Fusarium Species and Fumonisin in atoxigenic *Aspergillus flavus* (Aflasafe KE01) Treated Maize Fields in Lower Eastern Kenya

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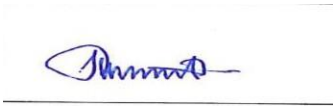
Signature:  Date: 1st August 2022

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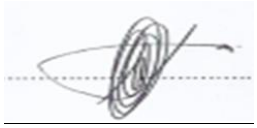
Signature  Date 1st August 2022

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DEDICATION

I would like to dedicate this thesis to my brother Gideon Maina Mbuthia and my mother Elizabeth Wanjiru Ndirangu, my Husband Bonface Muthuri Murianki for their support and encouragement during my studies and my daughters Zurielle Zawadi and Jenelle Kendi for inspiration to work harder.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
Bp	Base pairs
CFUs	Colony Forming Units
DNA	Deoxy Nucleic Acid
DON	Deoxynivalenol
ELEM	Leukoencephalomalacia
ELISA	Enzyme-Linked Immunosorbent Assay
EPZ	Export Processing Zone
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GDP	Gross Domestic Product
IITA	International Institute of Tropical Agriculture
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
MERCK	Medical Research Council, Kenya
MLN	Maize lethal necrosis
MOA	Ministry of Agriculture
MOH	Ministry of Health
PCNB	Pentachloronitrobenzene
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PES	Pulmonary oedema syndrome

Ppb	Parts per billion
SNA -	Synthetic Nutrient Agar

ABSTRACT

Contamination of crops with fumonisin leads to huge losses in cereals and negatively impacts on human health, animal productivity and international trade. Management of fumonisin contamination in maize has been a challenge since there are no effective management measures for *Fusarium* ear rots and lack of resistant maize varieties. This study was conducted with the objective of determining the efficacy of Aflasafe KE01 on the population of *Fusarium* species and fumonisin contamination of maize. The study was carried out in Wote, Kaiti, Kathiani and Nzambani sub counties of Makueni, Kitui and Machakos Counties, respectively. Twenty four maize fields were selected in each of the sub county where 12 fields were treated with Aflasafe KE01, while 12 fields comprised the untreated controls. The formulation comprised of four atoxigenic *Aspergillus flavus* on sterile sorghum seeds. Aflasafe KE01 was applied at a rate of 5 kg/ha and 10kg/ha by hand broadcasting in the maize fields two to three weeks before tussling of the maize. Soil samples were collected from each field prior to application of Aflasafe while maize grain samples were collected at harvest. *Fusarium* species were isolated from the ground maize and soil samples using pour plate method following serial dilution on low strength potato dextrose agar (PDA) and Spezieller Nahrstoffarmer Agar (SNA). *Fusarium* species growing were identified based on their cultural and morphological characteristics such as septation and sporophores, spore shape, pigmentation, mycelia color and colony pigmentation. Fumonisin in the maize samples was determined using Accuscan Pro-reader enzyme-linked immunosorbent assay (ELISA). The *Fusarium* species isolated from soil and maize samples at high frequency were *F. verticillioides, F. proliferatum, F. chlamydosporum, F. merismoides, F. semitectum* and *F. oxysporum. Fusarium verticillioides* was isolated at highest frequencies across the four sub counties. Maize samples from Aflasafe KE01 treated maize fields had significantly lower

population of the *Fusarium* species compared to untreated maize fields across all the four sub counties. The mean incidence of *F. verticillioides* was 43% and 40.9% in the maize grains sampled from fields treated with Aflasafe KE01 at the rate 5 kg/ha and 10kg/ha, respectively; while the control fields corresponding to the 5 kg/ha and 10kg/ha application rates had mean incidences of 63% and 54.8%, respectively. There was significant reduction of up to 68.7% in the fumonisin levels in maize samples from Aflasafe KE01 treated compared to the untreated maize fields. The population of *Fusarium* species and fumonisin levels were significantly different among the study areas following Aflasafe KE01 application. About 62.5% of the maize fields treated with 5kg/ha of Aflasafe KE01 met the European commission regulatory threshold of ≤2ppmfor total fumonisin as compared to about 45% from the control fields. The results showed that application of Aflasafe KE01 effectively displaced the toxigenic *Fusarium* species in maize resulting in reduction of fumonisin in the maize from the Aflasafe KE01 treated fields by up to 68% compared to samples from untreated fields. This indicates that Aflasafe KE01 is a potential biopesticide for the biocontrol of *Fusarium* species and fumonisin production in maize. Efficacy of Aflasafe KE01 on reducing fumonisin contamination of other key staples in Kenya should be evaluated. Key words: *Fusarium* species, fumonisins, Aflasafe KE01, maize, atoxigenic *Aspergillus* sp.

Key Words: Aflasafe, atoxigenic, Fumonisin, Fusarium, maize

CHAPTER ONE: INTRODUCTION

1.1 Background information

Maize (*Zea mays* L.) is a key crop to majority of Kenyan population and contributes up to 80% of the total cereals consumed (Kimanya *et al.*, 2008; FAO, 2000). It is grown on a wide agro-ecological zone with varying temperature and rainfall (Ureta *et al.*, 2013). In Kenya, maize is produced for both subsistence and commercial use where 30% is grown by large-scale farmers and 70% by smallholders (Guantai and Seward, 2010). Over 90% of the Kenyan populations depend on maize for food (Kirimi *et al.*, 2011; USDA, 2016). The per capita intake rate ranges from 98 to 100 kilograms converted to 2700 thousand metric tonnes per year (FAO, 2011; Olwande *et al.*, 2015).

In Kenya, the total area under maize production is about 1.4 million hectares and annually the production capacity averages 28 million tonnes (Government of Kenya, 2010; FAO, 2011). Over 38% of farmers in Kenya grow maize (FAO, 2016) and of this, small scale farmers produce about 70% of the overall production, however, they retain up to about 58% of their total production for household consumption (Olwande *et al.*, 2015). Large scale production by commercial producers is at 30% of total production (Guantai and Seward, 2010). In Kenya, maize is extensively grown in Uasin Gishu, Trans-Nzoia, Bungoma and Nakuru Counties. Small scale farmers' practice mixed cropping with subsistence crops like beans, potatoes and bananas. This method is common in the southern part of Nyanza, some parts of western and Rift Valley regions. Hybrid maize seeds supplied by seed companies give high yields compared to seeds sourced locally or from neighbours. The Kenya Agriculture and Livestock Research

Organization (KALRO) has developed a Katumani hybrid adapted to dry regions of Machakos, Kitui, Tana River and Isiolo counties (KALRO, 2008).

Maize is prone to degradation by mycotoxigenic fungi such as *Aspergillus* species, *Penicillium* species and *Fusarium* species which are ubiquitous in soils (Fandohan *et al.*, 2003; Kumar *et al.*, 2016; Koskei *et al.*, 2020). Mycotoxins are produced by fungi in products such as maize, wheat, groundnuts that are susceptible to mould infection (Wagacha and Muthomi, 2008; Bennett and Klich, 2003; Cinar and Onbasi, 2019). Aflatoxins, ochratoxins, deoxynivalenol, zearalenone, fumonisin, and T-2 toxins are some of the common mycotoxins (Tola and Kebede, 2016; Misihairabgwi *et al.*, 2019). Fumonisin produced by *F. proliferatum* and *F. verticillioides* is a main contaminant of maize-based food and feeds (Kamle *et al.*, 2019).

Some of this mycotoxin can be controlled by biological method that uses competitive exclusion. The science of biological control employs the principal of competitive exclusion in control of mycotoxin. This is made possible by the presence of *A. flavus* populations where some are toxigenic and others atoxigenic strains. This is achieved by identifying and successfully introducing harmless atoxigenic strains that show competitive advantage over the toxigenic strains. The atoxigenic strains of *A. flavus* effectively eradicate their highly toxigenic relatives and thereby lessen aflatoxin contamination (Agbetiameh *et al.*, 2019; Senghor *et al.*, 2019). Application of Aflasafe KE01, 2-3 weeks to tussling helps change the toxigenic *Aspergillus* strain profile to atoxigenic *Aspergillus* strains (IITA, 2009). The atoxigenic strains of *Aspergillus flavus* sporulate on maize grains that are their source of food and carrier material. These strains can produce spores for a period of three weeks after application and take control of the crop residues in the soil replacing the toxin producing strains. The spores produced are wind propelled and moved by vectors from soil surface to silk on maize ears dislodging the toxin producing

strains (IITA, 2009). Single application of Aflasafe is effective for up to 3 years and in several crops that will be planted on the same plot. Protection by Aflasafe is carried over from field to store thus protecting maize grains. This reduces aflatoxin contamination by 60-95% (IITA, 2009).

1.2 Problem statement

Maize is grown in areas with environmental conditions that are ideal for growth of most cereal pathogens such as *Fusarium* fungi most of which grow in temperatures around 25–35^{o}C and high humidity (Aldars-garcía *et al*., 2018). Mycotoxins produced by fungus has an effect to human and animals when they are consume in contaminated food or animal feed (Winter, 2019). Cereals stored in conditions with extreme temperatures and humidity can often have decay and be contaminated by mycotoxins (Bennett, 1987). The risk associated with mycotoxins increases with farming practices such as insufficient drying, poor storage practices and handling, and transport conditions (Marin, 2013).

Maize and product from maize are mostly infested with fumonisin. Fumonisin B, FB1, FB2, and FB3 which are the most abundant with FB1, FB2, and FB3 forms as the main food contaminants which cause major influences on health (Dall'Asta, 2016; Cendoya, 2018). Various fungal species produce mycotoxins which can infect maize (Zain, 2011). Fumonisins can affect produce before harvesting, at harvesting, during drying, storage and transportation (Rodrigues *et al.*, 2012; Fountain, 2015; Alshannaq, 2017; Welke, 2019; Varzakas, 2016).

