EFFICACY OF MAXIM XL 035 FS® AS A SEED DRESSER IN THE MANAGEMENT OF *ASPERGILLUS* SPECIES AND AFLATOXIN CONTAMINATION OF MAIZE

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my husband and soul mate George who supported me financially and morally, my sons Owen, Larry and Young for their tolerance and sacrifices that they had to endure during the entire period of my study and research. Finally it goes to my parents and friends for their continued support morally and psychologically, their persistent encouragement and prayers.

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ABBREVIATIONS

ANOVA – Analysis of Variance

CAN – Calcium Ammonium Nitrate

CCA – Coconut Cream Agar

DAP – Di-ammonium phosphate

ICRISAT –International Crops Research Institute for the Semi-Arid Tropics

ILRI – International Livestock Research Institute

KARI – Kenya Agricultural Research Institute

MDRB – Modified Dichloran Rose Bengal Agar

ABSTRACT

Aflatoxins are mainly produced by mycotoxigenic fungi and are a global food safety concern and human carcinogen. Maize is often contaminated with aflatoxin, making it unfit for human and animal consumption. Currently, there are inadequate tested effective controls for aflatoxin contamination of maize in Kenya. The objective of this study was to evaluate the efficacy of fungicide Maxim XL 035 FS® in management of *Aspergillus* spp, *Penicillium* spp. and *Fusarium* spp. and aflatoxin contamination of maize. This was done *in vitro*, green house and field conditions. In *in vitro* assay, concentrations (0.5µl/ml, 1.0µl/ml, and 1.5µl/ml) of Maxim XL 035 FS® were used, and efficacy determined against *A. flavus* L-Strain and S-Strain, *Pencillium* spp. and *F. oxysporum*. Sterile distilled water was used as a control. The fungicide (0.1µ) was placed at the centre of the Petri dish and fungicide in four quadrats equidistantly and incubated at 37°C. Radial growth of the test pathogens was measured from day two to determine the zone of inhibition. Greenhouse trial, concentrations (0.5µl/ml, 1.0µl/ml and 1.5µl/ml) of fungicide was assessed. Negative control had non treated seeds and inoculated soil; while positive controls had treated seeds and non inoculated soil. Five replicates each were used. Sampling was done every two weeks. Shoot wet weight and root wet weights were used as growth indicators. In the field, concentrations (0.5µl/ml, 1.0µl/ml and 1.5µl/ml) of fungicide slurry per kg/maize were used on seeds. Soil and maize debris from previous season were analyzed for microbial population. Maize tissues and kernels were sampled on 3rd, 4th and 5th months, at hard dough stage and at harvest and were analyzed for *Aspergillus* spp. incidence. Total aflatoxin level in kernels sampled at harvest was determined by ELISA method. The incidence of *A. flavus* S-strain and L-strain was high in soil and maize debris before planting; while the incidence of *A. caeletus*, *A. tamarii* and *A. alliaceus* was low. *In vitro* assay, Maxim XL 035 FS® had low activity (mean=11.2mm) against *Aspergillus* spp. while under green house conditions, the fungicide was

more effective (mean 6.9mm) against *A. flavus* L strain at lower concentrations of 0.5µl/ml, while it had no effect on the root and shoot wet weight. Dressing seeds before planting with 0.5µl/ml per kg of Maxim XL 035 FS$^{®}$ resulted in significant (p ≤ 0.05) decrease in total aflatoxin levels in kernels sampled at harvest. This resulted in a significant decline (40%) in levels of total aflatoxin in harvested kernels. High incidence of *Aspergillus* spp. in soil and harvested kernels indicates that *Aspergillus* inoculum is widespread in the environment, soil and kernels in the study area. The fungicide had some effect on the fungal population Since Maxim XL 035 FS$^{®}$ had some activity against various mycotoxigenic fungi, farmers should be encouraged to use it as a seed dresser.

<center>**CHAPTER ONE**</center>

1.0 INTRODUCTION

1.1 Maize and its role in food security

Maize is an important cereal crop particularly in Africa where many communities rely on it as their staple food (Nyoro *et al*, 2007). It is easy to cultivate, and adapts to a variety of ecological zones. Additionally, it has a variety of uses such as food, oil and manure (Nyoro *et al*, 2007). Maize production has to be increased for food security to be achieved in Kenya. It is the staple food crop for 96% of Kenya's population with 125kg per capita consumption providing 40% of the calorie requirements (Byerlee and Eicher, 1997).Current trends show that the country is struggling to achieve self sufficiency in maize. If the country is to be self sufficient, domestic production is required to grow at a rate of 4% (Byerlee and Eicher, 1997). Among factors that reduce vigor and yields is the attack of maize by a variety of organisms such as insects, parasites and fungi (Bruns, 2003), which results in heightened food insecurity and subsequent dependence on imports (Gok, 2010).

1.2 Fungi as disease causing pathogens

Fungi are some of the soil pathogens which attack seeds and seedlings causing wilts, decays and damping off (Horn *et al*., 2009). Improved agronomic care, rapid storage and controlled storage practices can reduce mycotoxin levels in food and feeds to non toxic levels (Bruns, 2003).

Majority of fungal species are cosmopolitan and occur in all of places because their reproductive structures, notably spores, are always floating in the air (Horn *et al*., 2009).

<center>1</center>

Fungi readily colonize several important crops such as maize and maize derived products, cottonseed, groundnuts, almonds, dried fig, spices, peanuts and tree nuts. This causes a variety of diseases in plants and degrades plant produce causing losses to farmers (Horn*et al.,* 2009).

Fungi in the genus *Aspergillus* comprise one important group which is responsible for inducing a variety of diseases in plants. When fungi from the genus *Aspergillus* attack humans they cause diseases collectively referred to as Aspergillosis (Horn*et al.,* 2009). The genus *Aspergillus* contains about 450 species and about 40 of them are deemed to induce aspergillosis in animals (Bennet, 2010). Some of the economically important species in the genus *Aspergillus* include *A. flavus, A. parasiticus, A. nomius, A. fumigatus*, *A. nidulans, A. oryzae, A. tereus and A. tamarii*. Aflatoxin production is the consequence of a combination of species, substrate, and environment (Bennet, 2010). The factors affecting aflatoxin production are mainly three: physical, nutritional, and biological factors. Physical factors include temperature, ph, moisture, light, aeration and level of atmospheric gases (Ruiqian *et al*., 2013). Aflatoxins are produced only between temperatures of 12 and 42^0C, and the optimal temperature is 25^0 to 35^0C (Ruiqian *et al*., 2013). Presence of CO_2 and O_2 influences mold growth and aflatoxin production. A 20% level of CO_2 in air depresses aflatoxin production and mould growth (Ruiqian *et al.,* 2013).

Negative effects of soil fungi to some extent can be reduced when seeds are dressed with fungicides and other relevant chemicals such as germination inducers before planting. Seed treatment prior to planting is an important agronomic care practice. This makes them grow fast and healthy and produce more in the long run (Solorzano and Malvick, 2009).

1.3 History of aflatoxins
The discovery of aflatoxins began immediately after an outbreak of a disease of turkeys of unknown etiology in England in 1960. The disease was called Turkey "X" disease and was

eventually attributed to a toxic groundnut meal imported from Brazil (Blount, 1961). From that point, an extensive effort to find the cause eventually elucidated that a species of mold, called *Aspergillus flavus*, was involved and the hepatotoxic products of this mold, found also as components in the toxic groundnut meal, were called aflatoxins (Richard, 2008).

The finding that the aflatoxins were carcinogenic caused concern over their occurrence in human foods and led to worldwide efforts to determine the relationships of these carcinogens to human disease and determine their occurrence in human foods as well as in animal feeds (Turner, 2010). The findings that the aflatoxins were immunosuppressive resulted in establishing that they were probable underlying causes to other diseases, mostly infectious in nature. Subsequent efforts revealed that aflatoxins can occur preharvest and therefore the aflatoxins were no longer only a storage problem (Richard, 2008).

Major crops such as maize kernels, peanuts, cottonseed, and certain tree nuts were frequently found to be contaminated. These findings brought into focus aflatoxin incidences. Aflatoxin reduction therefore became a large multidisciplinary scientific investigation into various aspects of concern such as eradication, control, analysis, epidemiology, and plant pathology as well as major efforts to determine the nature of animal disease caused by the aflatoxins (D'mello, 2003). Present-day investigations with aflatoxins continue with elimination as a major thrust based on knowledge of the biosynthetic pathway, genetics of host and pathogen, host-parasite-vector interactions, plant breeding, bio control, and selected agronomic practices (Richard, 2008).

1.4 Problem statement

Maize is the main food crop grown in Kenya, therefore the most important staple food for the majority of Kenyan population. The grain is vulnerable to degradation by mycotoxigenic fungi including *Aspergillus*, *Fusarium* and *Penicillium* (Muthomi *et al*, 2010). It is planted on 90% of all Kenyan farms (Mbithi and Van, 2000). The total acreage of land under maize in Kenya currently is 220,010Ha (Ministry of agriculture, 2013).

Aflatoxin production is affected by biotic, abiotic, and generic parameters. In storage, development of fungi, especially *Aspergillus* spp.*, F. oxysporum., penicillium* spp., is an unresolved problem exacerbated by the tropical climate, which promotes fungal growth. Aflatoxins are hazardous to animal and human health and contribute to food losses worldwide. *Aspergillus* spp. especially *A. flavus*, *A. parasiticus* and *A. nomius* attack crops producing aflatoxins which cause aflatoxicosis to humans. Aflatoxins have previously been detected in maize sampled before harvest and in storage. In 2004 during the worst known outbreak of aflatoxicosis in Kenya, 317 cases were reported and 125 people died (GOK, 2003). The minimum level of aflatoxin exposure required to cause aflatoxicosis is not known, but children are mostly predisposed (GOK, 2004). Chronic exposure to aflatoxins affects the incidence and severity of many infectious diseases in both animals and humans (Clare *et al.,* 2010). Public health officials sampled maize from the affected area and found concentrations of aflatoxin B_1 as high as 4,400 ppb, which was 220 times greater than the then Kenyan regulatory threshold of 20 ppb (Onsongo, 2004) and 440 times greater than the current regulatory threshold of 10ppb (KEBS 2007).

1.4.1 Justification of the study

So far there are very few known fungicides for control of *Aspergillus* spp. that causes aflatoxin contamination (Onsongo, 2004). MAXIM XL 035 FS® contains the broad spectrum seed treatment fungicide fludioxonil as its active ingredient combined with Mefenoxam. Fludioxonil belongs to the chemical class of pheylpyrroles, which has been derived from a natural antimycotic compound isolated from a soil bacterium.

Maxim XL 035 FS® is a fungicide that is less toxic to animals immobile in soil, does not accumulate in soil and does not cause skin sensitization (Syngenta, 2005). Thus this study aimed at testing the efficacy of Maxim XL 035 FS® on *Aspergillus* spp. and subsequent aflatoxin contamination of maize. Data generated will contribute to current efforts in identifying viable options in the management of *Aspergillus* spp. with a view to reducing aflatoxin contamination of maize.

1.5 Objectives

1.5.1 General objective

The main objective of this study was to evaluate the efficacy of Maxim XL 035 FS® as a seed dresser in the management of *Aspergillus* species, and aflatoxin contamination of maize

1.5.2 Specific objectives

i. To determine the occurrence and diversity of *Aspergillus* spp. in soil and maize debris in Kiboko Research sub-station.

ii. To evaluate the incidence of *Aspergillus* species, before and after application of Maxim XL 035 FS® in maize.

iii. To determine the effect of Maxim XL 035 FS® on aflatoxin levels of maize kernels at harvest.

1.6 HYPOTHESIS

H_O: Treatment of maize seeds with Maxim XL 035 FS® will have no effect on the inoculum levels of *Aspergillus* species and aflatoxin contamination of harvested kernels, before and after application of the fungicide.

H_A: Treatment of maize seeds with Maxim XL 035 FS® will significantly reduce the inoculum levels of *Aspergillus* species and aflatoxin contamination of harvested kernels, before and after application of the fungicide.

2.0 LITRATURE REVIEW

2.1 Economic importance of maize Kenya

Maize *(Zea Mays)* is the staple food for most households in Kenya (GOK, 2004) and an important livestock feed both as silage and as crop residue. It is also used industrially for starch and oil extraction (GOK, 2004). It is mostly produced by small scale farmers (GOK 2004). It is an important source of carbohydrate, protein, iron, vitamin B, minerals. Kenyans consume maize mainly as *ugali*[1]*,* porridge and green maize that is roasted or boiled. Several parts of the maize plant including the grain, leaves, stalk, tassel, and cob derive an economic value and are used to produce a large variety of food and non-food products (Owuor, 2010).

2.2 Constraints to maize production in Kenya

National maize production levels have been declining since 2006 from an all-time high of over 34 million bags in 2006 to about 25 million bags in 2008. This is attributed to factors such as drought, the post-election violence of 2007/2008, and high input costs in 2008. In 2009, the failure of about 35-45 per cent of the long-rains crop led to a huge production shortfall (GOK, 2010).

Constrains to maize production in Kenya include: continued over-reliance on rain-fed agriculture; limited agro-processing/value addition; inefficient marketing systems; losses due to pests and diseases; poor handling and high costs of production due to high cost of inputs (fertilizer, seeds, and fuel);poor rural infrastructure(such as roads/railway, energy, market sheds), (AATF, 2010); limited access to affordable credit facilities; under funding/investment in Agriculture sector and the global scene that directly and indirectly affects maize production in

the country through export bans on cereals by surplus countries which then reduce food volumes available in world market and lead to high cost of import goods (Kiome, 2009).

2.3 Mycotoxins in Maize crops

Maize, one of the principal crops grown for human food and livestock feed, is highly predisposed to several key mycotoxins (D'mello, 2003). Although numerous toxic fungal metabolites can be found in maize, management has focused on the few mycotoxins that occur with greater frequency or are associated with particularly undesirable consequences. These include aflatoxins mainly produced by *A. flavus* and *A. parasiticus* (Wagacha and Muthomi, 2008; Hell and Mutegi, 2010).

Occurrence of the toxins is affected by climate and geography, but each of these mycotoxins occurs across a substantial proportion of the maize-producing areas of the world, sometimes with serious economic repercussions (D'mello, 2003). This phenomenon has raised both awareness and research opportunities in relation to mycotoxins in maize. Effects of mycotoxins on this crop have been recognized for centuries, yet management of their occurrence and the associated outcomes remains costly and inadequate. Most tactics aiming at mycotoxin prevention are essentially disease management practices whose goal is to reduce infection of the plants or grain by toxigenic fungi. Mitigation of mycotoxin problems can include tactics for reducing mycotoxin concentration or simply diverting contaminated grain into uses with a greater tolerance for contamination (Munkvold., 2003a).

[1] *Ugali* (also sometimes called **sima**, **sembe** or **posho**) - is a dish of maize flour (cornmeal) cooked with water to a porridge- or dough-like consistency. It is the most common staple starch featured in the local cuisines of the eastern African Great Lakes region and Southern Africa

Contamination starts in the field and is exacerbated when crops are damaged by drought or insect infestation, or when produce comes into contact with soil and is not properly dried (IFPRI, 2011). Contamination is often unavoidable, and many African countries, do not regularly test maize for aflatoxins, leading to the sale and consumption of contaminated and suspect grain (IFPRI, 2011). In many developing countries, widespread subsistence farming systems, lack of irrigation, and inadequate drying and storage facilities impede the prevention and detection of aflatoxin in crops (International Food Policy Research Institute, 2011).

2.4 Major groups of mycotoxins and associated fungal species

2.4.1 Aflatoxin

These are toxins produced by *Aspergillus* species, such as *A. flavus* and *A. parasiticus*. The umbrella term aflatoxin refers to many different types of toxins produced including four major aflatoxins which are B_1, B_2, G_1, and G_2 (Groopman and Wogan, 2011). Aflatoxin B_1, the most toxic, is a potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in many animal species (Turner *et al*., 2009). Aflatoxins are largely associated with commodities produced in the tropics and subtropics, such as cotton, peanuts, spices, pistachios, maize (Turner *et al.,* 2009), barley, and tree nuts (Lisker *et al*., 1993; Richard and Abbas, 2008). Other fungi that produce aflatoxins are *A. alliaceus, A. auricomus, A. fumigatus, A. ochraceus A. pseudotamarii* and *A. niger.*

In peanuts, aflatoxins can be produced at both the pre- and post- harvest stages (Anon, 2013). Due to the adverse effects associated with aflatoxin contamination especially in maize and peanuts, many countries have strict regulatory control measures, especially with regard to tolerance levels in food and fodder. Many governments, for example Kenya and Malawi, have recently scaled up awareness raising campaigns regarding aflatoxin contamination. In Kenya,

acute cases of aflatoxin outbreaks, mainly from maize (the staple food) or its products have been reported (Shepard, 2003). Acute outbreaks in the country have overshadowed chronic (and often sub-clinical) incidences of aflatoxin poisoning, which are more pervasive and have adverse effects on human health (Marasas *et al*., 2008; Wild and Turner 2002). Reporting of toxicity in Kenya has also not been systematic and only incidences of high mortality are reported (Ngindu and Kenya., 1982; Nyikal *et al*., 2004), as chronic incidences are usually attributed to other causes, in addition to there being no monitoring system.

