aflatoxin contamination at the farm level.
ii. The government of Kenya should support and assist in deployment and up-scaling of Atoxigenic *A. flavus* (Aflasafe KE01) to different aflatoxin prone regions of the country.
iii. Efficacy of Atoxigenic *A. flavus* (Aflasafe KE01) on aflatoxin contamination of other key staples in Kenya should be evaluated.
iv. Treatment of large acreage under maize production with Atoxigenic *A. flavus* (Aflasafe KE01) in aflatoxin hotspots in Kenya should be explored.

6.3 Further research

Multi-seasonal studies should be conducted to determine the period of survival of Atoxigenic *A. flavus* (Aflasafe KE01) strains beyond the one year investigated in the current study. Studies on Efficacy of Atoxigenic *A. flavus* (Aflasafe KE01) strains in storage to be investigated. Efficacy of this biocontrol product in consecutive seasons to be studied.

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APPENDIX

Appendix I: Preparation of stock solutions

Rose Bengal

Five hundred milligram of rose Bengal (Sigma-Aldrich Company, St' Louis, USA) was weighed and transferred to a 100 ml volumetric flask and dissolved in 30 ml of 100% ethanol. The final volume was brought to 100ml with sterile distilled water and stored at ambient temperature (23 ± 2°C) (Garber *et al*., 2012).

Streptomycin and chloramphenicol

One gram of streptomycin sulphate (Duchefa Biochemie, Haarlem, Netherlands) was weighed and transferred to a 100 ml volumetric flask. The final volume was brought to 100 ml with sterile distilled water. The solution was filter sterilized and aliquot of 5 ml put into sterile 5 ml falcon tubes and stored in a freezer (-4°C). Five grams of chloramphenicol was 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 ± 2°C) (Garber *et al*., 2012).

Dichloran

Two hundred and fifty milligram of dichloran was dissolved in 50 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 ± 2°C) (Garber *et al*., 2012).

A&M micronutrients

Manganese sulphate (0.11 g) and 0.5 g $(NH_4)_6Mo7O_{24}*4H_2O$ was 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 $ZnSO_4*7H_2O$, 0.7 g $Na_2B_4O_7*10H_2O$ and 0.3 g $CuSO_4*5H_2O$ was added. Each of the above ingredients was completely dissolved before adding the next. The solution was transferred to 1 litre volumetric flask and the final volume brought to 1 litre with sterile distilled water. The resulting solution was put in aliquot of 5-10 ml in pre-sterilized screw cap tubes and stored in a freezer (-22 ºC) (Garber *et al*., 2012).

Appendix II: Annual average temperature (°C), precipitation (mm) and relative humidity (%) in Kathiani and Wote Sub Counties in 2013 and 2014

Sub County	Year	Min.temperature (°C)	Max.temperature (°C)	Precipitation (mm)	Min.relativehumidity (%)	Maximum relative humidity (%)
Kathiani	2013	17.22	27.43	352.6	46.92	93.14
Wote	2013	17.33	27.66	237.7	45.83	93.02
Kathiani	2014	16.73	27.49	223.2	45.64	92.99
Wote	2014	16.98	27.7	245.3	46.17	93.57

Source: http://me.awhere.com

Appendix III: Mean square table of factors affecting the proportion of members of *Aspergillus* section *Flavi* in maize grains

Source of variation	df	Mean squares		
		AFL	AFS	AP
Season	1	5609.9*	5143.7*	9.7^{ns}
Sub County	1	44.2^{ns}	784.5^{ns}	456.6^{ns}
Treatment	1	59547.3***	49892.4***	426.3^{ns}
Season*Sub County	1	849.3^{ns}	1056.1^{ns}	11.0^{ns}
Season*treatment	1	476.6^{ns}	290.2^{ns}	21.0^{ns}
Sub County*treatment	1	1289.1^{ns}	255.4^{ns}	396.4^{ns}
Season*Sub County*treatment	1	993.6^{ns}	715.7^{ns}	22.7^{ns}
Error	86	1008.6	1120.1	154.9
Total	93			

Appendix IV: Mean square table of factors affecting the proportion of members of *Aspergillus* section *Flavi* in soil during the 2014/2015 cropping season

Source of variation	df	Mean Squares			
		AFL	AFS	AP	AT
Sub County	1	1650.0^{*}	11046.8^{***}	4388.1^{***}	3.1^{ns}
Treatment	1	4604.0^{***}	2898.0^{*}	103.5^{ns}	14.6^{ns}
Sub County*Treatment	1	1457.3^{*}	156.4^{ns}	478.9^{ns}	13.6^{ns}
Error	43	246.9	445.5	187.9	22.8
Total	46				

Appendix V: Mean square table of factors affecting the recovery of Aflasafe KE01 from maize grains

Source of variation	df	Mean Square
Season	1	19038.7^{***}
Sub County	1	177.2^{ns}
Treatment	1	39362.9^{***}
Season*Sub County	1	95.7^{ns}
Season*treatment	1	715.9^{ns}
Sub County*treatment	1	23.8^{ns}
Season*Sub County*treatment	1	31.2^{ns}
Error	87	761.0
Total	95	