These fungi cause both qualitative and quantitative losses on cereals. The qualitative effect may have an impact on health, livestock productivity and both local and global trade (Marin, 2013). Mycotoxin also causes mycotoxicosis disease such as ergotism, alimentarytoxic aleukia, aflatoxicosis which may either be critical or prolonged (Tola, 2016) and effects different human

body organs and can cause death (Winter, 2019; Ostry, 2017), cancer, endocrine disorders (Ben Taheu *et al.,* 2019). Human beings get exposed to mycotoxins through contact, absorption, and breathing (Pittet, 1998). Worldwide, mycotoxin contamination causes quantitative loses as the estimated value of maize, wheat and peanuts averaging $932 million annually (Benkerroum, 2020).

The climatic conditions in lower Eastern region of Kenya are ideal for growth of most pathogens. *Fusarium verticillioides* and *Moniliforme* have been identified in Kenya with predominance in Makueni Nandi, and western Kenya (Kedera *et al.,* 1999; Kang'ethe *et al.,* 2017a). These regions also record high incidence of *Aspergillus, Fusarium* and *Penicillium* species resulting in mycotoxins production such as aflatoxins, fumonisin and Zearalenone (Kilonzi *et al.,* 2014). A timely fungicide application reduces infection and mycotoxin contamination. However, there is lack of effective fungicide to control fumonisin producing *Fusarium*. Additonally, application of fungicides to control fungi in maize has been regulated due because they are carcinogenic, have severe residual toxicity, long degradation period and pollute the environment. This has created a great need to focus on methods for the control of mycotoxins such as using resistant varieties, harvest management and post-harvest methods. However, there is lack of maize genotypes that are resistant /tolerant to fumonisin contamination.

1.3 Justification

Generally, mycotoxin contamination is a major problem in maize and its products especially in Machakos, Makueni and Kitui with conducive environmental conditions over the years thus becoming a major concern (Mutiga *et al.,* 2014). According to a survey done in Eastern and western Kenya, *Fusarium* species are common contaminants of maize in these areas (Koskei *et al.,* 2020). This has necessitated the need to identify appropriate methods of controlling

mycotoxin in maize and other crops especially in these regions. To effectively prevent or minimize future mycotoxin contamination and reduce long term exposure to mycotoxin such as aflatoxins, proper methods of controlling and reducing mycotoxin contamination are required. This has led to the need for research of alternative ways of mycotoxin management. Some of this mycotoxin can be controlled by a biological method that uses competitive exclusion. The science of biological control employs the principal of competitive exclusion in control of mycotoxin. This is made possible by the presence of *A. flavus* populations where some are toxigenic and others atoxigenic strains. The atoxigenic strains of *A. flavus* can effectively eradicate their highly toxigenic relatives and thereby reduce aflatoxin contamination.

1.4 Objectives

The broad objective of the study was to contribute to better management of *Fusarium* species and fumonisin contamination in maize through assessing effectiveness of atoxigenic *Aspergillus flavus* on the *Fusarium* species and fumonisin.

The specific objectives were:

i. To determine the population of *Fusarium* species in maize grains and soil from atoxigenic *Aspergillus flavus* treated and untreated maize fields.
ii. To determine efficacy of atoxigenic *Aspergillus flavus* application in reducing fumonisin contamination of maize grains at harvest.

1.5 Hypotheses

i. The population of *Fusarium* species is lower in maize grains and soil from in atoxigenic *Aspergillus flavus* untreated than treated maize fields.
ii. Fumonisin contamination is lower in maize harvested from in atoxigenic *Aspergillus flavus* (Aflasafe KE01) untreated field than the treated fields

CHAPTER TWO: LITERATURE REVIEW

2.1 Maize production in Kenya and in Lower Eastern Kenya

Maize is extensively grown and is used by thousands of people worldwide because of its high production capacity per ha and ability to grow in wide agro-ecological zone (Shiferaw *et al.*, 2011; Jaidka, *et al.*, 2019). Warm temperatures above 15°C and high precipitation of about 1,200 mm - 2,500 mm favor maize farming (Jaidka *et al.*, 2019). Maize can also be grown in areas recieving rainfall below 380 mm. Rich, well drained light loam soils are the most favorable soil for maize growth. However, maize in Kenya can be grown in a wide range of soils in many parts of the country (Ouma *et al.*, 2013). Large scale maize production with mechanization is done in the counties of Trans Nzoia and Uasin Gishu (Jaetzold *et al.*, 2009).

Lower Eastern regions of Kenya such as Kitui, Machakos and Makueni Counties are semi-arid and the land is majorly used for cultivation of food crops and livestock rearing (Omoyo *et al.*, 2015). Due to the low rainfall in the regions, the government of Kenya with the ministry of agriculture investigated on the appropriate maize hybrid varieties for these marginalized areas (Pixley and Bänziger, 2004; Pixley, 2006). Despite this effort, farmers are not ready for the bumper harvests and often maize go bad due to mycotoxin contamination even before it has been stored. In Kenya, maize is cultivated on an average area of 1.5 million hectares, with a productivity of about 1 ton ha^{-1}. The amount produced is less than the potential productivity of 6-8 ton ha^{-1} (Kiboi *et al.*, 2019).

2.2 Importance of maize

Maize *(Zea mays* L.) is used as human food, medicine, feed for animals, and raw material for industry (Galen and Larry, 2013). Majority of the developing countries utilize 50 to 55% of their

maize produce as food while industrialized countries use maize majorly as raw material for industrial products and livestock feed (IITA, 2001, Kumar *et al.,* 2007). For increased production of products such as meat, milk and eggs, maize has been used as feedstuff and fodder (Dei, 2017; Kiptot *et al.,* 2015). Nutritionally maize contains carbohydrate, protein, fiber and mineral iron and vitamin B thus making it a nutritious crop fit for animal and human consumption (IITA, 2001).

Other uses of maize include fuel, medicines, compost and unripe ears can be consumed as a vegetable in form of baby corn (Alakonya *et al.,* 2008; Abdulrahaman and Kolawole, 2008). Levulinc acid from maize is used as ingredient in antifreeze and that can be used in place of toxic petroleum (Julian *et al.,* 2012; Chen *et al.,* 2018). The stigma from female maize flower and silk are used as herbal supplements while ethanol is used as a biomass fuel. (Hasanudin *et al.,* 2012). Maize straw can also be used as source of energy

2.3 Constraints to maize production in Kenya

Kenya is one of the leading maize producers and consumers in both eastern and southern Africa region (Kornher, 2018). However, maize production in Kenya is majorly affected by biotic and abiotic factors such as drought, extreme temperatures, low soil fertility, land degradation, high soil acidity, flooding and salinity (Mucheru-Muna *et al.,* 2010). Inadequate storage facilities and poor storage conditions in Rift valley and lower Eastern Kenya have led to losses amounting to 5-10% of the total production loss as a result of mycotoxin contamination which is influenced by weather (Koskei *et al.,* 2020). Maize crops grown in warm climates are affected by aflatoxin and infection mainly occurs when the temperatures rises concomitantly with drought (James and Zikankuba, 2018).

The main storage pests include maize weevil (*Sitophilus zea mais* Motsch) and larger grain borer (*Prostephanus truncatus* Horn). The storage pests have been implicated in causing up to 90% of yield losses (Likhayo *et al.,* 2004). Annually estimated losses due to post harvest pests are estimated at 1.8 million bags of maize. Chemical and biological control of these pests have had little impact in eradicating storage losses (Midega *et al.*, 2016).

There is a projected sharp increase in demand for maize grain globally by 2023 (International Grain Council, 2019). However, there has been reduced production of maize in the recent years with the crops yield going far below the annual requirement of about 39 million bags (Kibaara, 2005;). Kenya average yield is 2 t/ha which is less than the estimated possible yield of 6 t/ha which has been attributed to poor weather, low yielding maize varieties and poor modern production technology (Kiboi *et al.*, 2019). The deficits in maize production are met through imports (Republic of Kenya, 2008).

Ear rot disease which is a major cause of decline of Kenya's maize production and its quality is caused by *Fusarium* species and factors like rainfall, low soil fertility and arthropod pests is a contributes to its occurrence (Pfordt *et al.*, 2020). The disease invades more than half of maize grains before harvest and enters the food chain in cereal crops (Wu *et al.*, 2014). Other major diseases of maize in the country that causes significant lose include leaf blight and rust grey leaf spot, maize smut (Kinyua, 2003) and *Maize streak virus* and the maize lethal necrosis (MLN) (MOA, 2012; Wangai, 2012). Fumonisin which was first discovered in 1988 and is a common contaminant of maize-based food and feeds through discoloration and reduced nutritional value due to mycotoxin contamination (Omotayo *et al.*, 2019).

2.4 *Fusarium* species affecting maize

Various diseases negatively affect the yield of maize, by affecting the leaves, stalks and ears. The diseases are caused by various pathogenic fungi. The *Fusarium* species for example causes the ear rots that result in poor grain quality and mycotoxins contamination (Marin *et al.*, 2013). This pathogen contaminates cereals, oil seeds, spices and tree nuts (Schisler *et al.*, 2002; Marasas *et al.*, 2010). Studies show that *F. proliferatum, F. verticillioides, F. oxysporum* and *F. graminearum* as the major phytopathogenic *Fusarium* species associated with maize crops (Lane *et al.*, 2018; Balendres *et al.*, 2019). *Fusarium verticillioides, F. proliferatum, F. graminearum* and *F. anthophilum* are the most common species contaminating maize (Czembor *et al.*, 2015; Ahangarkan *et al.*, 2014). Species in the *Gibberella fujikuroi* species complex like *F.verticillioides, F. proliferatum* and *F. subglutinans* primarily cause stalk and ear rot diseases in maize (Janse van Rensburg *et al.*, 2015; Chilaka *et al.*, 2017). Worldwide, *Fusarium verticillioides* is commonly isolated from diseased maize and maize-based commodities (Shephard, 2006; Bii *et al.*, 2012; Castanares *et al.*, 2019; Guo *et al.*, 2014). Fewer isolated species are *F. solani* and *F. oxysporum* (Tsehaye *et al.*, 2016).