2.4.2 Ochratoxin

Ochratoxins are toxins that come in three secondary metabolite forms, A, B, and C. All are produced by *Penicillium* and *Aspergillus* species (Richard, 2007). The three forms differ in that Ochratoxin B (OTB) is a non chlorinated form of Ochratoxin A. Ochratoxin C (OTC) is an ethyl ester form of Ochratoxin A. Major species that produce ochratoxins are *A. alliaceus, A. auricomus, A. glaucus, A. melleus* and *A. niger. Aspergillus ochraceus* is found as a contaminant of a wide range of commodities including beverages such as beer and wine (Richard, 2007). *Aspergillus carbonarius* is the main species found on vine fruit, which releases its toxin during the juice making process. Ochratoxin A (OTA) has been labeled as a carcinogen and a nephrotoxin, and has been linked to tumors in the human urinary tract, although research in humans is limited by confounding factors (Richard, 2007).

2.4.3 Citrinin

Citrinins are toxin that were first isolated from *Penicillium citrinum*, but has been identified in over a dozen species of *Penicillium* and several species of *Aspergillus* (Howlet, 2008). Some of these species such as *Penicillium camemberti*are used to produce human foodstuffs such as

cheese while *Aspergillus oryzae* is used in the production of sake, miso, and soy sauce (Turner, 2004). Citrinin is associated with yellow rice disease in Japan and acts as a nephrotoxin in all animal species tested (Peraica *et al,* 1999). Although it is associated with many human foods (wheat, rice, corn, barley, oats and rye), its full significance for human health is unknown. Citrinin can also act synergistically with Ochratoxin A to depress RNA synthesis in urine kidneys (Fox and Howlet, 2008).

2.4.4 Ergot Alkaloids

Ergot alkaloids are compounds produced as a toxic mixture of alkaloids in the sclerotia of species of *Claviceps* spp. which are common pathogens of various grass species. The ingestion of ergot sclerotia from infected cereals, commonly in the form of bread produced from contaminated flour, cause ergotism the human disease historically known as St. Anthony's Fire (Turner *et al*, 2007). There are two forms of ergotism: gangrenous, affecting blood supply to extremities, and convulsive, affecting the central nervous system (Richard, 2007). Modern methods of grain cleaning have significantly reduced ergotism as a human disease; however it is still an important veterinary problem. Ergot alkaloids have however been used pharmaceutically (Turner *et al*, 2007).

2.4.5 Patulin

Patulin is a toxin produced by the *Penicillium expansum*, *Aspergillus*, and *Paecilomyces* fungal species (Stinson *et al,* 1978). *P. expansum* is especially associated with a range of moldy fruits and vegetables, in particular rotting apples and figs. It is destroyed by the fermentation process and so it is not found in apple beverages, such as cider (Stinson *et al,* 1978). Although patulin has not been shown to be carcinogenic, it has been reported to damage the immune system in

animals (Turner, 2008). In 2004, the European Community set limits to the concentrations of patulin in food products. They currently stand at 50 μg/kg in all fruit juice concentrations, 25 μg/kg in solid apple products used for direct consumption and at 10μg/kg for children's apple products, including apple juice (WHO, 2003).

2.4.6 *Fusarium* toxins

Fusarium toxins associated with *Fusarium* spp. are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize (Turner, 2008). They include a range of mycotoxins such as fumonisins, which affect the nervous systems of horses and may cause cancer in rodents; trichothecenes which are most strongly associated with chronic and fatal toxic effects in animals and humans; and zearalenone which is not correlated to any fatal toxic effects in animals or humans (Omurtag, 2008). Some of the other major types of *Fusarium* toxins include: beauvercin and enniatins, butenolide, equisetin, and fusarins (Turner, 2008)

2.5 Aflatoxins and aflatoxigenic fungi

A. flavus and *A. parasiticus* have long been recognized as major contaminants of organic and non-organic items (Gourama and Bullerman 1995). *A. flavus,* a common soil fungus, can destroy a wide range of agricultural products. Some strains of *A. flavus* produce aflatoxins, which are carcinogenic toxins that induce liver cancer in laboratory animals (Bullermann and Gourama 1995). *A. flavus*, *A. parasiticus* and *A. nomius* share the ability to produce aflatoxins (Gourama and Bullerman 1995). Identification of A. *flavus* species group is mainly based on the color, macroscopic and microscopic characteristics of the fungus. *A. flavus* growth and aflatoxin biosynthesis depend on substrate, moisture, temperature, pH, aeration, and competing microflora. The growth of *A. flavus* and aflatoxin production are unavoidable in nature (Lloyd, 1995).

Aflatoxins are considered natural contaminants of food and feed (Lloyd, 1995). The ideal control approach of aflatoxin contamination is prevention of mold growth and aflatoxin production (Hassan *et al.,* 1995).

2.6 Aflatoxin producing *Aspergillus* species

2.6.1 *Aspergillus flavus*

This is a fungal pathogen, which causes post-harvest disease in cereal grains and legumes (Shepard, 2003). Post-harvest rot typically develops during harvest, storage, and/or transit. *A. flavus* infections can occur while hosts are still in the field (pre-harvest), but often show no symptoms (dormancy) until post-harvest storage and/or transport (Payne, 1998). Many strains produce significant quantities of toxic compounds known as aflatoxins, which when consumed are toxic to mammals. *A. flavus* is also an opportunistic human and animal pathogen causing aspergillosis in immunocompromised individuals (Shepard, 2003). Species within the *A. flavus* group (referring to both *A. flavus* and *A. parasiticus*) are responsible for producing various types of aflatoxins (Cotty, 1998)

Aspergillus flavus can be categorized into the S strain and L strains on the basis of sclerotial morphology (Donner *et al,* 2009). On average, S strain isolates produce greater quantities of aflatoxins than do L strain isolates (Machida & Gomi., 2010). For example, S-strain isolates of *A. flavus* produce Aflatoxin B1 and B2 (Kurtzman *et al*., 1987; Egel *et al*., 1994). The most common strains are the S and L strains (Donner, *et. al.,* 2009). Typical or L strain isolates vary widely in aflatoxin-producing ability and a significant amount of L strain isolates are non-aflatoxigenic (produce no aflatoxins). The L strain produces fewer, larger sclerotia and on average, less aflatoxins (Garber and Cotty 1997; Cardwell, 2002). S strain isolates, on the other hand, have a tendency to produce greater quantities of smaller sclerotia (average diameter <400

µm) and also produce high levels of aflatoxins. Non-aflatoxigenic S strains are rare (Cardwell, *et. al.,* 2002). Within the S strain, some isolates, termed Sb, produce only B aflatoxins, while others, termed Sbg, produce both B and G aflatoxins (Cotty, *et. al.,* 1999).

2.6.2 *A. parasiticus*

Aspergillus parasiticus is a mold known to produce aflatoxin, although strains of it that do not produce this carcinogen exist (Diener *et al*., 1987). *A. parasiticus* produces the polyketide mycotoxin aflatoxin (AF), one of the most mutagenic and carcinogenic natural compounds described to date (Bok *et al,* 2006). The fungus is common to cereal grains and peanuts (Bryden, 2009), and also produces the G toxins (Diener *et al*., 1987; Klitch and Pitt, 1988).

2.6.3 *A. nomius*

A. nomius is an aflatoxigenic species phenotipically similar to *A. flavus* (Kurtzman *et al*, 1987). *A. nomius* have only evolved from *A. flavus* and *A. tamari* (Kurtzman *et al*, 1987). *A. nomius* are producers of both B and G aflatoxins but also produce sterigmatocystin (ST) which is carcinogenic (Marklinder *et al.,* 2005). The *A. nomius* isolates are good producers of both B and G aflatoxins and also important producer of aflatoxins in Brazil nuts (Olsen *et al*, 2008). *A. nomius* is morphologically similar to *A. flavus* (Vaamonde *et al.,* 2003).

2.6.4 Other *Aspergillus* spp.
A. fumigatus is one of the most ubiquitous of the air borne saprophytic fungi. *A. fumigatus* is exceptional among micro-organisms in being both a primary and opportunistic pathogen as well as a major allergen (Galagan *et al*, 2005). Its conidia production is prolific, and so human respiratory tract exposure is almost constant. *A. fumigatus* is isolated from human habitats and vegetable compost heap. The interaction of *A. fumigatus* and other airborne fungi with the

immune system is increasingly linked to severe asthma and sinusitis (Galagan *et al,* 2005). *A. terreus* also cause resistant invasive aspergillosis.

Aspergillus niger is the causal agent of a crown rot of peanut. Their main impact on agriculture is in saprophytic degradation of products both before and after harvest and in production of mycotoxins. Since members of the genus are more heat tolerant and xerophilic than most other fungal genera, they are very common food and feed spoilage organisms. Virtually all of the common *Aspergillus* species have been recovered from agricultural products (Domsch *et al.*, 1980; Pitt and Hocking, 2009; Perrone *et al*, 2007). The 'koji molds' (*Aspergillus oryzae, Aspergillus sojae* and *Aspergillus awamori*) have been used for more than a thousand years to produce a number of Asian foods and beverages including sake and soy sauce (Hara *et al* ., 1992).

2.7 Identification of *Aspergillus* species

The genus *Aspergillus* is easily identified by its characteristic conidiophores but identification of individual species is difficult. Macro morphological features used in identification include: mycelia color, conidial color, colony diameter, colony reverse colour, exudates production and presence of cleistothecia and sclerotia (Rodrigues, 2007). On the other hand micro morphological features used in identification include: nature of seriation, shape and size of the conidia vesicle, stipe morphology and presence of hull cells (Maliha, 2008). Morphology of the cleistothecia and ascospores are other crucial micro morphological features used in identification. Identification of *Aspergillus* species is easily achieved by use of taxonomic keys (Rodriques *et al.*, 2007).

A. flavus, A. parasiticus and *A. nomius*, the major producers of aflatoxin can be identified through various ways. *A. nomius* resembles *A. flavus* but it produces small bullet shaped sclerotia while *A. flavus* produces globose sclerotia. On the other hand *A. parasiticus* has phialides only (uniseriate conidial head) while *A. flavus* has metulae and phialides (biseriateconidial head) (Maliha *et al.,* 2008). Conidia of *A. flavus* will have relatively thin walls and the conidia shape will vary from spherical to elliptical while conidia of *A. parasiticus* are more spherical, echinulate and spinose. One effective way of differentiating *A. parasiticus* from *A. flavus* is to culture the fungi on Czar Dox Agar. The colonies of *A. flavus* will appear yellow in colour while those of *A. parasiticus* are dark green (ivy green) as reported by (Maliha *et al*, 2008; Rodrigues *et al.*, 2007).

2.7.1 Factors affecting occurrence and production of aflatoxins in maize

Growths of fungus and aflatoxin contamination are the consequence of interactions between the infection and colonization of the substrate, and also the type and amount of aflatoxin produced by the fungus (Marin *et al*., 2001). However, a suitable substrate is required for fungal growth and subsequent toxin production, although the precise factor(s) that initiates toxin formation is not well understood. Water stress, high-temperature stress, and insect damage of the host plant are major determining factors in mold infection and toxin production (Munkvold, 2003a). Similarly, specific crop growth stages, poor fertility, high crop densities, and weed competition have been associated with increased mold growth and toxin production (Munkvold, 2003a). Aflatoxin formation is also affected by growth of other molds or microbes.

Preharvest aflatoxin contamination of peanuts and maize is favored by high temperatures, prolonged drought conditions, and high insect activity; while postharvest production of

aflatoxins on maize and peanuts is favored by warm temperatures and high humidity (Cuero *et al*., 1991). Occurrence of aflatoxins is influenced by certain environmental factors hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, the susceptibility of commodities to fungal infestation during pre harvest, storage and processing periods (Sangare-Tigori *et al*., 2006).

Since fungicides are widely used to control crop diseases caused by fungi, it is pertinent to assess efficacy with respect to mycotoxin production (D`mello *et al*., 1998). Since fungal infection of plant products is often preceded by insect damage, there is interest in the effectiveness of an insecticide to reduce infestation, infection and mycotoxin production (Brown *et al*. 1995). World Health Organization (WHO) showed a direct correlation between colonization with *A. flavus* and AFBI (Aflatoxin B1) contamination of maize kernels (WHO, 1995).

2.8 Implications of aflatoxins on human and animal health

The 1980's and 1990's were globally fatal decades. In India, at least 400 people were affected by eating infected maize and 104 of them died (Mehan *et al*, 1991). 1981, 12 people were also killed by high intake of aflatoxins in Kenya (Mehan and Mc Donald, 1991). In Southeast Asia, 19 patients died after eating contaminated rice and pasta contracting jaundice; 17 of them died of hepatitis, 14 died because of liver failure and 7 because of renal failure (Mehan *et al.,* 1991). In biopsies, high concentrations of aflatoxin were found in liver, lungs and other organs (Hendrickse, 1999). It has been well documented that chronic aflatoxin exposure causes hepatocellular carcinoma (HCC), generally in association with hepatitis B virus (HBV). Maize contaminated with aflatoxin has been implicated in deadly epidemics in Kenya majorly in 1981 where there was recorded a major human fatality (Hendrickse, 1999). A. *flavus* S strain is associated with lethal aflatoxicoses which caused the death of more than 125 people in 2004. In

Makueni district 2 families also were affected by aflatoxin poisoning after consuming contaminated maize, from which 8 of 12 sick members died (Ngindu and Kenya 1982).

The 2004 outbreak of acute aflatoxicosis in Kenya was one of the most severe episodes of human aflatoxin poisoning in history. A total of 317 cases were reported by 20 July 2004, with a case fatality rate of 39% (Nyikal, *et. al.*, 2004; Lewis, *et. al.,* 2005). This epidemic resulted from ingestion of aflatoxin contaminated maize.

2.8.1 Effects of aflatoxin on human health

The effects of chronic exposure to aflatoxin in Africa are common. However concern still remains for the possible adverse effects resulting from long term exposure to low levels of aflatoxins in the food supply (Hell *et al,* 2008). Evidence of acute aflatoxicosis in humans has been reported from many parts of the world, namely the developing countries, like Kenya, Taiwan, Uganda, India, and many others (The freedictionary, 2013). Aflatoxicosis is characterized by vomiting, abdominal pain, pulmonary eodema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart (Williams *et al.,* 2003). Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control (Robinson *et al*., 1992).

African communities and populations are exposed to aflatoxins before birth and throughout their lives with serious impact on their health (Williams *et al*., 2003). Aflatoxins are the most potent natural carcinogenic substances and they have been linked with a higher prevalence of hepatocellular cancer in Africa (Strosnider *et al*., 2006). There is a very high risk of Hepatitis B

and Hepatitis C carriers to develop liver cancer when humans are exposed to aflatoxin (Williams *et al.*, 2003). Chronic exposure to aflatoxins has much wider health effects than these rare acute poisonings (Williams *et al.*, 2004). Aflatoxins have been linked to immune suppression (Turner *et al.*, 2005; Jiang *et al.*, 2005). Children in areas of high aflatoxin exposure have been found to have stunted growth (Gong *et al.*, 2004).

Aflatoxins have both carcinogenic and hepatotoxic actions, depending on the duration and level of exposure. The human gastrointestinal tract rapidly absorbs aflatoxins after consumption of contaminated food, and the circulatory system transports the aflatoxins to the liver (Fung and Clark 2004). From 1 to 3% of ingested aflatoxins irreversibly bind to proteins and DNA bases to form adducts such as aflatoxin B1–lysine in albumin (Skipper and Tannenbaum 1990) causing liver toxicity (Tandon *et al.*, 1978).

Ingestion of higher doses of aflatoxin can result in acute aflatoxicosis, which manifests as hepatotoxicity or, in severe cases, liver failure and death (Fung and Clark 2004; Etzel 2002). Such deaths have been recorded in Kenya yearly since the major outbreak of July 2004 that resulted into 317 cases of aflatoxins poisoning and 125 deaths (Azziz-Baumgartner *et al.*, 2005; Muture and Ogana, 2005; Muthomi *et al.*, 2009; Nyika *et al.*, 2004). Consequently, many people are chronically exposed to aflatoxins in their food and are at risk of serious health problems. Acute exposure to high levels of aflatoxins can result in liver failure and rapid death. Chronic exposure, in both humans and animals, exacerbates infectious diseases and can lead to cancer, liver cirrhosis, weakened immune systems, and stunted growth in children.

2.8.2 Effects of aflatoxin on animal health

Susceptibility of animals to aflatoxins varies considerably depending on species, age, sex, and nutrition (Annon, 1989). Aflatoxins cause liver damage, decreased milk and egg production, recurrent infection as a result of immunity suppression, in addition to embryo toxicity in animals consuming low dietary concentrations (Annon, 1989). While the young of a species are most susceptible, all ages are affected but in different degrees for different species (Groopman *et al.,* 1994). Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced productivity, reduced feed utilization and efficiency, anemia, and jaundice (Pier and Richard, 1992). Nursing animals may be affected as a result of the conversion of aflatoxin B1 to the metabolite aflatoxin M1 excreted in milk of dairy cattle (Pier and Richard, 1992). The induction of cancer by aflatoxins has been extensively studied. Aflatoxin B1, aflatoxin M1, and aflatoxin G1 have been shown to cause various types of cancer in different animal species (Amstrong and Collopy 1992). However, only aflatoxin B1 is considered by the International Agency for Research on Cancer (IARC, 1987) as having produced sufficient evidence of carcinogenicity in experimental animals to be identified as a carcinogen.