Fusarium verticillioides is an endophytic fungus which forms a long long-term relationship with maize plants as they grow (Glen and Yates, 2008; Oldenburg *et al.*, 2017) and causes symptoms such as severe rotting, wilting and chlorosis and retarded growth of plants grown in *F. verticillioides*-infested soil (Robertson *et al.* 2006; Gai *et al.*, 2018). Infection occurs through the silk or injuries, or infected seeds causing grain rot (Gai *et al.*, 2018).

2.5 Fusarium mycotoxins contaminating maize

Infections by *Fusarium* species incite an imperfect establishment of the crop and, subsequently, lower yields. However, the most important concern is the contamination of grains with

mycotoxins. In Kenya, maize grains have been infected by *F. verticillioides*, and *F. subglutinans*, which produces deoxynivalenol, and nivalenol, zearalenone, fumonisins and moniliformin. The most common fumonisin is Fumonisin B which comprises of approximately 75% total fumonisin content (Braun and Wink, 2018). Zearalenone is produced by *Fusarium graminearum, F. semitectum, F. equiseti, F. crookwellense and F. culmorum* (Beukes *et al.*, 2017) while zearalenone is a secondary metabolite and a non-steroidal estrogenic mycotoxin (Awad *et al.*, 2013). *Fusarium tricinctum, F. moniliforme, F. oxysporum, F. sporotriochioides* and *F. laterium* may similarly produce zearalenone (Ismaiel and Papenbrock, 2015; Tralamazza *et al.*, 2016). *Fusarium verticillioides* produces more than 17900 µg/g of fumonisin B1 whereas 31000 µg/g fumonisin B1 is produced by *F. proliferatum* cultures *(*Rheeder *et al.*, 2002). In the field, fumonisin contamination follows seed to cob and furthest to grain by the systemic movement in stalk. Fumonisin is spread by air where conidia are blown to silk (Ako *et al.*, 2003; Munkvold, 2003). Fumonisin also causes plant diseases such as maize ear rot and pink ear rot (Parsons and Munkvold, 2012).

2.6 Factors influencing mycotoxin contamination of maize

Environmental conditions, insect infestation, pre – post harvest handling of maize affects its infection with *Fusarium* species and contamination by fumonisins. The growth of the fungus and and its subsequent production of mycotoxin is affected by factors including rainfall during cultivation, high humidity and temperature, poor timing of harvest, inappropriare drying and poor storage. Climatic factors such as temperature and humidity are factors that impact the development of *Fusarium* diseases in cereals (Brennan *et al.*, 2003). Temperature, humidity and rainfall change just towards harvesting and at harvesting enhance development of *Fusarium* diseases and fumonisin in maize (Boutigny *et al.*, 2012; Ediage *et al.*, 2015; Hove *et al.*, 2016;

Czembor *et al.*, 2015). The interaction between water and temperature influences germination, growth, sporulation of the fungus and mycotoxin production (Sanchis & Magan, 2004). Dry weather and fluctuations in wet and dry cycles affects growth of mycotoxigenic fungi resulting in high mycotoxin level. Raw maize damaged by insects and stored under improper conditions shows high levels of fumonisins (Kamala *et al.*, 2016; Leggieri *et al.*, 2020). Agronomic and storage practices such as variety selection, storage, and processing also affect level of contamination (Zhang *et al.*, 2014). Mycotoxin contamination can be reduced by application of good agricultural practices in the entire maize value chain such as production, harvesting, storage, transportation, marketing and processing (Blandino *et al.*, 2009). Timely harvesting, rapid drying to low moisture level creates unfavorable condition for fungal growth and proliferation reduce fungal infection in the field and in the stores (Walker *et al.*, 2018; Monda and Alakonya, 2016). When the kernels are not dried adequately in the field, mechanical drying using machine or directly by the sun is used to attain the 13% moisture levels to stop the growth of mould and subsequent contamination by mycotoxin during storage (Blandino et al., 2009).

2.7 Effects of fumonisin on human and animal health

There is a lot of concern from mycotoxins due to their risk as food contamiants and human health. Fumonisins occur globally, majorly in maize crops (Wei et al., 2013). The fumonisins become toxic by obstructing the sphingolipid biosynthesis. High-level exposure of fumonisin occurs through ingestion, inhalation and absorption through the skin and may result in instant death, human oesophageal cancer and nervous disorders (Wagacha and Muthomi, 2008; Queiroz *et al.*, 2012; Berthiller, *et al.*, 2013; KEPHIS, 2006; Gong *et al.*, 2016). For human consumption of maize flour, Food and Drug Administration (FDA) guide industries by ensuring fumonisin concentrations is below the harmful levels of between 2 and 4mg/ ppm (CFSAN, 2001a, 2001b).

Fumonisin promotes diseases with fumonisin B1 and fumonisin B2 being the most poisonous mycotoxin to humans. It causes diseases such as human esophageal cancer, liver cancer, gastrointestinal cancer, leukoencephalomalacia, edema, rat neural tube defects and cardiovascular problems (Wild and Gong, 2010; Kimanya, 2015; Ostry *et al.*, 2017). The health effects of mycotoxin contamination to human include skin irritation, gastrointestinal disorders, trachycardia, oedema and haemorrhages (Mathison, 2013).

2.8 Management of mycotoxins in maize

It is difficult to determine mycotoxin contaminated maize since it appears just like the normal grain without outward physical signs of fungal infection. Mycotoxin in maize cannot be destroyed by heat during convectional food processing because they are resistant to heat (Betran and Isakeit, 2003; CAST, 2003; Murphy *et al.*, 2006). Therefore, other methods of mycotoxin are required.

2.8.1 Chemical control

Chemical control has been widely used in control of most diseases on crops. Chemical efficacies vary in control of pathogenic *Fusarium* species on maize and other crops. The pesticide is used to protect maize grains against *Fusarium*, and these include thiram, captab and fludioxonil or mefenoxam (Blandino *et al.*, 2009). Fungicides has been used in the field for mycotoxin control, nevertheless they are associated with stress reaction within the plant that may increase mycotoxin contamination. Chemicals have the ability to manage mycotoxins. A study by Medina et al (2007) showed that natamycin produced by *Streptomyces natalensis* has a powerful action at low concentrations against *A. carbonarius*.

2.8.2 Physical and cultural method

A combination of these two methods in addition to chemical control may help in management of soil health. Practicing sanitation, intercropping, mulching, addition of organic manure, planting disease-free material, extermination of diseased plants, utilization of resistant cultivars, can be used to manage mycotoxins in maize (Hell and Mutegi, 2011; Levkor and Var, 2017). During pre-harvest, modify the crop growth conditions in order to avoid infections. Modifications can be done through fertilizer application, rotation, reducing plant population, early planting, timely irrigation and scouting (Baliukoniene *et al.*, 2011; Baliukoniene *et al.*, 2011; Abbas *et al.*, 2012). Drying of maize grains is important when the kernels are inadequately dried in-field. Therefore mechanical drying using machine or directly by the sun should be used to attain the low moisture content that will hinder growth of moulds and later mycotoxin contamination in storage (Blandino *et al*., 2009). Milling and dehulling of the maize grains is also effective in the reduction of mycotoxin contamination (Pietri *et al*., 2009).

Moisture and temperature accompanied by drying are considered the most vital aspects for controlling fungal growth. Proper irrigation and pesticide application to crops prevents plant stress and damage by insects respectively, is an important pre-harvest control strategy. Control of temperature of stored grain is crucial to prevent fungal growth. Once matured, maize should be allowed to dry in the field to attain the right moisture content. Additionally, timely planting has been showen to be a good approach towards minimizing the risks of contamination by mycotoxins (Blandino *et al*., 2009).

2.8.3 Good agricultural practice

Mycotoxin contamination can be reduced by application of good agricultural practices from production to processing (Blandino *et al.*, 2009). Practices such as timely harvesting and drying

of agriculture produce to low moisture level creates unfavorable condition for fungal growth and proliferation reduce fungal infection in the field and in the stores (Walker *et al.*, 2018; Monda and Alakonya, 2016).).

Insect management can also be used as a means of reducing mycotoxin contamination. Insect damage increase the levels of mycotoxin contamination during transport, where infected maize spores infect the clean ones. Insects also create wounds through feeding (Chulze, 2010). The increased moisture content via condensing of moisture from respiration and low temperatures also increases mycotoxin contamination. (Suleiman *et al.*, 2013; Zunino *et al.*, 2015; Lane and Woloshuk, 2017). Physical treatment through traditional processing such as winnowing, sorting, crushing, washing and de-hulling of maize grains is effective in minimizing mycotoxin (Fandohan *et al.*, 2005; Wagacha and Muthomi, 2008). Proper sanitation and cleaning the stores before loading with new produce help reduce mycotoxin contamination levels (Armarcius and Zikankuba, 2018).

2.8.4 Biological control

Biological control employs use of beneficial micro-organisms to controls mycotoxins contamination (Atehnkeng *et al.*, 2016). These microorganisms reduce or suppress toxins to no or levels that do not affect the consumers. Another method of mycotoxin control could be interactions among fungi for instance an interaction between fumonisin producers *F. verticillioides* and *F. proliferatum*, and DON producer *F. gramenearum*. The presence *F. gramineanum*, significantly reduces the population of *F. verticillioides* and *F. proliferatum* and at the same time reduce the production of fumonisin Bl (FBI) (Lauren *et al.*, 2004). This method uses competitive exclusion which is made possible by the presence of *A. flavus* populations where some are toxigenic and others atoxigenic strains. Application of non-toxigenic inoculum

in the soil ensures competition for infestation sites on the plant with toxigenic strains. This is achieved by identifying and successfully introducing harmless atoxigenic strains that show competitive gain over the toxigenic strains. The atoxigenic strains of *A. flavus* virtually eliminate their highly toxigenic relatives and as a result minimize mycotoxin contamination (Agbetiameh *et al.*, 2019; Senghor *et al.*, 2019). Application of such products 2-3 weeks to tussling helps shifts the *Aspergillus* strains profile from toxigenic to atoxigenic strains (IITA, 2009). Within 3-5 days post-application, the atoxigenic strains sporulate on the sorghum grains which act as their substrate and carrier material. The atoxigenic strains produce spores for up to three weeks on field after applications which colonize the organic matter and plant residues in place of the toxin producing strains. Atoxigenic strains produce spores that are blown by air to silk on maize ears displacing the toxin producing strains (IITA, 2009). For a period of three years, one application of Aflasafe is effective in several crops that will be planted on the same plot. Protection by Aflasafe is carried over from field to store thus protecting maize grains. This reduces aflatoxin contamination by 60-95% (IITA, 2009).

IITA isolated and identified four atoxigenic strains of *A. flavus* in locally grown maize, that have been employed to formulate Aflasafe KE01. This product has been approved and registered in Kenya and is being disseminated to local maize farmers. The atoxigenic strain ensures that there is no exchange of genetic material with the toxigenic isolates that can generate progenies producing mycotoxins (Cotty 2006). The atoxigenic isolates inhibits the development and growth of the toxigenic fungal species.

2.8 Methods of fumonisin analysis

Enzyme linked immunosorbent assay (ELISA) has been widely used to analyse for fumonisins (Zheng *et al.,* 2006; Krska *et al.,* 2007), mostly performed in a 96-well plate, that can allow

simultaneous analysis of many samples (Singh and Mehta, 2020) and incubated for about 0.5 - 2 hours and the developed color is usually measured spectrophotometrically (Schneider *et al.,* 2004). Other approaches have also been developed, for example the flow-injection or flow-through immunoassays having multi-well microtitre plate used in ELISA with electrochemical or spectrophotometric detection (Piermarini *et at.,* 2009). They use immobilized proteins to separate antigen 19 antibody complexes and detect only the free marker (Zheng *et al,* 2006). Mycotoxin tagged liposomes have been prepared to develop a flow-injection liposome immunoanalysis (FILIA). Also, various immunostrips or immunodipsticks, have been developed for mycotoxin testing in the field, using either membrane with immobilized antibodies or dry reagents on the test strip (Schneider *et al.,* 2004; Zheng *et al,* 2006; Prieto-Simon *et al,* 2007).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Determination of population of *Fusarium* species in soil and maize grains in atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

3.1.1 Description of the study area

The study was conducted in Kitui County in Nzambani Sub County, Machakos County in Kathiani Sub County and Makueni County in Wote and Kaiti Sub Counties in the lower Eastern parts of Kenya. The regions lie in the latitude between 4^{o}N to 4^{o}S, longitude 34^{o} to 41^{o}E. These regions receive an average rainfall of between 150 mm to 650 mm p.a. Machakos County receives average annual rainfall of 500 to 700mm per year. Kitui County receives an average rainfall of between 500 and 1050 millimeters per annum which is not evenly distributed in the year. The soils in these areas are sandy to loamy sand texture with low organic matter contents, low water retaining capacity and low plant nutrients thus making is susceptible to erosion (Sombroek *et al*, 1982; Keating *et al*, 1992; Gachimbi *et al.*, 2007). Makueni County has several agro-ecological zones (AEZs) with altitudes ranging from 790 - 1770mabove the sea level and receives about 600-1050mm of average annual rainfall (Karanja, 2006; Jaetzold *et al.*, 2010).

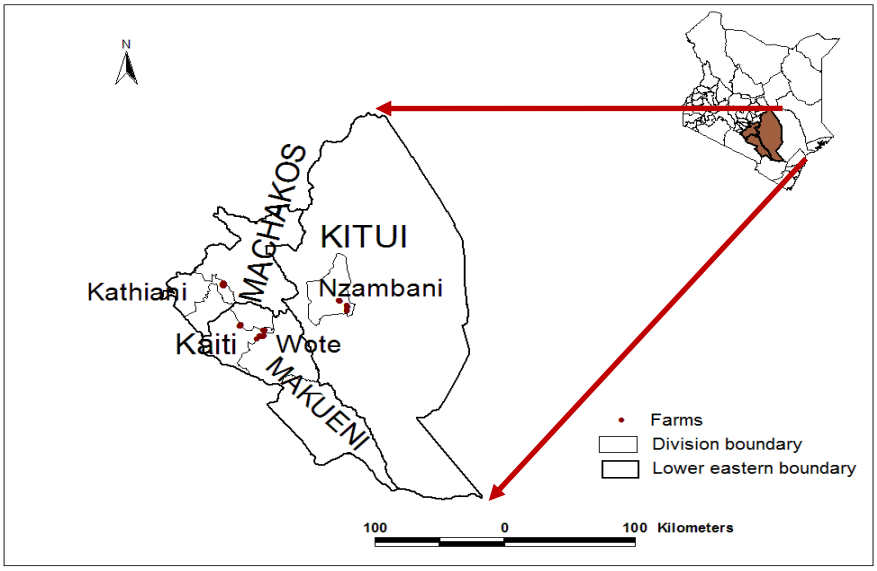

Figure 1: Map showing the field trials sites.

3.1.2 Experimental design and application of atoxigenic *Aspergillus flavus* (Aflasafe KE01)

Selection of the study sites was purposive and based on areas where maize is commonly cultivated, have a history of aflatoxin contamination and have low risk of maize crop failure. This information was obtained from the Ministry of Agriculture and published research works (Muthomi et al., 2009). The farms were selected randomly within each sub county with each farm having a minimum of 2 acres except where it was not possible to get such farms in a particular sub county.

The experiment was conducted using the maize planted by the farmers willing to take part in Each of the four sub counties had 24 maize fields where 12 fields were treated with Aflasafe KE01 while the other 12 were control fields. Within each area control fields were a minimum of 100m from the treated fields. Aflasafe KE01 was obtained from the International Institute of Tropical Agriculture (IITA). Six of the individual farmers' farms were treated with Aflasafe KE01 at an application rate of 5 kg/Ha while the other six was treated with 10 kg/Ha. Aflasafe

KE01 was broadcast by hand in the selected fields 2-3 weeks prior to tussling of maize. The experiment was carried out in one maize cropping season across the four sub counties. The data collected from the experiment were the population of Fusarium species in the soil and grain samples; and the fumonisin levels in the maize grains.

3.1.3 Collection of soil and maize cob samples

Soil samples and the maize cobs were sampled from the maize fields to determine the incidence of *Fusarium* species in the soil. Additionally, the maize cob sampled was used to quantify the amount of fumonism present in the grains. Samples of soil were collected from the farms to a maximum depth of 2cm using a spoon 2 to 3 weeks before flowering of maize just before application of Aflasafe KE01 and from the control fields. The spoon was surface sterilized with 70% ethanol before using it in another field. During sampling, the boarder rows (5 meters) of every maize field were avoided. Soil samples were then taken at an interval of 5-10 meters in a zigzag manner from the various locations in the field and then mixed to form a composite sample from which approximately 0.5kg sample was taken. Maize cobs were sampled at harvest ensuring a minimum of eight cobs from each farm. The cobs were picked from the farm using a zigzag approach. The bags were properly labeled with the farmer details and sampling date and closed firmly. The samples were then transported in a cool box to the laboratory for analysis with 48 hours after collection.

3.1.4 Handling of soil and maize samples

Soil lumps were crushed gently using a hammer and plant debris removed by hand. The soil was then sieved through sieve number 20, standard testing sieves (0.833mm opening) and air dried at 23 ± 3°C. The maize cobs were sun dried avoiding contact with the ground. Thereafter the dry maize was manually shelled by hand and dried in an oven at 45°C for two days and then crushed

to a fine powder with a Bunn coffee mill grinder (Bunn omatic Corporation, Spring Field Illinois, USA). The ground maize sample was thoroughly mixed and separated into two samples which were used for isolation and identification of mycotoxigenic fungal species while the other was for fumonisin analysis. The samples were stored in the refrigerator at 4 $^{\circ}$C.

3.2 Isolation of *Fusarium* species from soil and maize kernels

Species of *Fusarium* were isolated from the ground maize and soil samples using serial dilution method and plating on low strength potato dextrose agar (PDA) amended with mineral salts and antimicrobial agents (Muthomi, 2001). Low strength potato dextrose agar was prepared by weighing PDA (17g) in a litre of sterile distilled water. The media was adjusted by adding mineral salts in proportions of 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4$ and 0.5g KCl plus antibiotics (0.05g chloramphenical, 50mg tetracycline, 50mg penicillin and 50 mg streptomycin) to suppress growth of fast-growing fungi and bacteria (Muthomi, 2001). The media was autoclaved at 120℃ at a pressure of 15 p.s.i in an autoclave for 20 minutes and cooled to 45℃. After cooling, 50mg of antibiotics penicillin, tetracycline, and streptomycin was added per liter of the medium and mixed thoroughly. Approximately 20ml of the molten PDA was dispensed in 9 cm Petri dishes and allowed to solidify overnight. Full strength potato dextrose agar was prepared by weighing 39g PDA in a litre of sterile distilled water and autoclaved at 120℃ at a pressure of 15 p.s.i.

One gram of each ground maize and soil samples was weighed and put into 10 ml of sterile distilled water in a 40 ml glass vial and vortexed for three minues at 1750 rpm on a vortex mixer (Velp Scientifica, Europe). One milliliter of the mixture was drawn using a micropipette and mixed with 9ml to form the first dilution of 10^{-1}. One milliliter of the first dilution was measured and dissolved in 9ml of sterile distilled water to form the second dilution of 10^{-2}. This was done

up to the third dilution of 10^{-3}. One milliliter aliquot of the second and third dilutions (10^{-2} and 10^{-3}) of soil and ground maize samples was plated on the low strength PDA in triplicate. The plates were incubated at room temperature (23 ± 3°C) for 5-7 days during which the soil and maize samples showing growth of a particular fungus was recorded and different fungal colonies recorded. *Fusarium* isolates from low strength PDA were sub-cultured on PDA and incubated at 25℃ for 14 days. *Fusarium* species growing on PDA were identified based on their cultural characteristics.