2.9 Testing for aflatoxins

Various methods are suggested for testing levels of aflatoxin and depend on factors such as cost effectiveness, precision, and number of samples being analyzed. Equally important is the sampling strategy as this significantly affects the margin of error in analysis of results (ICRISAT, 2007). Pascale and Visconti (2008) have summarized the various methodologies available for mycotoxin analysis including Thin Layer Chromatography (TLC), Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Liquid Chromatography/Mass Spectrometry (LC/MS), Enzyme-Linked Imunosorbent Assay (ELISA),

and rapid tests. Enzyme-Linked Imunosorbent Assay procedures are the most widely used serological tests for aflatoxin analysis due to their simplicity, adaptability and sensitivity (ICRISAT, 2007). Enzyme-Linked Imunosorbent Assay procedures allow for analysis of multiple samples which is ideal for screening purposes. High Performance Liquid Chromatography has the advantage of being highly sensitive and has good selectivity, and is easily automated. Specialized apparatus is required for an HPLC separation because of the high pressures and low tolerances under which the separation occurs. If the results are to be reproducible, then the conditions of the separation must also be reproducible. Thus HPLC equipment must be of high quality; it is therefore expensive. However, this method has a major challenge due to its high cost, making it unsuitable for routine analysis.

2.9.1 Black light test

Black light test is a quick preliminary test that is a visual inspection for the presence of a greenish gold florescence under a black light. However, yellow green fluorescence under a black light does not indicate the presence of aflatoxins. Thus, blacklight testing is not considered to be a reliable method for detecting aflatoxins, the results must be verified by laboratory analysis (Vincelli *et al,* 1995).

2.9.2 Commercial test kits with immunoassay or ELISA techniques

These are available for on-site tests for aflatoxin. Immunoassay analysis is based on the detection of specific proteins found in aflatoxins using antibodies to identify these proteins. The tests are very specific for aflatoxin, but they require operator training and practice to be accurate. Some tests determine only the presence or absence of aflatoxin; others can quantify, within a range, the amount of aflatoxin present (ICRISAT, 2007). If a lot of maize is rejected based on the results of an immunoassay test Kit, the results should be confirmed by laboratory analysis. It is very

important that the entire sample is ground before removing small sub samples for the test kit (ICRISAT, 2007). Subdivision of samples prior to grinding is a major source of error.

2.9.3 ELISA Assay procedure

The cytokine ELISA (Enzyme-Linked Immuno Sorbent Assay) is a specific and highly sensitive method for quantitative measurements of cytokines or other analytes in solutions. A specific monoclonal antibody (mAb) able to capture the cytokine of interest is coated on a micro titer plate. A second mAb, used for detection, binds a different epitope on the cytokine. The detection mAb is labeled with biotin, which allows subsequent binding of a Streptavidin-conjugated enzyme. Any unbound reagents are washed away. When substrate is added, a color reaction will develop that is proportional to the amount of cytokine bound. The concentration of cytokine is determined by comparison with a standard curve with known concentrations of cytokine. The sensitivity of an ELISA depends mainly on the affinity of the antibodies and on the amplification system used. The detection limits for cytokine ELISAs are commonly in the lower pictogram/ml range (AOAC, 1995; Gathumbi, 2001).

2.9.4 Aflatoxin analysis by High Performance Liquid Chromatography (HPLC)

Aflatoxins are extracted with dichloromethane:water (10:1 v/v). Gel permeation chromatography (GPC) using a column packed with Bio-beads S-X3 and dichloromethane:hexane (3:1v/v) as the eluent is used for clean-up of extracts prior to separation and quantification of aflatoxins by HPLC. The eluent fraction containing the aflatoxins is concentrated by evaporation under reduced pressure and the aflatoxins separated by reverse phase HPLC on the different limits of detections (LoDS) column (AOAC, 1995).

2.10 Regulation of aflatoxin contamination of maize

The Food and Drug Administration (FDA) has established an "action level" of 20 ppb for aflatoxins in maize in the United States of America (FDA, 2009). This is the level at which federal agencies may take action, including seizure of the maize or prohibition of its sale (Abbas and Shier, 2009). Further it does not accept maize with 20 ppb or more of aflatoxin unless they have a known use for the particular level of aflatoxin (Abbas, 2009). In Kenya, the safe limit for maize and peanuts for total aflatoxin was 20ppb but, this has recently changed to 10ppb of total aflatoxin in peanuts or maize (Kenya Bureau of Standards, 2007).

2.11 Strategies for aflatoxin control and prevention

These strategies can be broadly divided into: stopping the infection process (host plant resistance, biocontrol); control of environmental factors (temperature, rainfall, relative humidity, evapotranspiration, soil type) including efforts to build predictive models; crop management strategies (good agricultural practices (GAP), pre- and post-harvest management); post-harvest strategies (harvesting, drying, storage, use of plant extracts and preservatives) and decontamination (sorting, processing). Many new strategies that enhance host plant resistance against aflatoxin involving biotechnologies are being explored (Brown *et al*., 2003). These approaches involve the design and production of maize plants that reduce the incidence of fungal infection, restrict the growth of toxigenic fungi or prevent toxin accumulation (Brown *et al*., 2003). In the long term, identification of compounds that block aflatoxin biosynthesis would significantly enhance mycotoxin control.

Field management practices that increase yields may also prevent aflatoxin (Fandohan, 2010). They include use of resistant varieties, timely planting, fertilizer application, weed control, insect

control and avoiding drought and nutritional stress (Hell and Mutegi, 2010). Other options to control aflatoxin producing fungi in the field include use of atoxigenic fungi to competitively displace toxigenic fungi, and timely harvest (Hell and Mutegi, 2010). Post-harvest interventions that reduce mycotoxins are rapid and proper drying, sorting, cleaning, drying, smoking, post harvest insect control, and the use of botanicals or synthetic pesticides as storage protectant (Fandolan, 2010).Another approach is to reduce the frequent consumption of 'high risk' foods (especially maize and groundnut) by consuming a more varied diet, and diversifying into less risky staples like sorghum and millet (Hell *et al*, 2010).

Chemo-preventive measures that can reduce mycotoxin effect include daily incorporation of sodium calcium alumino-silicates into the diets (Hell and Mutegi, 2010). Detoxification of aflatoxins is often achieved physically (sorting, physical segregation, flotation etc.), chemically (with calcium hydroxide, ammonia) and microbiologically by incorporating pro-biotics or lactic acid bacteria into the diet. There is need for efficient monitoring and surveillance with cost-effective sampling and analytical methods. Sustaining public education and awareness can help to reduce aflatoxin contamination (Brown *et al*, 2003).

2.11.1 Prevention of mould contamination and growth

Prevention of mould growth during the storage process includes elimination of air as quickly as possible and managing the silo to minimize air infiltration into the ensiled mass during storage and feed out (Adegoke and Letuma, 2013). Rapid filling, harvesting at the correct moisture level, adequate compaction, covering exposed surfaces, and rapid feed out will go a long way in minimizing mould growth and potential mycotoxin contamination in silos. Moisture and temperature reduction of shelled maize minimizes mould growth. High levels of moisture and

temperatures, together with damage to kernels, are the main reasons for the growth of molds in shelled maize. Mould growth is negligible when maize moisture content is below 13% (Heathcote and Hibbert, 1978).

Method of limiting mould growth for maize immediately after harvest is by maintaining maize temperatures as low as possible under some form of aeration or maintains maximum ventilation as possible. Maize immediately after harvest contains 12 to 14 percent moisture (Codes Alimentarius Commission, 2002). Cultural practices most likely to produce a vigorous maize crop should be followed and maize should fully matures before harvesting. If maize is fully mature, there is less infection and mould growth while ears are still on the plant (Kabak *et al*, 2006). Damage to kernels should be kept to a minimum level. Causes of damage include: high-moisture shelling, high speed operation of Sheller, drying process, loading and unloading, equipment and Insect damage in field and in storage (Adegoke and Letuma, 2013). Caution in blending lots of maize that differ substantially in either quality or moisture content must be used (Ware, 1998).

2.11.2 Rapid drying

Moisture and temperature influence the growth of toxigenic fungi in stored commodities (Bennet, 2010). Aflatoxin contamination can increase 10 fold in a 3-day period, when field harvested maize is stored with high moisture content (Hell *et al*., 2008). The general recommendation is that harvested commodities should be dried as quickly as possible to safe moisture levels of 10 – 13 % for cereals. Achieving this through simple sun-drying under the high humidity conditions of many parts of Africa is difficult. Even, when drying is done in the

dry season, it is not completed before loading grains into stores (Mestre *et al*., 2004) and products can be easily contaminated with aflatoxins.

2.11.3 Biological control

Another potential means for toxin control is the biocontrol of fungal growth in the field (*Yin*, 2008). Numerous organisms have been tested for biological control of pathogens including bacteria, yeasts, and non toxigenic (atoxigenic) strains of the causal organisms (*Yin et al*., 2008) of which only atoxigenic strains have reached the commercial stage. These strains have been shown to reduce aflatoxin concentration in both laboratory and field trials, reducing toxin contamination by 70 to 99% (Atehnkeng *et al*., 2008). A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin has gained provisional registration (AflaSafe®) to determine efficacy in on-farm tests and candidate strains have been selected for Kenya and Senegal (Atehnkeng *et al.,* 2008).

2.12 Characteristics of Maxim XL 035 FS®

Maxim XL 035 FS® is a phenylpyrrole fungicide for the control of seed and soil borne diseases in maize (Syngenta, 2005). Maxim XL 035 FS® contains the broad spectrum seed treatment fungicide fludioxonil as its active ingredient combined with Mefenoxam. Fludioxonil belongs to the chemical class of pheylpyrroles, which is derived from a natural antimycotic compound isolated from a soil bacterium. It is characterized by its broad activity and unique mode of action. It interferes with transport mechanism in fungal cells interacting at various points in the lifecycle of the fungus i.e. conidial germination, germ tube and mycelial growth. It is a non-volatile, dust free formulation which is important for operator safety and ensures that the treatment remains on the seed. It has active ingredients that have been classified as reduced risk by the Environmental

Protection Agency (E.P.A.) in the USA meaning that they pose fewer risks to human health and

the environment than existing alternatives.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Field study was conducted at KARI Kiboko Research Centre, while *in vitro* and greenhouse experiments were carried out at the school of Biological Sciences, University of Nairobi. Kiboko is located in sub-locality, Kiboko locality and lies 37°40′E, 2°21′S in Makueni County with an altitude of 975m above sea level. Makueni County borders Taita Taveta, Kitui and Machakos counties (Figure 1). Kiboko Research Centre receives between 545mm and 629mm of rainfall coming in two seasons. Long rains are from March to April and short rains from November to December. Temperatures are as high as 31°C during the day and low (12°C) during the night, with mean temperatures of 28.6°C and mean annual minimum temperature of 16.5°C. The area is covered by red clay soil, with an average rainfall of 1000mm per annum. The main food crops grown in the region are maize, beans, pigeon peas, and cow peas. Tomatoes, kales, onions, brinjals, okra and mangoes are also grown for domestic consumption (GOK, 2006). Kiboko Research Substation was suitable for the study because of the previous outbreaks of aflatoxicosis in the region as well as previous reports on high population of *Aspergillus flavus* S-strain in the region.
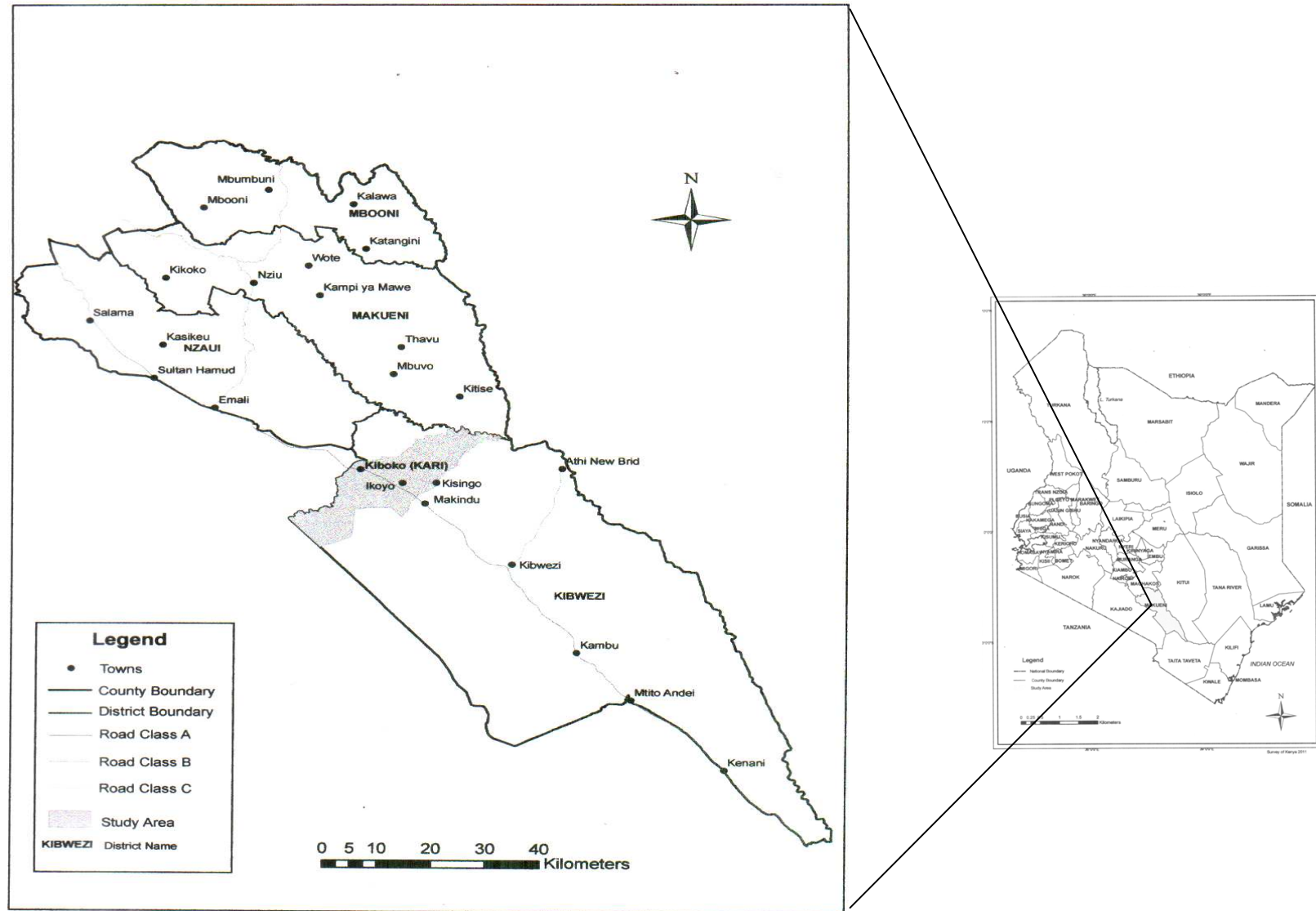
Figure 1: Map of Kenya showing Makueni County. Courtesy of University of

University of Nairobi, Geography Department

3.2 Assessment of the efficacy of Maxim XL 035 FS® under *in vitro* conditions

Various concentrations of Maxim XL 035 FS® were prepared and then tested for their efficacy on *A. flavus* (S strain and L strain), *F. oxysporum* and *Penincillium* spp. under *in vitro* conditions at the mycology Laboratory at the School of Biological Sciences, University of Nairobi.

3.2.1 Preparation of different concentrations of Maxim XL 035 FS®

Serial dilution was done by dissolving 1ml of Maxim XL 035 FS® in 9ml of distilled water to get a stock solution that was diluted by a factor of ten. From the stock solution, 0.5µl was obtained and mixed with distilled water up to the mark of 1ml. Another 1.0µl was obtained from the stock solution and mixed with distilled water up to a mark of 1ml. Lastly 0.5µl was obtained from the stock solution and mixed with distilled water up to 1ml mark in a measuring cylinder. The concentrations of the fungicide were 0.5µl/ml, 1.0µl/ml and 1.5µl/ml. The different concentrations were used against each test pathogens.

3.2.2 Test pathogens

The test pathogens whose growth period was three days were *A. flavus* S-strain and L-strain, *Penicillium* spp. and *F. oxysporum* used in the study were retrieved from the Culture Collection Centre at the Plant Pathology laboratory at the Department of Plant Science and Crop Protection, University of Nairobi. *A. flavus* S-strain and L-strain were provided by Dr. Bruce Horn of USDA through ICRISAT. The test pathogens were then grown on Potato Dextrose Agar (PDA), and incubated at room temperature (23 ± 2^{0}C) for 2-3 days. Cultures of other fungi were also isolated from soil samples from maize field in Kiboko Research Sub-station and cultured in the laboratory on Modified Dichloran Rose Bengal Agar (MDRB).