3.3 Identification of *Fusarium* species

Different *Fusarium* species growing on low strength PDA was identified based on the cultural, morphological and biological characteristics such as septation and sporophores, spore shape, pigmentation, mycelia color and colony pigmentation and those showing similar characteristic was given a specific code and then sub-cultured on PDA and SNA. Spezieller Nahrstoffarmer Agar (SNA) medium was prepared by adding mineral as stated in section 3.2.1 plus 0.2g glucose, and 20g agar in one liter of distilled water (Nirenberg, 1981). Spezieller Nahrstoffarmer Agar (SNA) was autoclaved for 20 minutes at 121^0C at a pressure of 15 p.s.i and approximately 20 ml was dispensed in 9cm Petri dish in the biological safety cabinet. *Fusarium* species sub-cultured on SNA were incubated under UV light for sporulation facilitation. *Fusarium* species growing on PDA and SNA were identified following cultural (pigmentation), morphological (mycelia color and colony pigmentation) and biological (septation and sporophores, spore shape) characteristics (Seifert, 1996; Nirenberg, 1981). Then, the morphological characteristics of various *Fusarium* isolates were used in identification to species level based on the manuals by (Nelson *et al.,* 1983; Leslie and Summerell, 2006). The formula by Gonzalez et al., (1999) was used in determining the relative isolation frequency of each *Fusarium* genus

$$\text{Frequency (\%)} = \frac{\text{Number of samples in which a species occurred}}{\text{Total number of samples}} \times 100$$

Microscopic identification of *Fusarium* species on SNA was based on macro-conidial shape, septation, widest part of macro-conidia, length of apical cell, relative abundance of microconidia in aerial mycelium, micro-conidia in chains or heads, micro conidial shape and conidiophores in aerial mycelium (Seifert, 1996).

The number of colonies growth in each serial dilution was counted and from this the colony forming unit per gram (CFU/g) of soil or maize samples was determined using the following formula:

$$\text{CFU/g} = \frac{\text{Number of } \textit{Fusarium} \text{ colonies per ml plated}}{\text{Total dilution factor}}$$

3.4 Analysis of fumonisins in maize kernels

AccuScan Neogen Reveal Q+ was used for fumonisin analysis. A sample of 10g of ground maize was mixed with fifty ml of 65% ethanol then shaken vigorously in a centrifuge for three minutes and allowed to settle and then sieved using a Whatman No. 1 filter paper. Red and clear sample cups were placed into a labeled sample rack. Two hundred microliter of the sample diluents was placed in the red dilution cup was mixed with 100 µL sample extract in sample cup and was mixed by a pipette up and down five times, then transferred into a new well labeled sample cup. The new reveal Q+ for fumonisin test strip with the sample end down was placed into the sample cup with the test strip coming into contact with the liquid and the timer set for 6 minutes. The reveal Q+ test strip was removed after it had developed for 6 minutes from the sample cup and read within 1 minute by fully inserting in the Accuscan pro reader with the

sample end first and the result side facing outside. The cartridge with the strip was inserted upside down into Accuscan pro which automatically analyzed the cartirage. The test strips were read in the Reveal AccuScan or Reveal AccuScan III Reader within one minute of completion of the 5 minute incubation. Reaveal Q+ was designed for quantitative analysis of fumonisin with a limit of detection that range from 0.3 to 6 ppm. Samples with quantities above 6 ppm were determined through serial dilution and the result acquired multiplied by the number of dilutions. After each dilution, the above procedure on taking reading from the Reveal AccuScan III Reader was followed.

3.5 Data analysis

The data on the *Fusarium* species population, percent incidence, and fumonisin level was subjected to analysis by GenStat 15th edition to determine significant difference in incidence of *Fusarium* species in Aflasafe KE01 treated and untreated maize fields. Differences between treated and untreated fields were separated, using Fishers protected LSD ($P \leq 0.05$). The fumonisins guidance level categories by Food and Drug Administration (FDA) of ≤ 2 is low, 2-4 is medium and >4 is high fumonisin level (above acceptable level in maize) was used to analyze fumonisins.

CHAPTER FOUR: RESULTS

4.1 Population and diversity of *Fusarium* species in soil and maize grains in atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

Major *Fusarium* species isolated from soil and maize samples were *F. verticillioides*, *Fusarium proliferatum, F. chlamydosporum, F. merismoides, F. semitectum* and *F. oxysporum*. *Fusarium verticillioides* produced white aerial mycelium which was tinted with purple color and the underside was dark purple on PDA while their microconidia were formed in chain. *Fusarium oxysporum* had a white aerial mycelium and sometime has purple tinge on PDA. The basal part was creamy to tan orange in color while the underside was dark purple. It produced chlamydospores and microconidia are borne on false head (Figure 4.1).

Fusaruim verticillioides had an aerial white mycelium on PDA. It grows rapidly and was tinged with purple. When sporodochia was present it was tan to orange in color. The undersurface was dark purple in color. Its microconidia were formed in chains on monophialides. *Fusarium avenaceum* had scarce microconidia with very a long, slender with more than three septate and thin walled. On PDA it had a dense white-tan aerial mycelium with a dark brown color. *Fusarium semitectum* had a rapid growing aerial mycelium that was tan in color with very few microconidia. The macroconidia were borne in aerial mycelium and were spindled shaped and slightly curved. Poliphialides were borne on the aerial mycelium. *Fusarium graminearum* in mature cultures had a dense aerial mycelium which turned yellow and presence of sporodochia in thick walled and mature culture with the carmine red undersurface. Its macroconidia were distinctively septate, with the basal cell distinctly foot shaped.

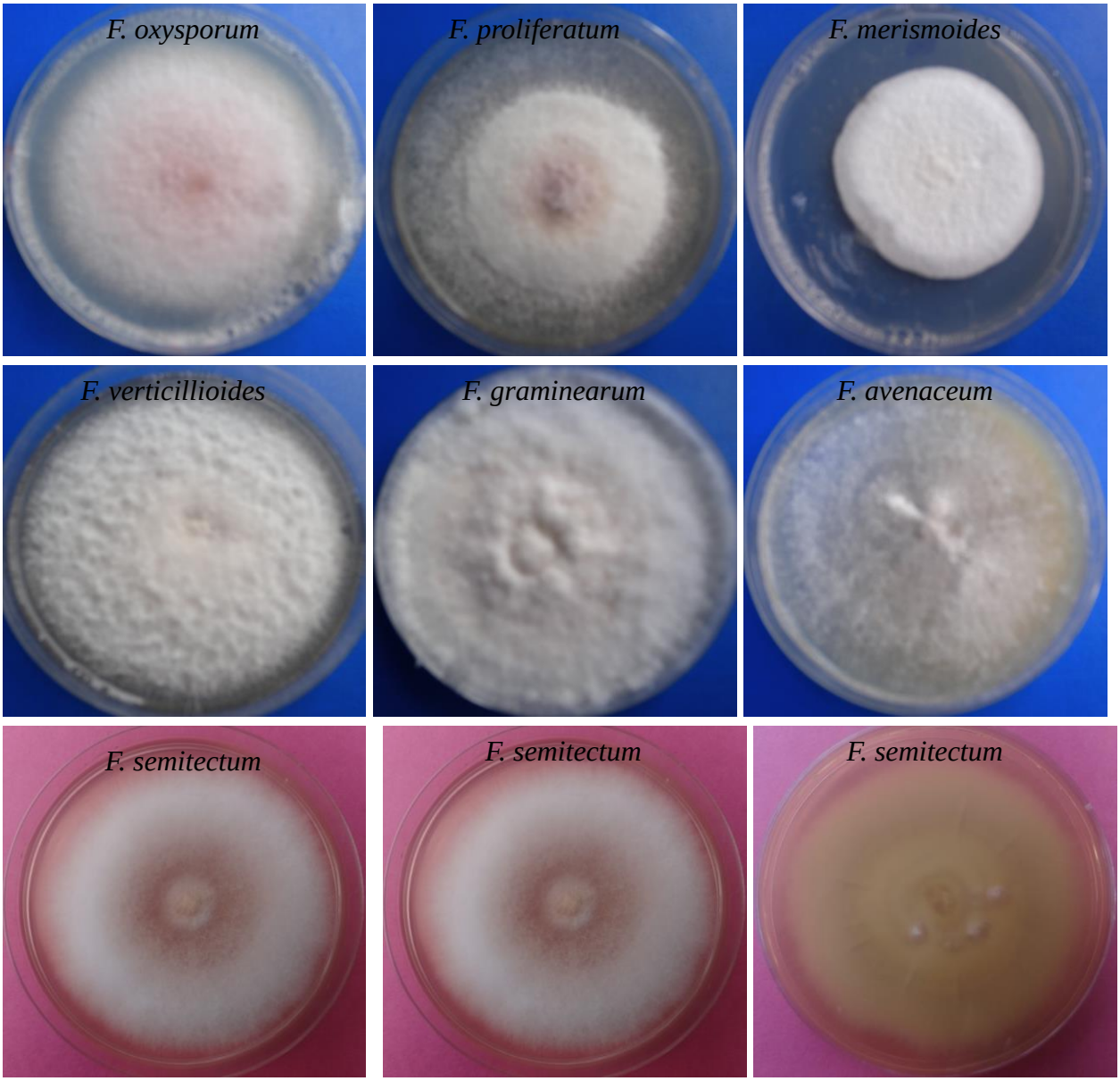

Figure 4. 1: Growth on PDA of *Fusarium* species isolated from soil collected from maize fields

Fusarium verticillioides was the most predominant one isolated from maize sample from all the sub counties. It had abundant microconidia which were single celled, oval shaped, non-septate microconidia. *Fusarium proliferatum* had abundant micro conidia that were club shaped with a flattened base which occurred in long chains and its basal cell is foot shaped with branched polyphialides and monophialids. The rare species was *Fusarium chlamydosporum* which had

spindle shape microconidia which was either septate or non-septate with macroconidia that were sickle shaped with a basal cell that is foot shaped and had abundant rough wall chlamydospores (Figure 4.2).

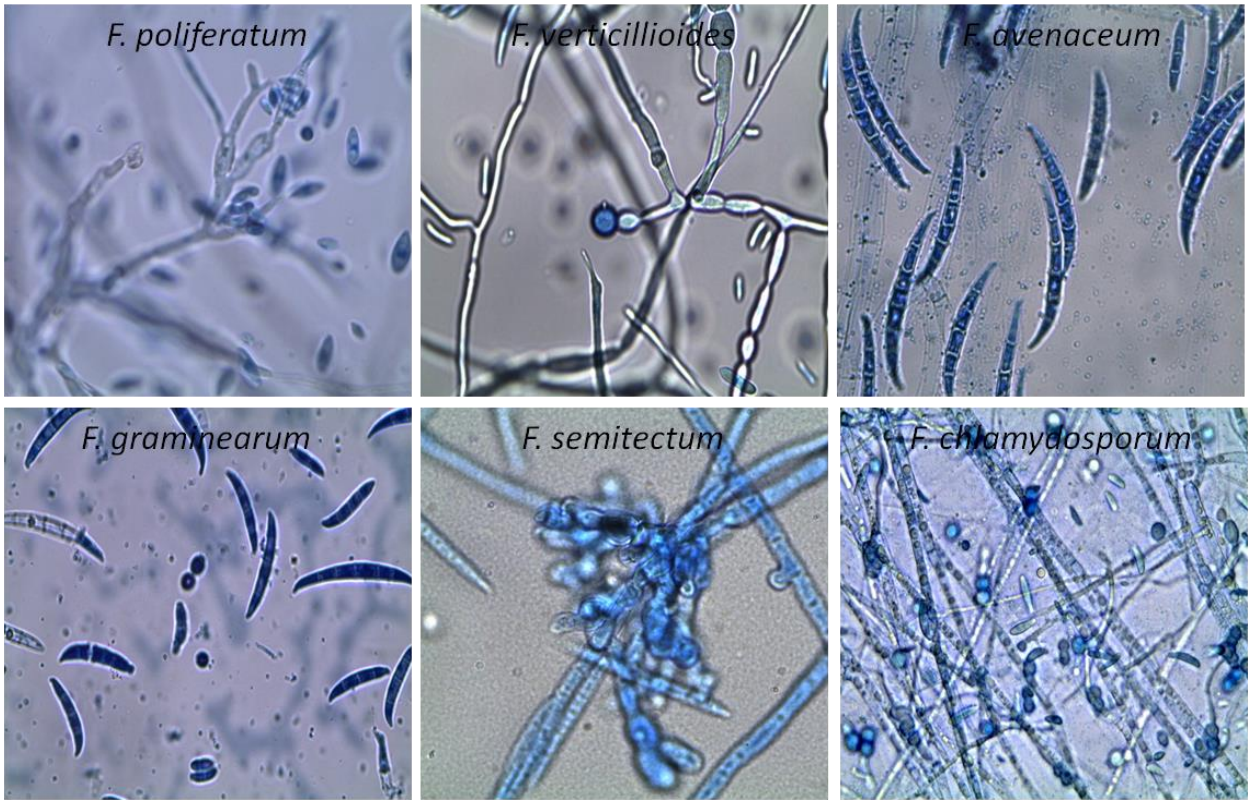

Figure 4. 2: Various conidial types of different *Fusarium* species isolated from maize grain and soil samples from fields in lower Eastern Kenya. X 100 magnification.

4.2 Population of *Fusarium* species in soil sampled from Lower Eastern Kenya from atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

The major *Fusarium* spp. isolated from soil samples in order of decreasing incidences were *Fusarium verticillioides* which was the most predominant *Fusarium* species in all the four sub counties with 65%, followed by *Fusarium proliferatum* at 18%, *Fusarium avenaceum* at 8%, F. merismoides 6%, F. *chlamydospora* 2%, *F. oxysporum* 0.9%, while *Fusarium graminearum*

0.3% was the least predominant (Table 4.1). The baseline population of the fungal population in lower Eastern Kenya varied among the different *Fusarium* species with *F. verticillioides* being the highest species isolated across the four sub counties. *Fusarium graminearum* was the least isolated *Fusarium* species across all the four sub counties (Table 4.2).

Table 4. 1: Frequency (%) of *Fusarium* spp. Isolated from soil samples from maize fields before treatment with 5kg/ha and 10 kg/ha of Alasafe KE01 in Lower Eastern Kenya.

Application rate of Aflasafe KE01	Sub county	F.v	F.p	F.a	F.m	F.c	F.g	F.o
5 kg/ha	Kaiti	65.3a	21.2a	4.0b	19.6a	2.9a	0a	0.0a
	Kathiani	64.3a	25.6a	5.8b	6.5b	1.7a	0a	1.0a
	Nzambani	69.7a	18.7a	8.2b	1.5c	1.5a	0a	0.0a
	Wote	57.8a	16.6a	16.7a	1.9c	1.5a	0.3a	0.8a
	Mean	64.3	20.5	8.7	6.3	1.9	0.1	0.5
	LSD ($P \leq 0.05$)	16.2	16.8	8.4	4.1	3.8	0.4	1.0
	C V %	27.8	10.9	19.8	19.6	18.8	17.2	28.6
10 kg/ha	Kaiti	74.1a	19.0a	1.5b	3.1bc	2.2a	0.0a	0.0a
	Kathiani	70.1a	21.1a	5.5b	1.1c	0.0a	0.5a	1.7a
	Nzambani	68.0bc	11.6a	6.8b	4.5bc	4.6a	1.0a	3.5a
	Wote	57.1bc	13.6a	14.3a	10.0a	3.3a	1.2a	0.5a
	Mean	67.3	16.3	7.1	4.7	2.5	2.5	2.5
	LSD ($P \leq 0.05$)	14.8	13.6	7.4	8.4	5.6	1.9	3.7
	C V %	2.1	5.1	20.4	7	20.6	18.7	25.5

Means with same letter(s) within a coloum for each application rate are not significantly different. F.v – *Fusarium verticillioides,* F.p - *Fusarium proliferatum,* F.a - *Fusarium avenaceum,* F.c - *Fusarium chlamydosporum,* F.m - *Fusarium merismoides,* F.g - *Fusarium graminearum,* F.o - *Fusarium* o*xysporum*

Table 4. 2: Baseline number (CFU/g) of *Fusarium* spp. from soil from different maize farms before treatment with 5kg/ ha and 10 Kg/ha application rates in four sub counties in lower Eastern Kenya.

5Kg/ha	*Fusarium* species	Kaiti	Kathiani	Nzambani	Wote	Mean
	F. proliferatum	3472b	3195b	4722b	9445b	5208
	F. avenaceum	2222b	1805b	819b	2083b	1732
	F. chlamydosporum	8473b	13334b	5084b	9167b	10139
	F. merismoides	1806b	1528b	954b	1250b	1384
	F. graminearum	0b	0b	0b	139b	35
	F. oxysporum	0b	695b	834b	1389b	729
	F. verticillioides	34306a	44723a	32500a	39306a	37708
10Kg/ha	*F. proliferatum*	34862a	44028a	34167a	43611a	34931
	F. avenaceum	11528b	1528b	1721b	5834b	5153
	F. chlamydosporum	8750b	16112ab	7084b	14306b	11563
	F. merismoides	278b	467b	2500b	1667b	1228
	F. graminearum	556b	139b	278b	417b	347
	F. oxysporum	556b	139b	278b	695b	417
	F. verticilloides	34862a	44028a	34167a	36943a	37500
	Mean	10119	12266	8936	11875	
	LSD ($P \leq 0.05$)	23476	25561	27098	28295	

Means followed by different letter(s) within a column for each application rate are significantly different at $\alpha \leq 0.05$

The incidence of the fungal pathogen varied significantly ($P \leq 0.05$) in the maize field treated with Aflasafe KE01 and untreated maize field especially in *F. verticillioides, F. proliferatum and F. oxysporum.* Maize samples from Aflasafe KE01 treated (5 kg/ha) maize fields recorded lower incidence of the *Fusarium* species compared to untreated maize fields (Figure 4.3A). A significant difference ($p \leq 0.05$) was observed in the incidence of the fungal pathogen between the maize grain samples from maize field treated with Aflasafe KE01 (10 kg/ha) and untreated maize field especially in the prevalence level of *Fusarium verticillioides, F. proliferatum and F.*

oxysporum. Aflasafe KE01 treated maize fields had significantly lower incidence of *Fusarium verticillioides, F. proliferatum and F. oxysporum* compared to the untreated maize fields (Figure 4.3B).

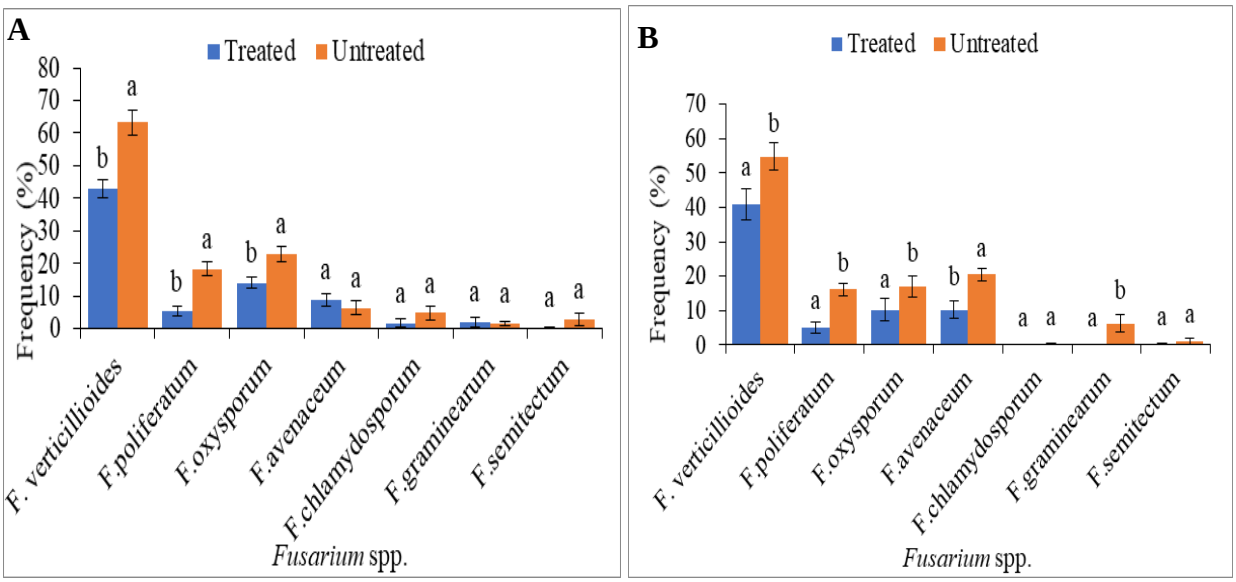

Figure 4.3: Frequency (%) of *Fusarium* spp. in maize grains sampled at harvest from fields treated with 5kg/ha (A) and 10kg/ha (B) and untreated maize fields. Error bars represent the standard error of the means. Means with different letters for each species are significantly different from each other at $\alpha \leq 0.05$.