3.2.3 Preparation of culture media

The culture media was prepared by dissolving 39g of Potato Dextrose Agar in one liter distilled water. The medium was autoclaved for 20 minutes at 121°C and a pressure of 15 psi, and cooled in a water bath at 45°C. To inhibit bacterial growth and ensure the medium was semi selective for *Aspergillus* section *Flavi,* 5 ml of 4 mg/ℓ dichloran (in acetone), 40mg/ℓ streptomycin (in 5 mℓ distilled water) and 1 mg/ℓ chlorotetracycline (in 10mℓ distilled water) was added to the medium through a sterile 0.25 μm syringe filter after cooling to 50°C. The medium was then poured on to 90 mm Petri plates aseptically in a lamina flow and allowed to settle and cool down in the lamina flow to room temperature (23 $\pm$ 2°C). The plates were then transferred to the refrigerator under aseptic conditions for 2 to 3 days before use.

3.2.4 Testing the efficacy of Maxim XL 035 FS$^{®}$ on test pathogens

The Petri dishes were divided into four quadrats and the test pathogens inoculated 2mm from the edge of the Petri dish on each quadrat. One drop of 1µl/ml Maxim XL 035 FS$^{®}$ was applied from the serial dilution of 0.5µl/ml ,1.0µl/ml and 1.5µl/ml, one drop at the center of the agar plate such that it did not spread out from the center. The plates were incubated at 37°C for ten days. A control test was included in the trial. Sterile distilled water was the control. The inhibition zone (mm) was measured from the second to the tenth day using a ruler.

3.3 Assessment of the efficacy of Maxim XL 035 FS$^{®}$ under greenhouse conditions

The greenhouse had a constant temperature of 28°C. The efficacy of different concentration of Maxim XL 035 FS$^{®}$ fungicide (0.5µl/ml, 1.0µl/ml and 1.5µl/ml) was also tested under green house conditions, where the experiment took six weeks.

3.3.1 Preparation of potting soil

Soil was collected from Chiromo forest from a depth of up to 10cm. The soil was packed in polythene bags and transported to the green house. All debris was removed by hand sorting and the soil placed in autoclave and autoclaved with saturated steam at 121°C for 20 minutes at a pressure of 15 psi. The soil was left to cool for twenty four hours and potted in two liter sterile pots. The soil was then checked for any presence of fungal pathogens by inoculation on PDA. Ten grams of Di-ammonium Phosphate fertilizer was mixed with soil in each pot before planting.

3.3.2 Preparation of seed dresser and its application on maize seeds

Slurry was prepared by making dilutions with distilled water to obtain different concentrations. Hybrid maize seeds of DH01 cultivar were then treated with Maxim XL 035 FS$^{®}$ at different concentrations (0.5µl/ml, 1.0µl/ml, 1.5µl/ml of the fungicide per kilogram of maize seeds). The maize seeds were poured on the slurry and mixed properly to coat the seeds uniformly. The coated seeds were shade dried for 30 minutes before sowing.

3.3.3 Preparation of fungal inoculum

Fungal spores of *A. flavus* L strain and S strains were acquired from seven day old pure cultures previously grown on PDA media and incubated at $37^{°}$C. Preparation of cell suspensions, each isolate was sub cultured at least twice to ensure viability. Inoculums suspensions of each isolate were prepared for each experiment from fresh, mature (3- to 7-day-old) cultures grown at 37°C on modified Dichloram Rose Bengal Agar plates. The fungal colonies were covered with approximately 1 ml of sterile distilled water, and the suspensions were made by gently probing the colony with the tip of a Pasteur pipette. The resulting mixture was withdrawn and transferred to a sterile tube. Heavy particles of the suspensions were allowed to settle, and the upper homogeneous suspensions were used for further testing. Spore suspension was prepared by

flooding the plate with 0.05% tween 80 and scraping of conidia with bend Pasteur pipette, with

5ml pipette transferred to glass wool filters in 12-15ml centrifuge tube to collect spores and catch

away mycelium. Spinned for 5minute at 800rpm/per 114g. Supernatant was discarded, pipette

spores in sterile water was washed; resuspend in 10ml. Triton was used to avoid clumping of

spores. Nine micro liters of triton was taken as suspension and added to tween 80 (0.05%) since

most fungal spores are hydrophobic, hence the need to ensure that the spores did not stick

together. A haemocytometer was used to count the spores and the concentration adjusted to $1 \times$

10^6 spores per milliliter of water.

3.3.4 Planting maize and application of fungal inoculum

The fungal inoculum was applied to the seeds by mixing the inoculum with the seeds. Ten maize

seeds were planted per pot. Control pots were planted with non treated maize seeds but soil was

inoculated with *Aspergillus flavus* S and L strains. The second control entailed treatment of seeds

with Maxim XL 035 FS® without inoculating the soil. The pots were arranged in a completely

randomized design, with each treatment being replicated five times (Table 1).

Table 1: Experimental layout and application rate (ml/kg) of Maxim XL 035 FS® on maize

Application rate of Maxim XL 035 FS®(ml/kg)				
0.5	1.0	1.5	Positive control	Negative control
Pot 19	Pot 5	Pot13	Pot 6	Pot 1
Pot 10	Pot 9	Pot 2	Pot 12	Pot 18
Pot 3	Pot 14	Pot 17	Pot 15	Pot 11
Pot 16	Pot 8	Pot 7	Pot 4	Pot 22
Pot 25	Pot 21	Pot 23	Pot 20	Pot 24

Positive control- Inoculated soil with seeds not treated with the fungicide

Negative control- Soil without pathogen fungicide and seeds not treated

3.3.5 Growth of maize plants and sampling

The maize plants were grown in the greenhouse at University of Nairobi, Chiromo campus. The maize seedlings were watered after every two days using a watering can. The experiment lasted for six weeks. Sampling was done three times, every two weeks after germination. Sampling at every stage was done both for soil and plant roots. After two weeks of germination, destructive sampling was done, and a total of five samples were taken for each treatment. The shoot wet weight and root wet weight were determined as indicators of growth.

3.3.6 Assessment of maize growth parameters

All parameters (wet weight, microbial population in the soil and tissues) were checked from single pots for all treatments and controls by complete destruction. The pots were chosen randomly from each treatment and controls. Five pots were checked and all parameters checked from each. This included changes in plant growth (wet weight), population of the fungi in the plant tissue and population of the fungi in the soil.

3.3.7 Isolation of fungal pathogens

Isolation of *A. flavus* L strain and S strains and other fungi present was done from plant tissues and soil samples. Serial dilution of the soil samples was done and the inoculation done in four replicates. For the plant tissues (roots), four pieces of approximately 1mm long were used per plate. The colonies were then identified, counted and recorded after 7-10 days. The experiment was carried out for six weeks. Sampling was done on the 2rd, 4th and 6th week. *A. flavus* L and S strains population in the root tissue and soil was determined.

3.4 Assessment of the efficacy of Maxim XL 035 FS® under field conditions

3.4.1 Seed treatment with Maxim XL 035 FS®

A slurry of Maxim XL 035 FS® which had an initial concentration of 500g/L was prepared at three concentrations; 50ml, 100ml, 150ml of Maxim XL 035 FS® per 100kg of seeds. The dilution was done using distilled water. This corresponds to 0.5µl/ml, 1.0µl/ml and 1.5µl/ml of fungicide per 1kg of maize seeds, respectively. The recommended dilution of the fungicide is one part of Maxim XL 035 FS® in four to nine parts of distilled water for management of fungi. The maize seeds were dipped into the fungicide slurry and mixed thoroughly. The seeds were then placed in a cool dry place and left to dry for thirty minutes.

3.4.2 Experimental layout and growth of maize

A Complete Randomized Block Design (CRBD) was used whereby the plots were selected randomly and treatments on the plots were assigned randomly. Each plot measured 15m × 20m and comprised of five rows. There were 16 plots with each treatment being replicated four times (Table 2). The treatment consisted of seed dressing of maize seeds with Maxim XL 035 FS® at the concentration of 50ml, 100ml and 150ml per 100kg of maize seeds. Control plots were planted with non-treated maize seeds. A total of two hundred maize seeds were planted per plot Di-ammonia Phosphate (DAP) fertilizer was applied at the rate of ten grams per pit during sowing. Two guard rows were planted round the experimental plots to limit the external interference. The maize growth period was six months.

Table 2: Allocation of treatments (Application rate of Maxim XL 035 FS$^{®}$ per kg maize seed) to field plots

	Application rate (ml/kg maize seed) of Maxim XL 035 FS$^{®}$			
	0.5	1.0	1.5	Control
	Plot 11	Plot 5	Plot 13	Plot 6
PLOTS	Plot 10	Plot 9	Plot 2	Plot 12
	Plot 3	Plot 14	Plot 1	Plot 15
	Plot 16	Plot 8	Plot 7	Plot 4

Control – maize seeds not treated with Maxim XL 035 FS$^{®}$

3.4.3 Sampling of soil and plant material

Sampling of soil and plant materials was done by random sampling method where by five samples were taken from each plot, four samples from the four corners of the plot and the fifth sample from the centre of the plot. A composite sample of 500g was taken from each plot. The samples were mixed thoroughly and transported to the Laboratory for microbial analysis in a cool box.

3.4.4 Isolation of fungal pathogens

Isolation of *A. flavus, Penicillium* spp. and *F. oxysporum A. caelatus, A. alliaceus, A. tamari A. parasiticus* and other fungi was carried out using Modified Dichloran Rose Bengal Agar (Horn and Dorner, 1998). The medium was prepared by mixing 10 g glucose, 2.5g peptone, 0.5 g yeast extract, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 20 g agar and 25 mg Rose Bengal in 1litre of distilled water. The final concentration of the ingredients was glucose 0.69g/L, peptone 0.17g/L, yeast extract 0.03g/L, KH_2PO_4 0.07g/L and $MgSO_4.7H_2O$ 0.03g/L. The pH of this medium was then adjusted to 5.6 using 0.01M HCl. The medium was autoclaved for 20 minutes at $121^{°}C$ and a

pressure of 15 psi, and cooled in a water bath at $60^{°}$C. To inhibit bacterial growth and ensure the medium was semi- selective for *Aspergillus* section *Flavi*, 5 ml of 4mg/ℓ dichloran (in acetone), 40mg/ℓ streptomycin (in 5mℓ distilled water) and 1 mg/ℓ chlortetracycline (in 10 mℓ distilled water) was added to the medium through a sterile 0.25 μm syringe filter after cooling to 50°C. The medium was then poured on to 90mm plates and allowed to settle for 2 to 3 days at room temperature before use.

Preparation of samples for plating was performed by thoroughly mixing the 500gram sample. One sub-sample (10g each) was weighed, mixed and kept aside for use. From the 10g ground samples, 3 replicates of 1.0 g each were placed in calibrated centrifuge tubes, into which 10 mℓ of 2 per cent distilled water was then added and mixed thoroughly. A volume of 0.1mℓ of the solution was then pipetted onto Modified Dichloran Rose Bengal medium in the 90mm Petri-plates under aseptic conditions. The plates were incubated for three days at $37^{°}$C, after which the colonies were identified and classified. Total colony counts and colony counts for *A. flavus* L-strain, *A. flavus* S-strain, *A. parasiticus*, *A. alliaceus*, *A. tamarii*, *A. niger, F. oxysporum* and *Penicillium* species per plate were recorded.
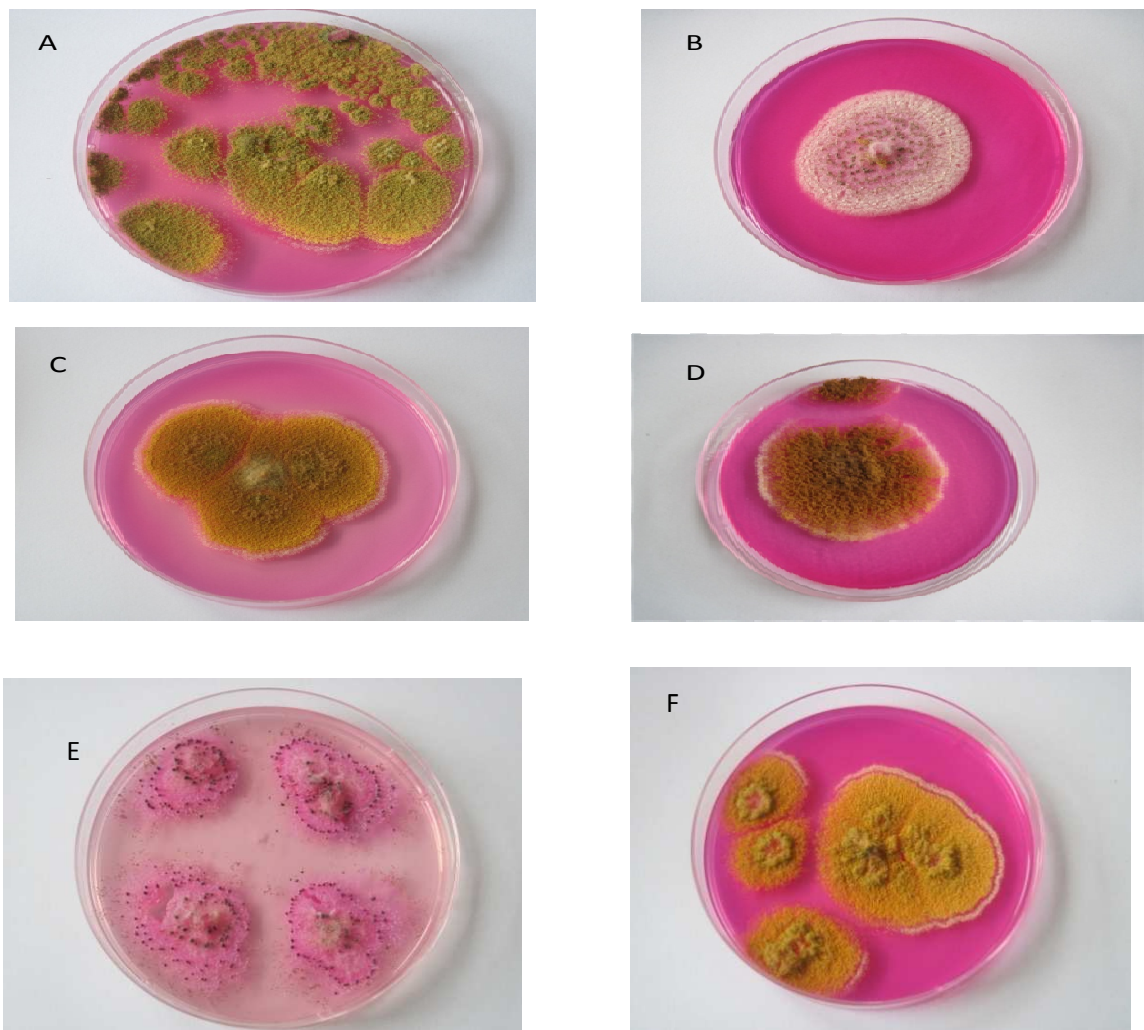
Figure 2: Reference cultures of various *Aspergillus* species on Modified Dichloram

  Rose Bengal Agar.

 (A) *A. flavus* L-Strain; (B) *A. flavus* S-Strain; (C) *A. caelatus*; (D) *A. tamarii*; (E) *A. alliaceus*;

(F) *A. parasiticus* cultures. Reference cultures were provided courtesy of Dr. Bruce Horn (USDA

Natural Peanut Research Laboratory, Dawson, Georgia, USA) and were used to assist in

identification of isolated fungi in *Aspergillus* section *Flavi*.

3.4.5 Sampling of maize grains at harvest

Grain samples were taken from crops growing in the field at harvesting stage. This is a period when the leaves and the kernels of the plant are very dry. A maximum of five maize samples were taken from each plot weighing 500g each. Random sampling was done where by samples were taken from each of the four corners of plots and one sample taken from the center of the plot. The five samples were then mixed thoroughly to make a composite sample from which the test samples were drawn each weighing 1g.

3.4.6 Determination of aflatoxin level in maize grain

1.0g sub-sample was drawn from each 100g sample and ground into a fine powder using a grinder. The ground sample was then sub-divided into two equal portions. The powder was triturated in 70 per cent methanol (v/v 70 ml absolute methanol in 30 ml distilled water) containing 0.5 per cent w/v potassium chloride in a blender, until thoroughly mixed. The extract was transferred to a conical flask and shaken for 30 min at 300 rpm. The extract was then filtered through Whatman No.41 filter paper and diluted 1:10 in phosphate buffered saline containing 500 $\mu\ell/\ell$ Tween-20 (PBS-Tween) and analyzed for aflatoxin with an indirect competitive ELISA kit. The aflatoxin quantities in every sample were then recorded (Gathumbi, 2001).

3.5 Statistical analyses

In order to determine the efficacy of Maxim XL 035 FS® at different concentrations, samples were grouped into experimental environments *(in vitro*, greenhouse, and field), different concentrations of Maxim XL 035 FS® and controls. For each concentration of the fungicide (0.5µl/ml, 1.0µl/ml, 1.5µl/ml fungicide per 1kg maize), the mean in each category was calculated, the actual colony counts converted into CFU/g substrate and plotted against different concentrations of the fungicide to obtain frequency distribution histograms. To test if the resulting frequency distributions were similar for different concentrations, the data was subjected

to t-test and analysis of variance (generalized linear model-GLM). Goodness of fit for the probability distribution models was assessed by analysis of deviance using GenStat14[th] edition. To identify factors associated with different levels of aflatoxin the samples were grouped into various different treatments. Categorical data analysis by means of contingency tables was used to assess for association between different concentrations of the fungicide and aflatoxin levels.