A five kg/ha rate application of Aflasafe KE01 significantly reduced the incidences of *F. verticillioides* and *F. proliferatum* in Kaiti sub county while in Kathiani sub county there was a significant difference in the incidence of *F. proliferatum* and *F. oxysporum* between the maize samples from Aflsasfe KE01 treated and untreated fields (Table 4.3). The incidence of *Fusarium verticillioides* varied significantly at ($p \leq 0.05$) in treated and untreated maize field in Kaiti and Kathiani sub counties but there was no significant difference at ($p \leq 0.05$) fields in Nzambani and Wote. *Fusarium proliferatum* and *F. avenaceum* varied significantly in maize field from Kaiti Sub County only. There were insignificant differences in the population of *F. oxysporum, F. avenaceum, F. chlamydospora, F. graminearum and F. semitectum* between treated and untreated maize fields in all the four sub counties.

There was a general decrease in the level of *Fusarium* species in the entire treated maize field as compared to the untreated fields (Table 4.3).

Table 4. 3: Frequency (%) of *Fusarium* spp. in maize grains sampled at harvest from fields treated with 5kg/ha and 10 kg/ha Aflasafe KE 01 in Lower Eastern Kenya.

Fusarium spp.	Treatment	5kg Aflasafe KE 01					10kg Aflasafe KE 01				
		Kaiti	Kathiani	Nzambani	Wote	Mean	Kaiti	Kathiani	Nzambani	Wote	Mean
F. verticillioides	Treated	23.7b	63.2a	74.2a	57.0a	63.3	43.5a	45.2a	73.6a	54.7a	54.3
	Untreated	58.8a	44.9a	62.7a	40.9a	43.1	41.7a	23.4b	75.4a	43a	45.9
F. proliferatum	Treated	1.8b	0.6b	4.9a	13.9a	5.3	4.1b	6b	4.8a	5.2a	5
	Untreated	31.0a	16.7a	0.0a	24.8a	18.1	34a	18.2a	2.8a	9.2a	16
F. oxysporum	Treated	36.0a	4.6b	1.4a	14.5a	14.1	27.8a	20.4a	4.6a	12.1a	16.2
	Untreated	39.7a	20.2a	3.4a	28.5a	23	15.9a	4.5a	18.4a	31.4a	17.6
F. avenaceum	Treated	2.5a	22.8a	8.3a	2.2a	9	23.3a	23a	11.4a	24.3a	20.5
	Untreated	5.4a	12.8a	3.7a	3.7a	6.4	8.3b	23.2a	0a	9.5a	10.3
F. chlamydosporum	Treated	0.0a	0.0a	0.0b	6.9a	1.7	0.5a	0a	0a	0a	0.1
	Untreated	0.0a	0.0a	19.1a	0.0a	4.8	0.1a	0a	0a	1.3a	0.4
F. graminearum	Treated	0.0a	7.9a	0.0a	0.0a	2	0.8a	0b	0a	0a	0.2
	Untreated	0.0a	4.3a	0.0a	2.2a	1.6	0a	20.7a	3.5a	1.2a	6.4
F. semitectum	Treated	0.9a	0.9a	0.0b	0.0a	0.5	0a	0a	0a	1.7a	0.4
	Untreated	0.2a	0.0a	11.1a	0.0a	2.8	0a	0a	0a	4.4a	1.1

Means followed by the same letter(s) within columns for each species in each sub county are not significant different (Fisher's protected LSD at $p \leq 0.05$).

4.3 Population of fungal pathogens in maize sampled from atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated farms in lower Eastern Kenya

The population of the fungal pathogen varied significantly ($p \leq 0.05$) in the maize field treated with Aflasafe KE01 and untreated maize field especially in *Fusarium verticillioides, F. proliferatum and F. oxysporum.* Maize samples from atoxigenic *Aspergillus flavus* treated (5 kg/ha) maize fields recorded lower population of the *Fusarium* species compared to untreated maize fields (Figure 4.4A). Application of Aflasafe KE01 at the rate of five kilogram per ha significantly reduced the incidences of *F. verticillioides* and *F. proliferatum* in Kaiti sub county while in Kathiani sub county, significant differences were observed in the incidence of *F. proliferatum* and *F. oxysporum* between the maize grains from treated and untreated fields (Table 4.4).

The population of *F. verticillioides* varied significantly at ($p \leq 0.05$) in treated and untreated maize field in Kaiti and Kathiani sub counties however, in Nzambani and Wote, there was insignificant differences ($p \leq 0.05$). *Fusarium proliferatum* and *F. avenaceum* varied significantly in maize field from Kaiti Sub County only. There were insignificant differences in the population of *Fusarium oxysporum, F. avenaceum, F. chlamydospora, F. graminearum and F. semitectum* between treated and untreated maize field in all the four sub counties. There was a general decrease in the level of *Fusarium* species in the entire treated maize field as compared to the untreated fields (Table 4.4).

Table 4. 4: Population (CFU/g) of fungal pathogens in maize kernels sampled from farms treated with 5kg/ ha in four sub counties in lower Eastern Kenya.

		5 kg/ha					10 kg/ha				
***Fusarium* spp.**	**Treatment**	**Kaiti**	**Kathiani**	**Nzambani**	**Wote**	**Mean**	**Kaiti**	**Kathiani**	**Nzambani**	**Wote**	**Mean**
F. verticillioides	Treated	43333a	23611b	14667a	27500a	30694	21660a	19444b	21889a	25278b	22069
	Untreated	57222a	66667a	59333a	37500a	52916	73889b	44722a	33722a	59722a	53014
F. Avenaceum	Treated	556a	11389b	1389a	1111a	3611	14167a	8889b	7500a	11111a	10417
	Untreated	7222a	28056a	556a	3056a	9723	10833a	31389a	0a	15278a	14125
F. Graminearum	Treated	0a	1111a	0a	0a	278	556a	0b	0a	1111a	417
	Untreated	0a	2778a	0a	1944a	1181	0a	21111a	1389a	2222a	6181
F. semitectum	Treated	278a	556a	0a	0a	209	0a	0a	0a	833a	208
	Untreated	278a	0a	0a	0a	1875	0a	0a	0a	8333a	2083
F. Chlamydospora	Treated	0a	0a	0b	17778b	4445	278a	0a	0a	0a	70
	Untreated	0a	0a	33229a	0a	8307	278a	0a	0a	1667a	486
F. Proliferatum	Treated	1944b	33056a	0b	50000a	21250	1944b	3056a	3056a	2222b	2570
	Untreated	99167a	278b	2222a	17500b	29792	55833a	43333b	556a	16944a	29167
F. Oxysporum	Treated	43056a	3056b	278a	19444a	25764	20000a	9722b	3889a	5000b	9653
	Untreated	80278a	50556a	4722a	56667a	38750	30278a	24444a	26667a	52500a	33472

Means followed by the same letter(s) within columns in each sub county are not significant different (Fisher's protected LSD at $p \leq 0.05$).

There was a significant difference ($p \leq 0.05$) in the population of the fungal pathogen between the maize grain samples from maize field treated with Aflasafe KE01 (10 kg/ha) and untreated maize field especially in the population of *Fusarium verticillioides, F. proliferatum and F. oxysporum.* Aflasafe KE01 treated maize fields had significantly lower population of *Fusarium verticillioides, F. proliferatum and F. oxysporum* compared to the untreated maize fields (Figure 4.4B).

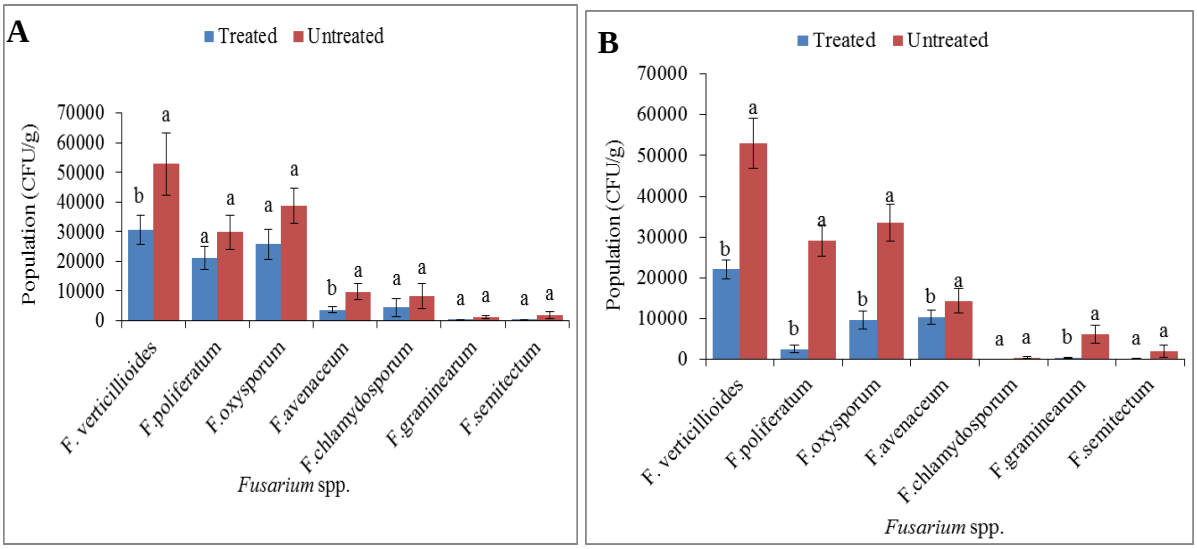

Figure 4. 4: The population (CFU/g) of fungal pathogens in maize sampled from farms treated with 5 kg/ha (A) and 10kg/ ha (B) of Aflasafe KE01 in lower Eastern Kenya. Error bars represent the standard error of the means. Means with different letters for each species are significantly different from each other at $\alpha \leq 0.05$.

4.4 Efficacy of field application of atoxigenic *A. flavus* (Aflasafe KE01) in reducing fumonisin contamination of maize grains at harvest

Maize sampled at harvest from Aflasafe KE01 treated fields were contaminated with varying levels of fumonisin. About 63.5% of the maize fields treated with 5kg/ha and 10kg/ha of Aflasafe KE01 met the threshold set by European commission (≤2ppm) for fumonisin (Table 4.5). A total of 66.6% of the maize samples met the standard set by the US food and drugs Administration (≤4ppm) while 33.3% was above the set standard of (≤4ppm) in the maize

samples from fields treated with 10kg/ha of Aflasafe KE01 (Table 4.5). Generally, there was a reduction in fumonisin level due to the application of Aflasafe KE01. There was significant difference in the fumonisin level in maize samples from Aflasafe KE01 treated and untreated maize fields from Kaiti and Nzambani using the 10 kg/ ha application rate with a 60% and 65.5% decrease in the fumonisin level respectively (Table 4.6).

Table 4.5: Proportions (%) of fumonisin contamination levels falling under different categories for maize sampled at harvest from treated and untreated fields with atoxigenic *A. flavus* (Aflasafe KE 01).

			Fumonisin level (ppb)			
Rate of Aflasafe	**Sub county**	**Treatment**	**≤ 2**	**2-4**	**>4**	**Range**
5kg/ha	Wote	Treated	83.3	0.0	16.7	0-6
		Untreated	66.7	16.7	16.7	0-11.5
	Kaiti	Treated	50.0	16.7	33.3	02-6.2
		Untreated	16.7	50.0	33.3	0-8.2
	Kathiani	Treated	16.7	50.0	33.3	0-6.5
		Untreated	16.7	33.3	50.0	0-8
	Nzambani	Treated	100.0	0.0	0.0	0-0.6
		Untreated	83.3	16.7	0.0	0-3.3
10kg/ha	Wote	Treated	83.3	0.0	16.7	0-4.1
		Untreated	100.0	0.0	0.0	0-0.6
	Kaiti	Treated	50.0	0.0	50.0	0-5.4
		Untreated	16.7	0.0	83.3	0-9.3
	Kathiani	Treated	50.0	16.7	33.3	0-7.5
		Untreated	83.3	0.0	16.7	0-7.5
	Nzambani	Treated	66.7	0.0	33.3	0-7
		Untreated	83.3	16.7	0.0	0-2.9

The fumonisin level categories are ≤ 2 ppb is low, 2-4 ppb is medium and >4 ppb is high.

Table 4.6: Fumonisin level (ppb) in maize kernels sampled at harvest from atoxigenic *A. flavus* (Aflasafe KE01) treated and untreated fields in Kaiti, Nzambani Kathiani and Wote sub counties

	Treatment	Sub county			
		Kaiti	Kathiani	Nzambani	Wote
5 Kg/ha	Treated	2.7a	3.7a	0.25a	2.5a
	Untreated	3.7a	4.2a	0.8b	3.1a
% Reduction[a]		**27.0**	**11.9**	**68.7**	**19.3**
10 Kg/ha	Treated	2.4a	1.8a	1.0a	0.5a
	Untreated	6.0b	3.5a	2.9b	0.9a
% Reduction[a]		**60**	**48.5**	**65.5**	**44.4**

Means were standardized before subjecting the data to analysis.

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

Means followed by the same letters within a column are not significantly different ($p \leq 0.05$) for each sub county.

CHAPTER FIVE: DISCUSSION

5. 1 Population of *Fusarium* species in maize grains and soil in atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

From this study, there was high contamination of maize samples with *Fusarium* species. This may be attributed to the conducive weather conditions of negligible rainfall and high temperatures pre -harvest stages. According to Görtz *et al.* (2010), small amounts of rainfall accompanied by high temperature in the early stages of maize development results in increased infection. Other authors have also reported that high levels of *F. verticillioides* infection are associated with drier, warmer climates (Cotty and Jaime-Garcia, 2007; Milani, 2013; Marin *et al.*, 2010; Magan and Medina, 2016). According to Doohan *et al.* (2003) and Munkvold (2003), climatic conditions directly affects the growth of fungus and its ability to produce and disperse inoculum. The climatic condition has an indirect effect on the vegetation type that might affect the survival of the fungus in the soil. From this study, it is evident that a range of *Fusarium* species infects maize kernels in lower Eastern Kenya. The observed widespread prevalence of different *Fusarium* species can be an indication that it is possible for the maize kernels to be contaminated by several mycotoxins other than fumonisin (Logrieco *et al.*, 2002).

In this study, atoxigenic *A. flavus* application significantly decreased the population and incidence of *F. verticillioides*, *F. graminearum* and *F. proliferatum.* This implies that the atoxigenic *A. flavus* strains present in Aflasafe KE01 reduced the population and incidence of these *Fusarium* species suggesting a competition for space or substrate between the fungi leading to a reduction in the frequency of *Fusarium* species (Kagot *et al.*, 2019). A similar work done in Brazil showed that the use of an atoxigenic *A. flavus* led to a reduction in the frequency of *F. verticillioides* (Reis *et al.*, 2020). Effective establishment of atoxigenic strains can exclude the

fumonisin-producing fungal strains or prevent their fumonisin-producing ability (Pereira *et al.*, 2011). The atoxigenic *A. flavus* strains found in Aflasafe KE01 excluded the toxigenic strains from the niche and competed for nutrients destined for synthesis of fumonisins.

Other fungal species have been reported to have antagonistic effects that inhibit the growth and proliferation of the *Fusarium* species (Wachowska *et al.*, 2013; Abdallah *et al.*, 2018; Samsudin and Magan, 2016). An earlier study reported that colonization and sporulation of toxigenic *F. verticillioides* and *F. proliferatum* was suppressed by non-pathogenic *Fusarium* species (Luongo *et al.*, 2005). A study by Wagacha and Muthomi (2008) suggested that atoxigenic strains of *F. proliferatum* and *F. verticillioides* would be effective bio-control agents for toxigenic strains since they have similar growth conditions, host plant and share the same ecological niche. In our study, the concurrent presence of *A. flavus* played a significant role in inhibiting *Fusarium* development on maize kernels the four sub counties.

5.2 Efficacy of field application of in atoxigenic *Aspergillus flavus* (Aflasafe KE01) in reducing fumonisin contamination of maize grains at harvest.

The higher fumonisin level noted in untreated fields is as a result of dominant occurrence of *F. verticillioides* and *F. proliferatum* in maize samples from untreated. *Fusarium verticillioides* and *F. proliferatum* are the major fumonisin producers (Chulze *et al.*, 2015; Tsehaye *et al.*, 2016). The infection of the maize grains in our study with fumonisin could be attributed to the high incidence of *F. verticillioides* and *F. proliferatum* (Misihairabgwi *et al.*, 2019). Additionally, the weather in the lower Eastern parts of Kenya provides appropriate conditions for the production of fumonisins in maize. The results from this study showed that maize grains sampled from Aflasafe KE01 treated maize farms had lower fumonisin levels than maize from untreated fields. This implies that the fumonisin level in maize grains was reduced due to the application of

Aflasafe KE01. Lauren *et al.* (2004) reported a reduction in the population of *F. verticillioides* and *F. proliferatum*, and significant inhibition of fumonisin Bl (FBI) production by the presence of *F. gramineanum*. In a study by Kaur *et al.* (2010) strains of non phytopathogenic *Fusarium* were combined with other biocontrol agents to obtain an effective reduction in fumonisin levels in crops. Other studies have reported a decrease in the fumonisin levels in maize as a result of the interaction of *A. flavus* and *Fusarium* species (Dwivedi and Enespa, 2013; Camiletti *et al.*, 2018; Giorni *et al.*, 2019; Reis *et al.*, 2020). Single application of Aflasafe has been shown to be effective for up to 3 years and in several crops that will be planted on the same plot (IITA, 2009). Therefore, Aflasafe KE01 should not be applied every season for effective control of the *Fusarium* species and fumonisin production in maize.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The diversity of the fungal population in lower Eastern Kenya varied among the different *Fusarium* species with *F. verticillioides* being the highest species isolated across the four sub counties. *Fusarium graminearum* was the least isolated *Fusarium* species across all the four sub counties. Findings from this study showed that application of atoxigenic *A. flavus* (Aflasafe KE01) effectively displaced the toxigenic *Fusarium* species in maize in lower Eastern Kenya. Furthermore, there was a reduction of up to 68 % in the fumonisin levels of in maize samples from atoxigenic *A. flavus* treated maize fields. This imply that atoxigenic *A. flavus* stains can be efficacious in managing fumonisin contamination in maize.

6.2 Recommendations

Based on our findings, the following recommndations are given;

i. The government through the ministry of agriculture and other stakeholders should support in up-scaling of Aflasafe KE01 to areas prone to fumonisin contamination
ii. Evaluate the efficacy of Aflasafe KE01 on reducing fumonisin contamination of other key staple crops
iii. Explore the treatment with Aflasafe KE01 of large acreage under maize production in fumonisin hotspots.

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