CHAPTER FOUR

4.0 RESULTS

4.1 Efficacy of Maxim XL 035 FS® under *in vitro* conditions

Different application rates of Maxim XL 035 FS® resulted in significantly (P≤0.05) different mean inhibition zones (Figure 5). The fungicide concentration of 0.5ml/ml resulted in the highest inhibition zone (Mean = 18.65mm), followed by 1.0µl/ml (Mean = 16.25mm) while concentration of 1.5µl/ml resulted in the lowest inhibition zone (Mean = 11.64mm).
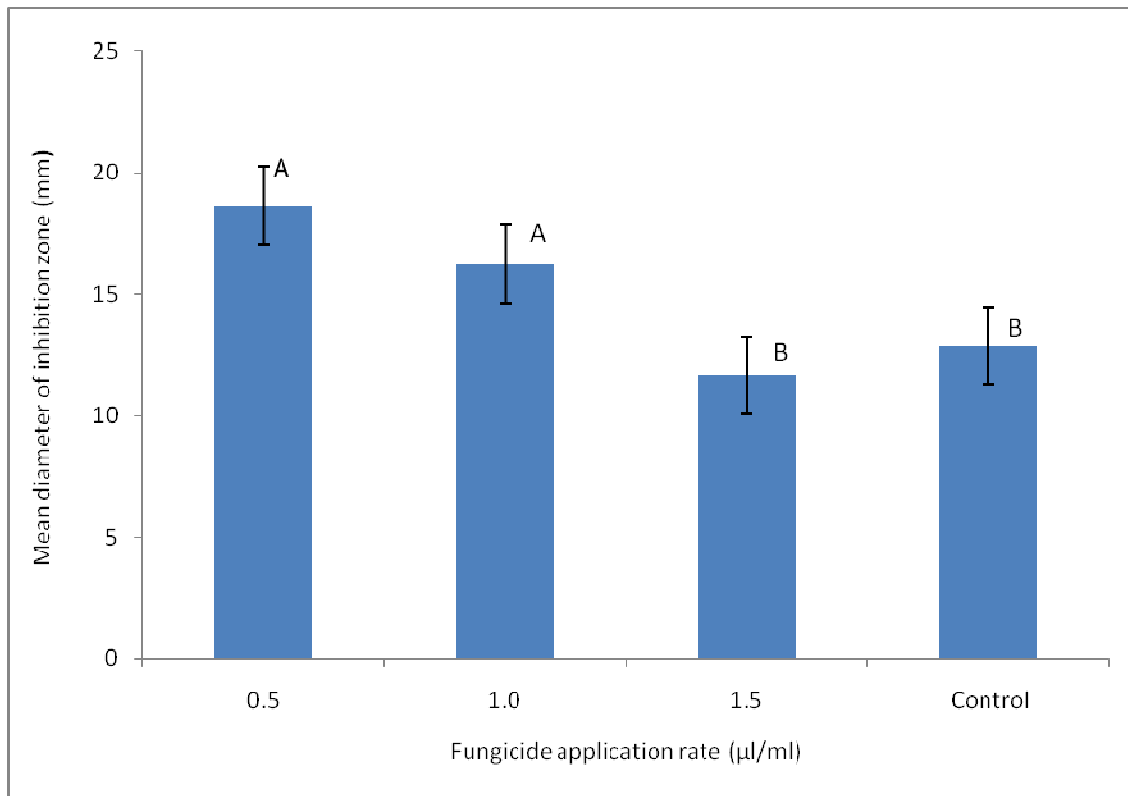
Figure 3: Mean diameter of inhibition zone (mm) at different concentrations (µl/ml) of Maxim XL 035 FS®.

Bars accompanied by similar letters are not significantly different (P≤0.05).

Error bars indicate the standard error of the means.

4.1.1 Effect of Maxim XL 035 FS® on selected fungal pathogens

There was a significant (P≤0.05) association between the effect of different concentrations of Maxim XL 035 FS® and the four test pathogens (Figure 6). The effect of Maxim XL 035 FS® was highest on *Penicillium* spp. which had a 19.22mm mean zone of inhibition followed by *F. oxysporum* (18.76mm) while the fungicide had a lower effect on *A. flavus* S-strain and *A. flavus* L-strain with means of 12.40mm and 11.98mm, respectively.
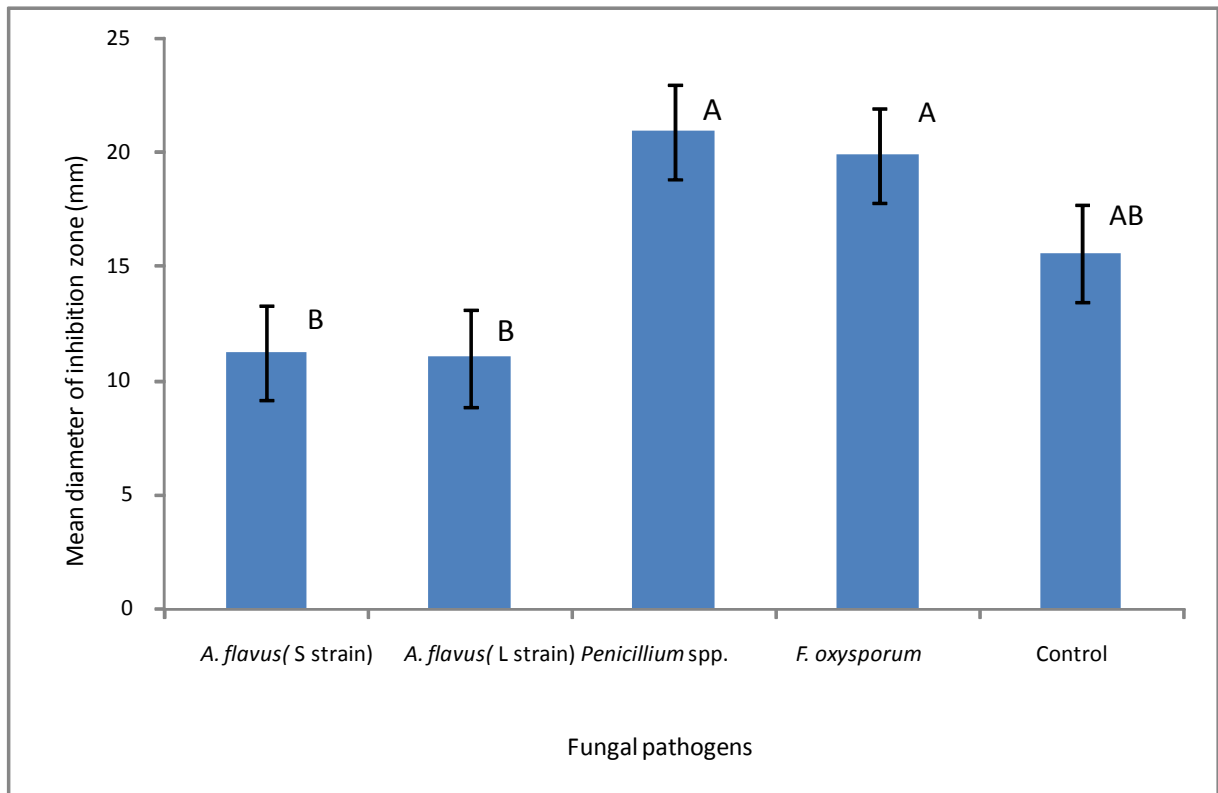
Figure 4: Mean diameter of inhibition zone (mm) of different test pathogens

Bars accompanied by similar letters are not significantly different (P≤0.05).

Error bars indicate the standard error of the means

There was a significant (P≤0.05) association between the diameter of inhibition zone and days of incubation (Table 3). Generally, the zone of inhibition decreased linearly with increase in the number of days. From the second day to the tenth day the mean diameter kept on reducing for the all test pathogens. The mean zone of inhibition decreased from 21.59mm (day two) to 18.93mm (day three), 17.04mm (day four), 14.69mm (day five), 13.09mm (day six), 12.40mm (day seven), 12.09mm (day eight), 11.91mm (day nine) and 11.98mm (day ten). In the first six days of incubation, there was a significant difference in the diameter of zone of inhibition while the differences in inhibition zone during the last two days were not significant.
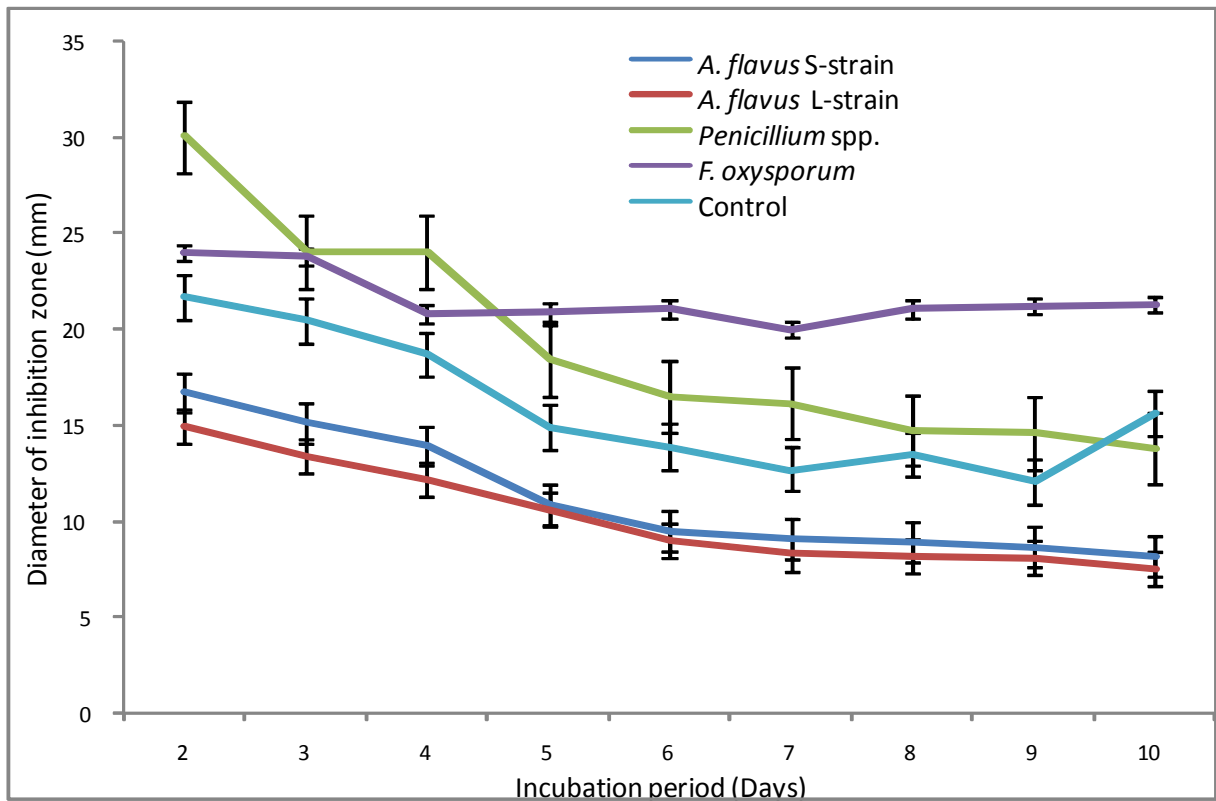
Figure 5: Diameter of inhibition zone (mm) of four test pathogens over a period of ten days.

Error bars indicate the standard error of the means for each of the sampling period.

Table 3: Inhibition zone (mm) of Maxim XL 035 FS® on *A. flavus* (L strain and S strains), *F. oxysporum* and *Penicillium* spp. for a

Period of ten days

Test pathogen	Fungicide concentration (µl/ml)	Incubation period (days)									Mean
		2	3	4	5	6	7	8	9	10	
A. flavus (S-strain)	0.5	18.3	17.5	16.7	13.1	12.4	12.0	11.9	11.5	10.4	13.8
	1.0	16.7	16.3	14.8	10.3	9.0	8.9	8.5	8.4	8.2	11.2
	1.5	18.7	13.6	12.5	12.0	8.5	8.0	8.0	7.8	7.6	10.7
	Control	13.0	12.8	11.4	8.3	8.1	7.4	7.0	7.0	6.4	9.0
	Mean	16.7	15.1	13.9	10.9	9.5	9.1	8.9	8.7	8.2	11.2
A. flavus (L-strain)	0.5	20.3	18.4	17.8	15.1	14.3	14.0	14.0	13.7	13.1	15.6
	1.0	20.0	19.9	15.8	13.7	10.7	9.6	9.6	9.6	8.7	13.1
	1.5	19.7	15.4	15.3	13.4	10.8	9.4	9.3	9.2	8.1	12.3
	Control	15.0	14.5	13.4	12.0	10.3	8.1	10.4	7.9	6.4	10.9
	Mean	15.0	13.4	12.2	10.6	9.0	8.3	8.2	8.1	7.5	10.3
Penicillium spp.	0.5	30.0	29.8	26.6	25.7	23.1	22.0	20.0	20.0	18.5	24.0
	1.0	30.0	22.7	21.3	20.2	16.6	16.0	16.0	15.8	15.5	19.3
	1.5	30.0	13.5	13.2	10.5	8.4	7.9	7.7	7.7	6.8	11.7
	Control	30.0	30.0	27.0	17.3	16.1	15.1	15.0	15.0	14.4	20.0
	Mean	30.0	24.0	22.0	18.4	16.1	26.5	14.7	14.6	13.8	20.0
F. oxysporum	0.5	28.7	27.3	22.9	20.5	19.7	18.8	17.9	17.7	17.7	21.2
	1.0	28.0	26.1	22.6	21.1	19.8	19.0	18.8	18.6	18.3	21.4
	1.5	17.0	14.8	11.9	11.8	10.9	10.1	10.0	10.0	9.7	11.8
	Control	25.0	24.6	22.9	22.1	21.0	20.0	19.7	18.6	28.3	22.5
	Mean	17.0	14.8	11.9	11.8	10.9	10.1	10.0	10.0	9.7	11.8
LSD (P≤0.05)		2.93	3.65	1.95	2.03	1.05	2.98	3.46	2.97	1.54	1.63

The mean inhibition zones of different test pathogens varied greatly at different fungicide concentrations (Figure 8). The lowest (mean=11.2mm) inhibition zone was observed on *A. flavus* S-strain while the highest was observed on *F. oxysporum* (mean=11.9mm) at the lowest fungicide concentration (0.5µl/ml). At the concentration of 1.0 and 1.5µl/ml, the lowest inhibition zone was observed in *A. flavus* S-strain while the highest was observed on *F. oxysporum*.
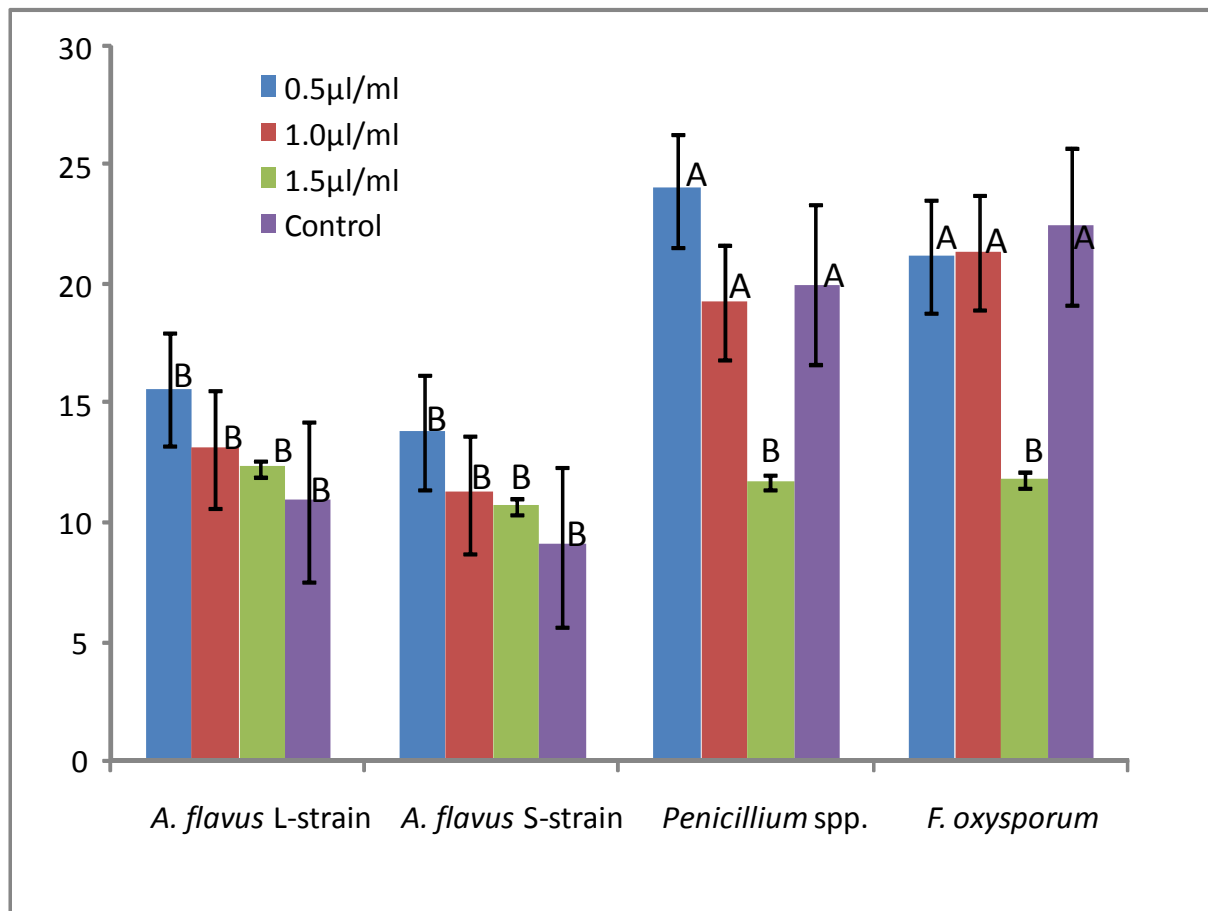
Figure 6: Mean diameter of inhibition zone (mm) of four fungi (CFU/g) tested against
different Concentrations of   Maxim XL 035 FS$^{®}$.

Bars accompanied by similar letters for each test pathogen are not significantly
different (P≤0.05).

Error bars indicate the standard error of means.

4.2 Efficacy of Maxim XL 035 FS$^{®}$ under greenhouse conditions

4.2.1 Effect of Maxim XL 035 FS$^{®}$ on shoot and root wet weight

Different application rates of Maxim XL 035 FS$^{®}$ resulted in significantly (P≤0.05) different
mean wet weights of maize seedlings (Figure 9). Application rate of 0.5µl/kg resulted in the

highest mean wet weight (mean=6.9g), followed by the application rate of 1.0µl/kg (mean= 4.79g) while application rate of 1.5µl/kg resulted in the lowest mean wet weight of 4.36g. There was no significant association (P≤0.05) between the effect of different concentrations of Maxim XL 035 FS® and the positive control.
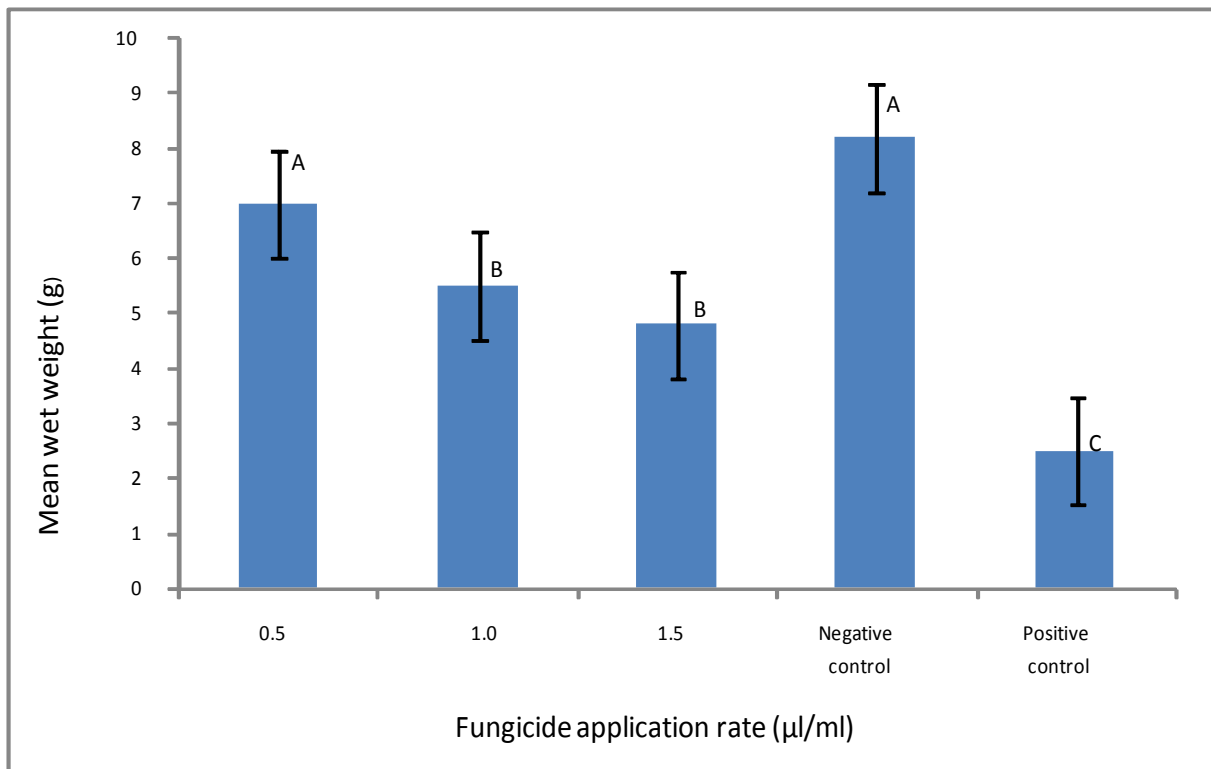
Figure 7: Mean wet weight (shoot and root) of the maize seedlings at different fungicide application rates.
Positive control – Inoculated with fungi but the seeds were not treated with Maxim XL 035 FS®.
Negative control – No inoculation with fungi, no application of Maxim XL 035 FS®.
Bars accompanied by similar letters are not significantly different (P≤0.05).

Error bars indicate the standard error of the means.

In the fourth and fifth week after germination, the shoot wet weights were higher than in the third week of germination (Table 4). The shoot wet weights of the controls were higher than

those of different fungicide application rates with the negative control having the highest (mean=2.88g).

Different application rates of Maxim XL 035 FS[®] also resulted in significantly (P≤0.05) different root wet weight (Table 4). Application rate of 0.5µl/kg resulted in the highest root wet weight (Mean=3.62g) while that of 1.5µl/kg had a mean of 1.6g. Application rate of 1.0µl/kg had the lowest root wet weight (Mean=1.01g).

The total wet weights after the third and fourth week were significantly different (P≤0.05), while similar weights determined at the fourth and fifth week after germination were not significantly different. The mean total wet weight for the third, fourth and fifth weeks were 4.57g, 8.52g and 9.99g, respectively.

Table 4: Shoot and root wet weight (g) of maize seedlings treated with different concentrations of Maxim XL 035 FS® and sampled three, four and five weeks after planting.

	Fungicide application rate (µl/ml)	Shoot wet weight (g)	Root wet weight (g)	Total wet weight (g)
a) Three weeks after planting				
	0.5	1.27	3.62	4.89
	1.0	1.97	1.01	2.98
	1.5	0.95	1.62	2.57
	Positive control	5.10	1.82	6.92
	Negative control	2.61	2.88	5.49
	Mean	2.38	2.19	4.57
b) Four weeks after planting				
	0.5	3.01	4.52	7.53
	1.0	2.26	2.61	4.87
	1.5	2.92	1.89	4.81
	Positive control	3.90	13.4	17.3
	Negative control	3.64	4.44	8.08
	Mean	3.15	5.37	8.52
c) Five weeks after planting				
	0.5	3.40	4.88	8.28
	1.0	3.30	3.21	6.51
	1.5	3.70	2.01	5.71
	Positive control	4.01	5.01	9.02
	Negative control	4.01	6.44	10.45
	Mean	3.68	6.31	9.99
	LSD (P≤0.05)	0.65	0.32	0.12

Positive control – Inoculated with fungi but the seeds not treated with Maxim XL 035 FS®

Negative control – No inoculation with fungi, no application of Maxim XL 035 FS®

4.2.2 Effect of Maxim XL 035 FS® on the population of selected fungal pathogens

Maxim XL 035 FS® had a significant (P≤0.05) effect on different fungal pathogens (Table 5). *A. flavus* L-strain had the highest isolation incidence three weeks after planting (mean=40.0 CFU/g), while *penicillium* spp. had the lowest (mean=31.0 CFU/g). The population of other non-inoculated fungi was 28.0CFU/g. At different growth stages, however, the incidences of different

fungal pathogens were not significantly different (P≤0.05). A pattern was noted whereby, the incidence of fungal pathogens decreased with the progress in growth stages.

Table 5: Incidence (CFU/g) of fungal pathogens in soil sampled at various growth stages from pots planted with maize seeds dressed with different concentrations of Maxim XL 035 FS[®].

Fungicide application rate (µl/kg)	*A. flavus* (L-strain)	*Penicillium* spp.	*F. oxysporum*	Others[a]
a) Three weeks after planting				
0.5	53	36	31	29
1.0	59	36	44	29
1.5	40	29	43	37
Positive control	34	28	46	24
Negative control	36	24	29	21
Mean	40	31	39	28
b) Four weeks after planting				
0.5	52	38	28	29
1.0	60	34	36	21
1.5	46	39	36	33
Positive control	34	42	25	24
Negative control	36	37	28	24
Mean	46	36	31	26
c) Five weeks after planting				
0.5	37	29	34	29
1.0	34	27	29	29
1.5	42	38	36	34
Positive control	36	25	24	27
Negative control	33	25	24	23
Mean	36	29	34	28
LSD (P≤0.05)	9.94	6.95	7.92	3.58

[a] Fungi which had not been inoculated in the soil.

Positive control – Inoculated with fungi but the seeds were not treated with Maxim XL 035 FS[®].
Negative control – No inoculation with fungi, no application of Maxim XL 035 FS[®].

Different application rates of Maxim XL 035 FS[®] resulted in significantly (P≤0.05) different mean incidence of the test pathogens (Figure 10). *A. flavus* L-strain had the highest re-isolation

incidence (mean=47.3CFU/g) at the application rate of 0.5µl/ml while *F. oxysporum* had the lowest incidence (mean=34.3CFU/g) at the same application rate. *A. flavus* L- strain showed the highest re-isolation incidence at all the application rates, while the re-isolation of *penicillium* spp. and *F. oxysporum* varied at different application rates.

Overall, at 0.5µl/ml concentration, the re-isolation incidence was lower for most fungal pathogens compared to the rest of the concentrations while at higher fungicide concentrations, the incidence of fungal pathogens was higher.
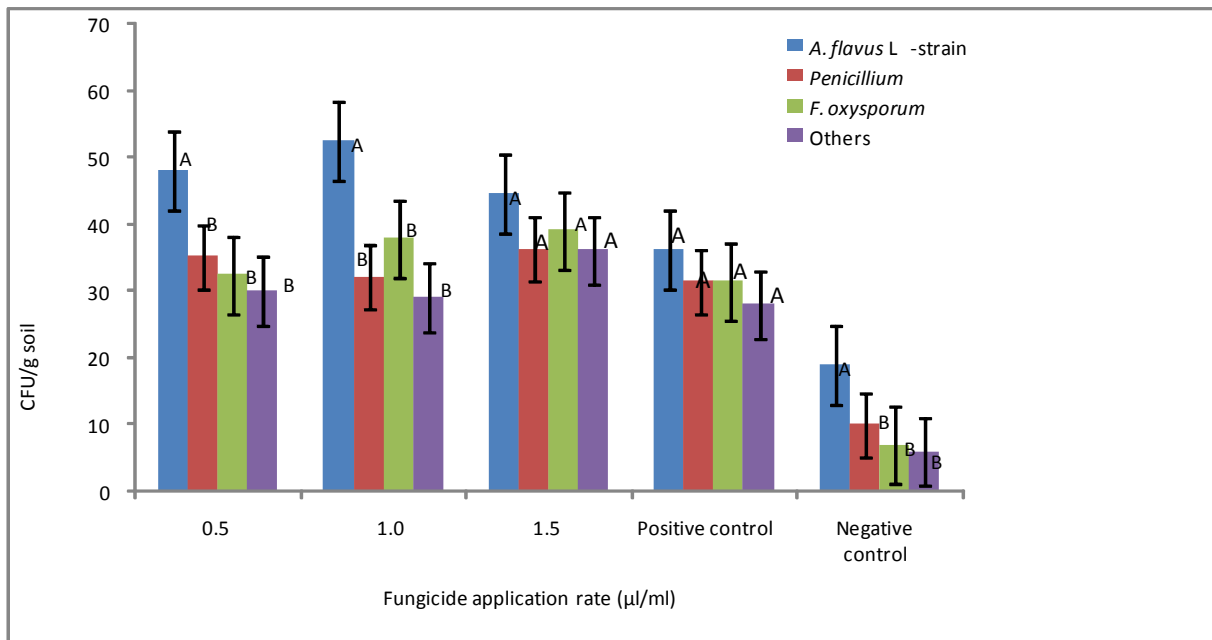
Figure 8: Incidence (CFU/g soil) of different fungal species in soil where maize seedlings were grown after treatment with various concentrations of Maxim XL 035 FS$^{®}$ under green house conditions

Positive control – Inoculated with fungi but the seeds not treated with Maxim XL 035 FS$^{®}$.

Negative control – No inoculation with fungi, no application of Maxim XL 035 FS$^{®}$.

Bars accompanied by similar letters for each application rate are not significantly different (P≤0.05).

Error bars indicate the standard error of the means.

At application rate of 0.5µl/ml, the isolation incidence of the fungi was lower compared to the rest of the concentrations four weeks after planting. However, five weeks after planting *A. flavus* L-strain exhibited less response to the fungicide compared to the other fungal pathogens (Table 6).

At four weeks after planting, the highest re-isolation incidence of the test pathogens was recorded in seedlings treated with 1.0µl/ml fungicide, while 0.5µl/ml resulted in the lowest fungal incidence (Table 6). Five weeks after planting, however showed a different trend whereby, treatment of seeds with a concentration of 0.5µl/ml resulted in the highest fungal incidence, while 1.0µl/ml resulted in the lowest incidence.

Table 6: Population (CFU/g) of fungal pathogens isolated from seedlings sampled at different growth stages of maize treated with Maxim XL 035 FS® at various concentrations

Fungicide application rate (µl/ml)	*A. flavus* L-strain	*Penicillium* spp.	*F. oxysporum*	Others
a) Four weeks after planting				
0.5	26	29	23	34
1.0	41	36	28	39
1.5	34	29	23	38
Positive control	31	39	22	29
Negative control	34	27	20	26
Mean	33	32	23	33
b)Five weeks after planting				
0.5	40	41	23	29
1.0	27	23	21	29
1.5	32	38	24	28
Positive control	28	40	25	33
Negative control	36	39	26	23
Mean	33	36	24	30
LSD (P≤0.05)	9.94	6.95	7.92	3.58

Positive control – Inoculated with fungi but the seeds were not treated with Maxim XL 035 FS®.

Negative control – No inoculation with fungi, no application of Maxim XL 035 FS®.

4.3 Maize crop under field conditions

Figure 11 shows the maize crop at different growth stages at KARI Kiboko Research Substation. The crop had been planted on a quarter an acre and the neighboring crops were legumes. The maize crop was grown under irrigation. The maize crop had been planted with different concentrations of Maxim XL 035 FS® fungicide. The fungicide had no effect on the growth and vigor of the crops, as all the maize plant grew at the same rate regardless of the dressing of seeds or not.

Figure 9: The maize crop at KARI Kiboko Research Substation at different growth stages.

(A) Maize crop three months after planting which marks the onset of flowering; (B) Maize crop at advanced flowering stage; (C and D) Maize crop during hard dough stage, four months after planting.

4.3.1 Population of fungi in maize debris

Figure 12 shows the incidence of various fungal pathogens in maize debris sampled before planting. *Aspergillus flavus* S-strain was the most commonly isolated fungi (68.9%) with *A. flavus* L-strain, *A. caeletus* and *A. alliaceus* also being isolated in high incidence. Other fungal

species commonly isolated from maize debris besides *Aspergillus* spp. were *Fusarium* and *Penicillium* spp. *Aspergillus parasiticus* was isolated from less than 20% of the maize debris.
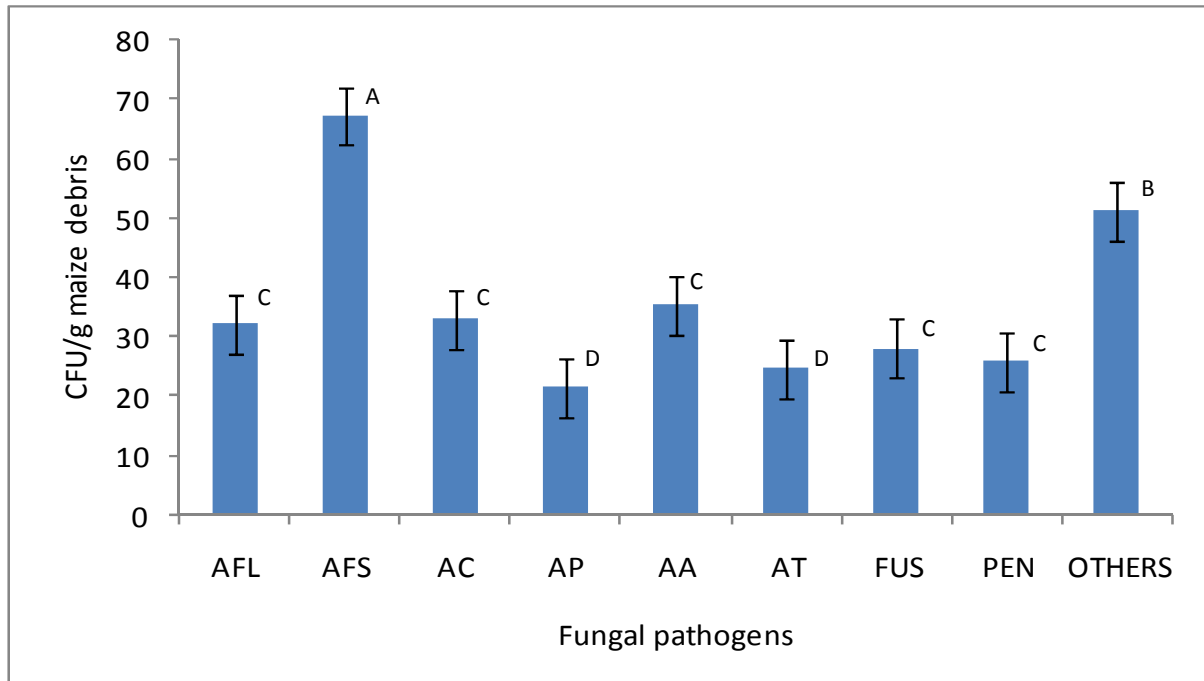
Figure 10: Incidence (CFU/g substrate $\times10^4$) of fungal pathogens isolated from maize debris

sampled before planting of the maize crop at Kiboko Research Substation.

Bars accompanied by similar letters are not significantly different (P≤0.05).

Error bars indicate the standard error of means.

AFL – *A. flavus* L-strain, AFS – *A. flavus* S-strain, AC – *A. caelatus*, AA – *A. alliaceus*, AT – *A. tamarii*, AP – *A. parasiticus*, FUS – *Fusarium* spp., PEN – *Penicillium* spp.

4.3.2 Population of fungi in soil

The population of the fungal pathogens in soil showed a significant difference (P≤0.05) at different growth stages (Figure 13). There was significantly (P≤0.05) higher population of fungal pathogens at three months after planting, while at the hard dough stage, the fungal population was statistically equivalent to population in soil sampled at harvest. The incidence of fungal pathogens before planting was the lowest.
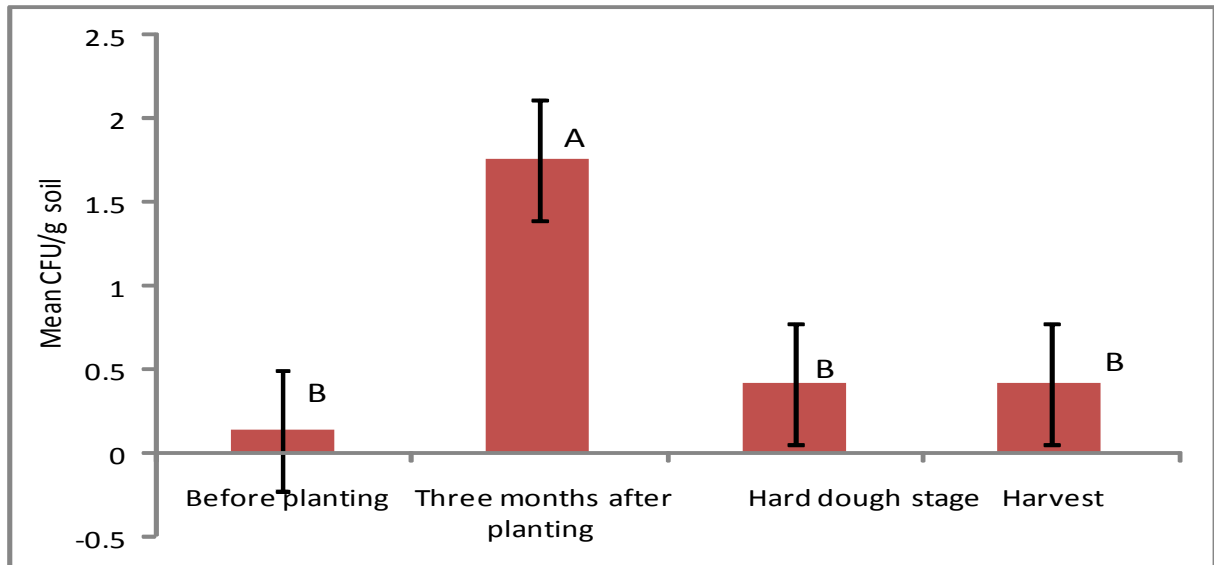
Figure11: Mean population of fungal pathogens (CFU/g soil $\times 10^4$) sampled at different growth

Stages of maize at Kiboko Research Sub-station

Bars accompanied by similar letters are not significantly different (P≤0.05).

Error bars indicate the standard error of means.

Table 7: Incidence (CFU/g soil $\times 10^4$) of fungal pathogens isolated from soil sampled at different growth stages of maize grown after treatment with various concentrations of Maxim XL 035 FS$^®$

Fungicide application rate (µl/ml)	AFL	AFS	AC	AA	AT	FUS spp.	PEN spp.	Others
a) Before planting								
0.5	53	70	37	37	49	37	30	48
1.0	39	58	41	37	39	30	24	40
1.5	40	56	35	34	37	21	24	44
Control	21	55	34	33	34	28	28	49
Mean	38	60	37	35	40	27	22	43
b) Three months after planting								
0.5	62	60	49	31	34	23	22	44
1.0	42	63	38	31	47	20	20	44
1.5	40	68	28	30	24	34	20	37
Control	41	39	26	31	25	23	31	46
Mean	46	50	35	31	33	25	24	43
c) Hard dough stage								
0.5	39	82	24	24	21	49	49	21
1.0	37	56	21	26	27	50	47	48
1.5	37	32	44	23	28	40	60	39
Control	37	66	44	34	29	45	55	37
Mean	38	59	33	32	26	46	53	36
d) At harvest								
0.5	38	91	29	33	28	51	41	29
1.0	25	99	21	34	27	62	41	40
1.5	29	49	37	42	28	38	58	34
Control	34	58	34	38	28	30	34	3^14
Mean	32	74	26	37	28	43	44	34
LSD (P≤0.05)	6.02	7.21	6.46	5.98	5.09	7.62	7.98	8.06

AFL – *A. flavus* L-strain, AFS – *A. flavus* S-strain, AC – *A. caelatus*, AA – *A. alliaceus*, AT – *A. tamarii*, AP – *A. parasiticus*, FUS – *Fusarium* spp., PEN – *Penicillium* spp.

There were significant (P≤0.05) differences in the isolation incidence of fungal pathogens. *A. flavus* S-strain was the most frequently isolated species ($49{\times}10^4$ CFU/g soil) followed by *A. alliaceus* at $29{\times}10^4$ CFU/g, while *A. tamarii* was the least frequently isolated (Figure 14). Other fungal pathogens isolated from soil included *Fusarium* spp. and *Penicillium* spp.
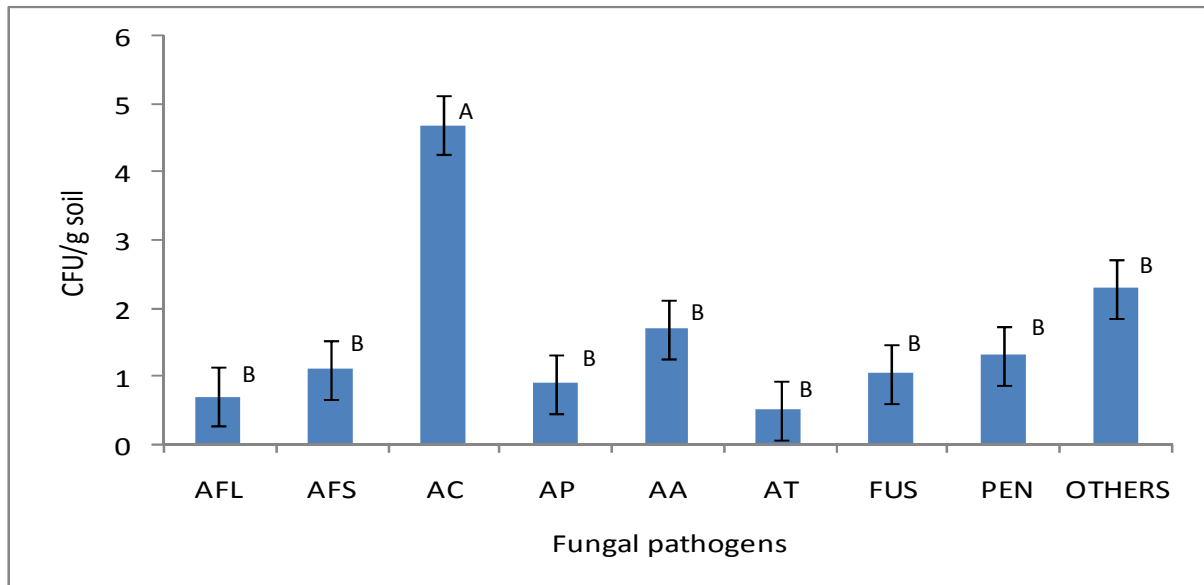
Figure 12: Incidence of fungal pathogens (CFU/g soil) in soil before planting at Kiboko Research Substation.

Error bars indicate the standard error of means.

Bars accompanied by similar letters are not significantly different (P≤0.05).

AFL – *A. flavus* (L-strain), AFS – *A. flavus* (S-strain), AC – *A. caelatus*, AA – *A. alliaceus*, AT – *A. tamarii*, AP – *A. parasiticus*, FUS – *Fusarium*, PEN – *Penicillium* spp.

Eight fungal pathogens were isolated from soil with *A. flavus* S-strain having the highest overall isolation incidence (Table 7; Figure 11). Table 7 shows that there was no significant difference (P≤0.05) between the fungal population and the fungicide application rate. At the application rate of 0.5ml/kg, the fungal incidence was higher than for the other concentrations. However, as the season progressed, the application rates had a significant (P≤0.05) correlation with the fungal

incidence. Regardless of the fungicide application rate, the most predominant species isolated from soil was *A. flavus* S-strain followed by *A. flavus* L-strain.

4.3.3 Population of fungi in maize tissues

Different application rates of Maxim XL 035 FS$^®$ resulted in significantly (P≤0.05) different incidence of fungal pathogens in maize tissues (Table 8). Application rate of 0.5µl/ml resulted in the highest overall fungal incidence (mean = 46.22×10^4 CFU/g); followed by application rate of 1.0µl/ml (mean = 38.9×10^4 CFU/g). Application rate of 1.5µl/ml resulted in the lowest fungal population. There was a strong association (P≤0.05) between application rate and the population of various fungal pathogens. The level of significance between the application rate and the fungal population was highest at hard dough stage.

Table 8: Incidence (%) of fungal pathogens isolated from stems and kernels (CFU/g) sampled at different growth stages of maize treated with Maxim XL 035 FS® at various concentrations.

Fungicide application rate (µl/ml)	AFL	AFS	AA	AS	AT	AP	FUS spp.	PEN spp.	Others
a) Maize tissues three months									
0.5	46	60	26	24	28	45	49	59	48
1.0	34	64	32	31	23	38	44	49	44
1.5	37	51	50	44	30	36	47	40	41
Control	29	55	57	39	35	29	34	46	24
c) Kernels at hard dough stage									
0.5	40	52	21	29	31	29	36	28	37
1.0	46	66	30	32	22	24	38	24	33
1.5	42	82	36	32	21	20	29	36	28
Control	40	56	37	32	20	27	30	33	34
d) Kernels at harvest									
0.5	39	48	29	20	22	26	35	23	35
1.0	40	58	21	28	28	20	33	23	30
1.5	38	74	36	26	22	20	27	37	26
Control	37	50	30	23	32	25	30	23	31
LSD (P≤0.05)	1.05	1.43	0.44	0.34	0.38	0.45	0.95	1.06	0.63

AFL – *A. flavus* (L-strain), AFS – *A. flavus* (S-strain), AC – *A. caelatus*, AA – *A. alliaceus*, AT – *A. tamarii*, AP – *A. parasiticus*, FUS – *Fusarium* spp., PEN – *Penicillium* spp.

At different growth stages, Maxim XL 035 FS® had different effects on the fungal pathogens (Figure 15). The population of fungal pathogens was higher at hard dough stage than at harvest. The action of Maxim XL 035 FS® against different fungal pathogens at different growth stages showed that the fungal population decreased as the season advanced.
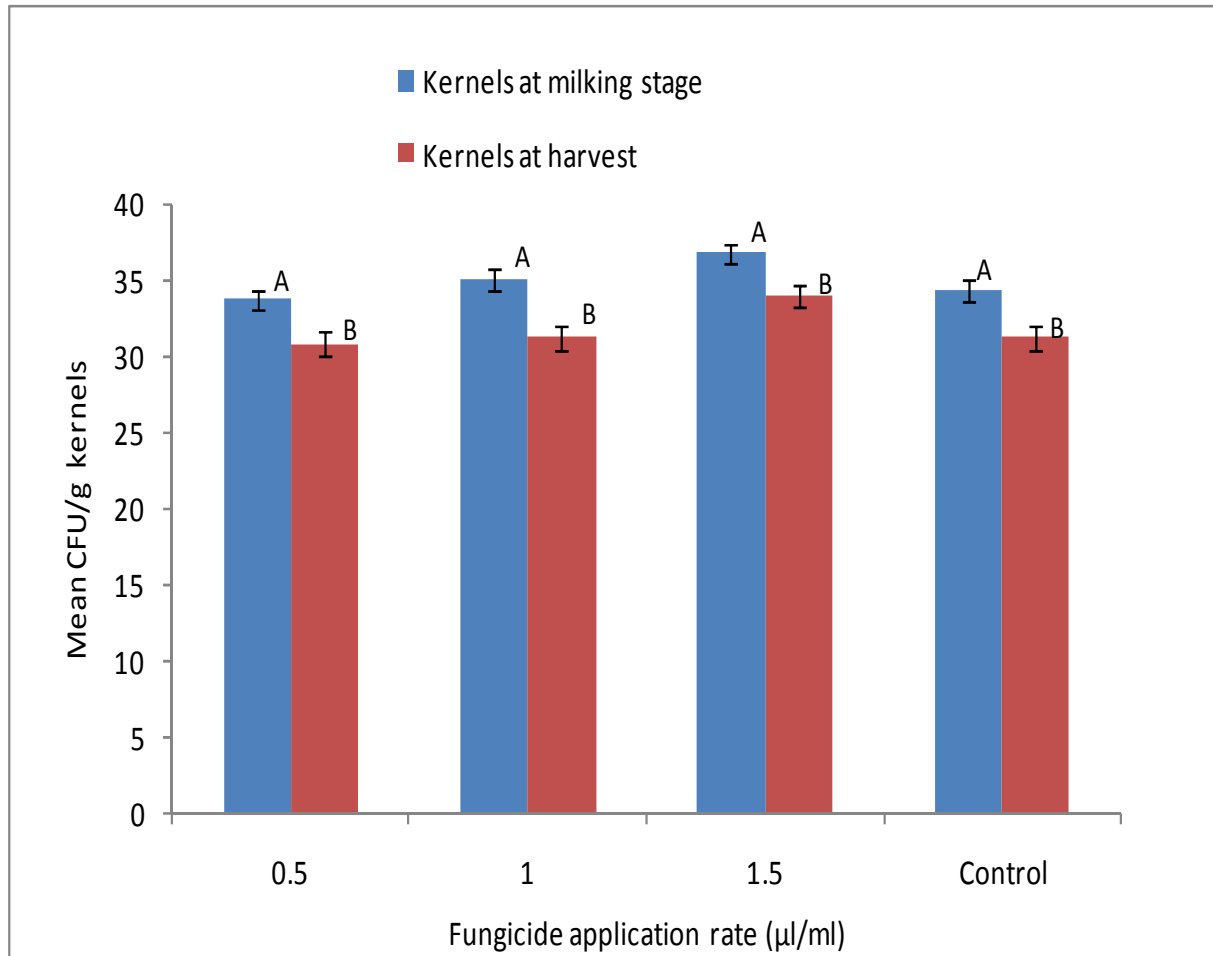
Figure 13: Incidence (CFU/g kernels) of different fungal pathogens in kernels sampled at hard

dough stage and at harvest.

Bars accompanied by similar letters for each application rate are not significantly

different (P≤0.05).

Error bars indicate standard error of means.

Seven fungal pathogens, *A. flavus* L-strain, *A. flavus* S-strain, *A. caelatus, A. tamarii, A.*

parasiticus, F. oxysporum and *Penicillium* spp. were identified from kernels sampled at harvest

(Figure 16). The predominant fungi were *A. flavus* L-strain, *A. flavus* S-strain, *Penicillium* spp.

and *Fusarium* while *A. parasiticus*, *A. tamarii* and *A. caelatus* were isolated in lower frequency.
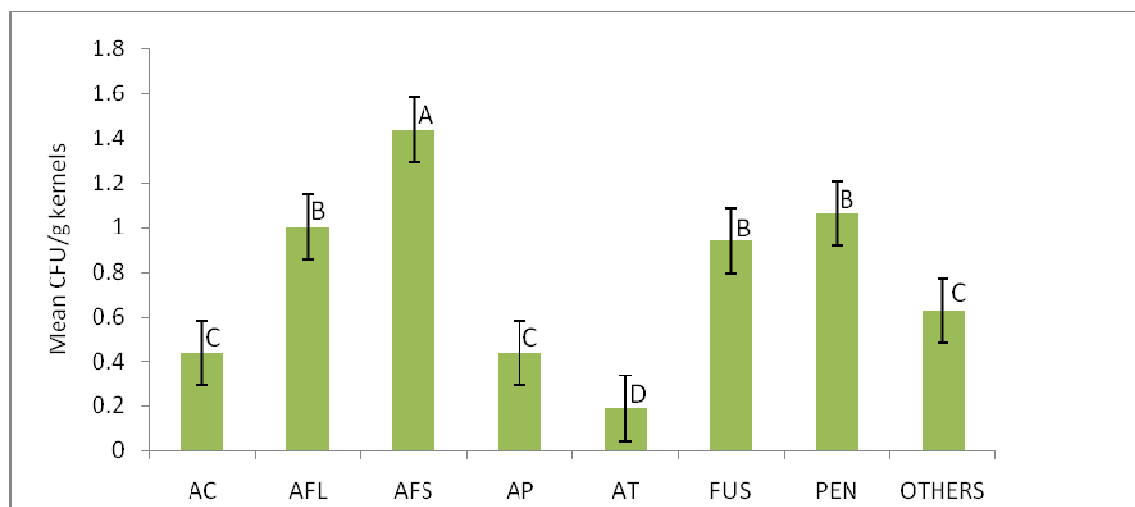
Figure 14: Mean population (CFU/g) of various fungi isolated from harvested kernels.

AFL – *A. flavus* (L-strain), AFS – *A. flavus* (S-strain), AC – *A. caelatus*, AA – *A. alliaceus*, AT – *A. tamarii*, AP – *A. parasiticus*, FUS – *Fusarium* spp., PEN – *Penicillium* spp.

Bars accompanied by different letters are significantly different (P≤0.05).

Error bars indicate the standard error of the means.

4.4 Aflatoxin levels in maize kernels

The levels of aflatoxin in maize kernels sampled at harvest ranged from 0 to 93.0ppb (Figure 15).

Overall, 44% of the samples had undetectable levels of aflatoxin while 56% had aflatoxin levels above the detection limit (2ppb). Kernels from maize plants treated with 1.5ml/kg of Maxim XL 035 FS[®] had significantly (P≤0.05) lower total aflatoxin concentration compared to kernels from plants treated with a concentration of 0.5 and 1.0ml/kg. However, the total aflatoxin levels in kernels from plants treated with the latter two concentrations were not significantly different and were significantly higher than the kernels from the non-treated control plants. There was no positive correlation between the population of fungal pathogens with the aflatoxin levels.
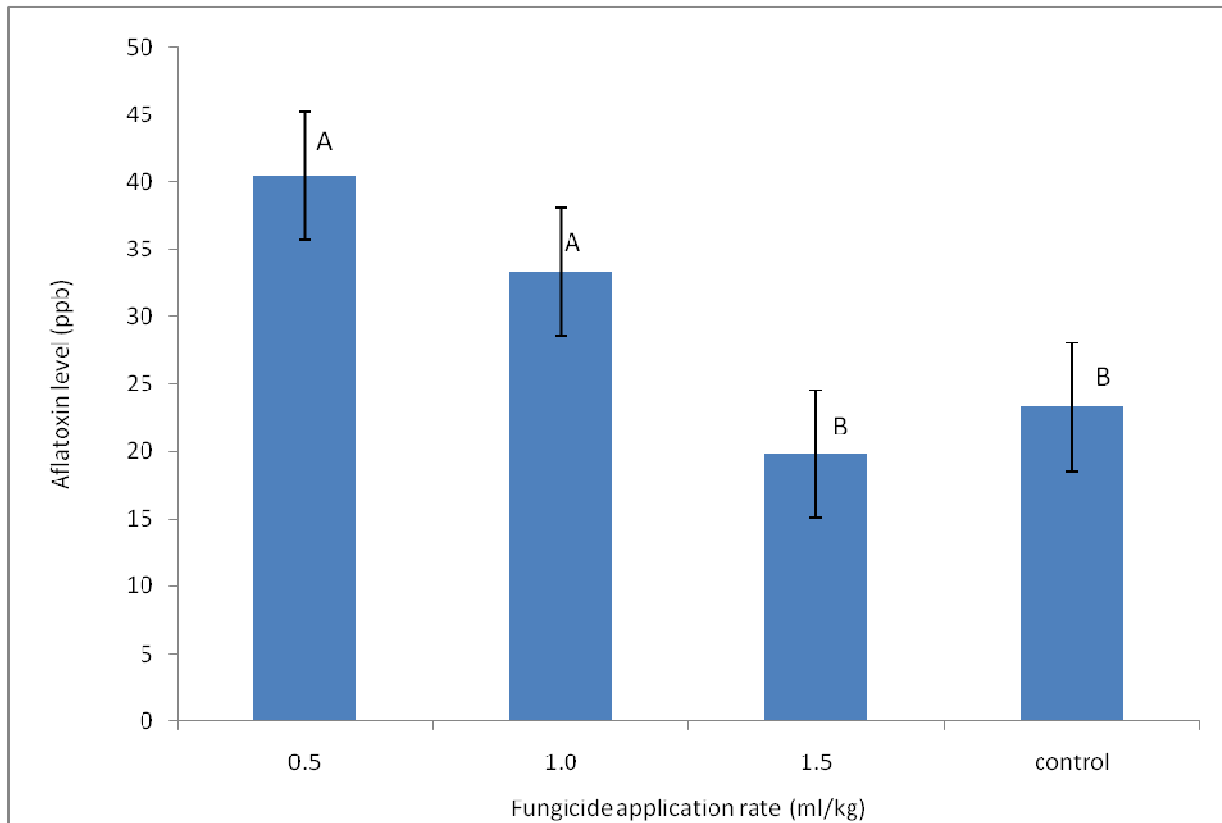
Figure 15: Total aflatoxin levels (ppb) in maize kernels sampled at harvest from maize

planted after treatment with different concentration of Maxim XL 035 FS[®].

Bars accompanied by different letters are not significantly different (P≤0.05).

Error bars indicate the standard error of the means.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

This study provides information on the occurrence of various *Aspergillus* species in soil sampled from a maize field in Kiboko Research Substation in Makueni County and their progression in maize crop. The efficacy of Maxim XL 035 FS® on *Aspergillus* species as well as aflatoxin contamination of maize kernels is also documented. The S-type *A. flavus* was the most prevalent pathogen associated with contamination of maize in Kiboko Research Substation, and therefore management strategies to reduce aflatoxin contamination of maize at the station should focus on this species. Researchers are seeking to better understand the causes of aflatoxin contamination and to identify cost-effective techniques to reduce the risk. Since Maxim XL 035 FS® is a phenylpyrrole fungicide that is relatively cheap, whose efficacy on the aflatoxin producing *Aspergillus* spp. is unknown. It was therefore necessary to investigate its effect on *Asper*Bandyopadhyay spp. and aflatoxin contamination of maize.

5.1.1 Efficacy of Maxim XL 035 FS® under *in vitro* conditions

The results from this study showed that Maxim XL 035 FS® had appreciable effect on the four test pathogens. The fungicide had the highest efficacy at lower concentration. The action of Maxim XL 035 FS® against different fungal pathogens showed that it was least effective against *A. flavus* L-strain followed by *A. flavus* S-strain. The fungicide had the greatest effect against *Penicillium* spp. and *F. oxysporum*. *A. flavus* is the most serious contaminant of maize and therefore limited activity of Maxim XL 035 FS® against this pathogen calls for a broader approach in managing the fungi. Additionally, the low efficacy of Maxim XL 035 FS® in controlling *A. flavus* implies that less exposure of harvested maize kernels to factors that favor aflatoxigenic *Aspergillus* is still paramount.

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There are several efforts in Kenya today focusing on reducing aflatoxin contamination in maize that include biocontrol (Bandyopadhyay, *et al*., 2011) and promotion of less susceptible maize varieties (Jagger, 2011). These efforts should also consider incorporation of Maxim XL 035 FS[®] as a fungicide in control of various fungal pathogens associated with maize and the importance of sensitization of actors in maize production on the importance of fungicides. Since Maxim XL 035 FS[®] showed some activity against *A. flavus* S-strain and L-strain although limited, the possible mechanism of action could be the activity of phenolic compounds that inhibit the mycelia growth and sporulation of *A. flavus*. Voriconazole fungicide is also a good antifungal agent against *Aspergillus* and *Fusarium* (Atehnkeng *et al*, 2008). When the fungicide was used at lower concentration, the inhibition zone was higher for *Aspergillus, Fusarium oxysporum* and *Penicillium* spp. (D'mello *et al,* 2003). This may have been due to the antagonistic nature of some of the *Aspergillus*.

5.1.2 Efficacy of Maxim XL 035 FS[®] under greenhouse conditions
This study showed that Maxim XL 035 FS[®] had no significant effect on the growth of maize.

It was evident that Maxim XL 035 FS[®] was effective at a higher concentration as it reduced the fungal incidence of *A. flavus* L-strain, *Penicillium* and *F. oxysporum* in the soil after inoculation. The higher the concentration of the fungicide, the lower the fungal population and the lower the fungicide concentration the higher the fungal population. This study also showed that Maxim XL 035 FS[®] was more effective on *A. flavus* L-strain at a higher concentration than at a lower concentration. A study done on the effect of Maxim XL 035 FS[®] (Fludioxonil and Metalaxyl-m) at the Gezira Research Station Farm in Sudan against the seedling disease of cotton, showed a significant improvement of crop standards and seed cotton yield over the untreated control, apparently due to substantial reductions in seed bed losses (Mahir *et al*., 1998). The cotton yields

of all tested rates of Maxim XL 035 FS® were comparable to that of the standard treatment of 1.0ml/L (D' Mello *et al*., 1998). Maxim XL 035 FS® had no significant effect on the growth of maize but had an effect on fungal pathogens and not on the germination and vitality of the maize crop. This indicates that the use of this fungicide at higher concentration could reduce the population of aflatoxin producing fungal population.

5.1.3 Efficacy of Maxim XL 035 FS® under field conditions

This study showed that there were many fungal species present in the study area, eight of which were identified. Of importance were aflatoxins producing *Aspergillus* spp. as well as other fungal genera that are known to produce mycotoxins. The predominant species in maize debris before planting were *A. flavus* S-strain, *A. flavus* L-strain and *A. alliaceus* with an incidence of 68.9% for *A. flavus* S-strain and L-strain. In the soil before planting, the fungus with the highest incidence was *A. caelatus*, but after planting the fungi isolated in the highest incidence were *A. flavus* S-strain and *A. flavus* L-strain.

The high incidence of *A. flavus-S* strain that produces aflatoxin (Cotty and Cardwell, 1999; Egel *et al.,* 1994) and in particular, the more potent aflatoxin B1 and B2, implies a risk of aflatoxin contamination of maize from Kiboko Research Substation. In as much as the incidence of *A. flavus* L-strain was high, it did not lead to a positive correlation with aflatoxin and this could be attributed to the fact that some of the *A. flavus* L-strains are known to be atoxigenic (Cotty and Cardwell, 1999; Egel *et al.,* 1994). Since the factors that trigger aflatoxin production are not well understood, vigilance in pre- and post-harvest handling of maize is needed to avert the risk of human exposure because the toxins can be produced at all stages (Horn *et al*., 1996). The confirmation of occurrence of other species that produce toxins, such as *A. tamarii* which produces cyclopiazonic acid (Horn *et al*., 1996) and *A. alliaceus* that produces ochratoxin A

(Bayman *et al.*, 2002), underscore the need to screen maize not just for aflatoxin, but also for other detrimental mycotoxins.

In the current study, several fungi were isolated from maize seeds after harvest including *A. flavus* L-strain, *A. flavus* S-strain, *A. tamarii, A. alliaceus, A. parasiticus, A. nomius, Fusarium* spp. and *Penicillium* spp. The high fungal population densities reported in Kiboko Research Sub-station means that households are not only exposed to the dreaded aflatoxins but also to other mycotoxins. This is comparable to the study done on the pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens, where the pesticide was seen to be effective at higher concentration (D' Mello *et al.*, 1998).

Maxim XL 035 FS® significantly inhibited mycelia growth of *A. flavus* at 250ppm and significantly decreased aflatoxin production at 100, 250 and 500ppm respectively (D' Mello *et al.*, 1998). Therefore in order to develop future strategies to ameliorate aflatoxin contamination in maize at Kiboko Research Substation, it is important to characterize the fungal population further. Mycotoxin contamination in maize depends on the coincidence of host susceptibility, environmental conditions favorable for infection, and, in some cases, vector activity (Munkvold, 2003). Because of the importance of timing in the events leading to infection, a change in planting date can significantly affect mycotoxin accumulation (Munkvold, 2003). Higher temperatures and drier conditions favour infection by *A. flavus* and the development of aflatoxin in maize prior to harvest (Diener *et al.*, 1987; Jones *et al.*, 1981) and aflatoxin contamination of maize frequently accompanies heat and water stress that may accompany drought (Guo *et al.*, 2005).

Aflatoxin levels in harvested kernels were high at the application rate of 0.5ml/kg as compared to 1.5ml/kg where the aflatoxin level was low (mean=24ppb). It was observed that the less

concentrated the fungicide, the higher the total aflatoxin levels. Thus, a concentration of 1.5ml/kg is recommended for use in reduction of aflatoxin levels in maize.

5.2 CONCLUSIONS

Aspergillus flavus more particularly *A. flavus* S-strain was the most common *Aspergillus* strain in Kiboko Research Substation. However *A. flavus* L-strain was also present in high incidence, which indicates the possibility of chronic exposure of maize consumers to aflatoxin produced by these strains. The influence of aflatoxins on human populations in Kenya over the past decade demonstrates a clear need for tools to manage contamination of locally produced maize. Given the widespread nature of *A. flavus* and its associated risk of aflatoxin contamination of maize, it is desirable to include a fungicide intervention in the field.

The incidence of aflatoxin producing fungi including *A. flavus* S-strain and L-strain was high; however, the incidence of other pathogens of *Aspergillus* section *Flavi* such as *A. caeletus*, *A. tamarii* and *A. alliaceus* was low. The presence of *Aspergillus* species in the soils and on harvested kernels indicates that *Aspergillus* inoculum is widespread in both the environment, soil and kernels in the study area. The inoculum in soil, crop and maize debris acts as the primary source of inoculum that infects maturing maize crop. Thus, elimination of inoculum sources such as infected debris from the previous harvest may prevent infection of the crop.

In addition, since Maxim XL 035 FS$^{®}$ has been found to have some activity against aflatoxigenic fungi and reduction of aflatoxin levels in maize kernels, farmers should be encouraged to use it as a seed dresser. The fungicide was effective at the highest concentration tested than at lower concentrations.

The aflatoxin levels of harvested kernels ranged from 0 to 93ppb. Maxim XL 035 FS$^{®}$ was effective against aflatoxin contamination as 60% of the samples at harvest were suitable for

human consumption based on the KEBS standards of ≤10 ppb for aflatoxin total (KEBS, 2007). The reason for carrying out the study under three conditions was to compare the results under controlled and uncontrolled conditions. Further it was to compare the results when other fungi are present and when they are absent like in the greenhouse and the field conditions.

5.3 RECOMMENDATIONS

i. The high incidence of *A. flavus* S-strain, which usually produces aflatoxin B1 and B2, underscores the need for more vigilance and implementation of preventive measures that reduce the risk of aflatoxin accumulation in contaminated maize.

ii. The isolation of mixed cultures of fungi shows that it is likely that maize in Kiboko is contaminated with more than one type of mycotoxin. Further studies are required to determine if this is the case. Planting improved maize cultivars, combined with good crop management and post-harvest handling practices should be explored to deter the proliferation of fungal species and reduce the risk of mycotoxins contamination.

iii. Further studies should also be done to ascertain whether Maxim XL 035 FS$^®$ can reduce the population of other fungal pathogens such as *A. parasiticus, A. nomius* and *A. alliaceus* that produces mycotoxins in maize.

iv. Results from the current study on effect of Maxim XL 035 FS$^®$ on *Aspergillus* species under different conditions did not show a consistent pattern. Further studies are required to establish a consistent effect of the fungicide against these fungi.

v. The impact of Maxim XL 035 FS$^®$ on *Aspergillus* was not as high hence the need to evaluate other fungicides against *Aspergillus.*

5.4 Further studies

Recently, the tolerance level for total aflatoxin in maize and other food commodities by the Kenya Bureau of standards was lowered from 20μg/kg to 10μg/kg. As a possible control measure, research on the use of Maxim XL 035[®] on other mycotoxin producing fungi in maize needs to be pursued. Further research on Maxim XL 035[®] to ascertain its reduction of fungal pathogens that are harmful to the maize crop, as this would be a cost effective means of managing aflatoxins in maize. The research should be built on the high presence of *A. flavus* S-strains found in the region.

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