EFFICACY OF SELECTED FUNGICIDES ON FUNGI ISOLATED FROM MYCOTOXIN CONTAMINATED MAIZE IN KENYA

JOSEPHAT KIPYEGON TONUI

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Efficacy of selected fungicides on fungi isolated from mycotoxin contaminated maize in Kenya

Josephat Kipyegon Tonui

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DECLARATION

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

Signature…….. Date……..

Josephat Kipyegon Tonui

This thesis has been submitted for examination with my approval as University Supervisors.

Signature…….. Date……..

Dr. Christine Bii

KEMRI, Kenya

Signature…….. Date……....

Prof. Ciira Kiiyukia

BMAK, Kenya

DEDICATION

I dedicate this work to my loving and caring parents Mr. John and Mrs. Betty Matingwony who have always supported and encouraged me in my studies. I also dedicate it to my brother Gilbert and my sisters Sheila, Valarie and Sharon. May the fruits of this achievement bring you joy and happiness.

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ABREVIATIONS AND ACRONYMS

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
AJFAND	African Journal of Food, Agriculture, Nutritionand Development
Aw	Water Activity
CDC	Centers for Disease Control
DMIs	Sterol demethylation inhibitors
DON	Deoxyivalenol
ERC	Ethical Review Committee
SSC	Scientific Steering Committee
FB1	Fumonisin B1
GARJAS	Global Advanced Research Journal of Agricultural Sciences
HIGS	Host induced gene silencing
IITA	International Institute of Tropical Agriculture
KEMRI	Kenya Medical Research Institute
Ks	Kisumu
Kt	Kitale
LPCB	.Lactophenol cotton blue
Mbs	Mombasa

Mc	Machakos
Mg	Magnification
miRNA	microRNA
NOI	Niche Overlap index
Nrb	Nairorbi
OTA	Ochratoxin A
ppb	parts per billion
QoI	Quinone outside Inhibitor Fungicides
RNA	Ribonucleic acid
RNAi	Interference of Nucleic Acids
SDA	Sabourauds Dextrose Agar
siRNAs	Small interfering RNAs
USDA-ARS	United States Department of Agriculture-Agriculture Research Service
Wp	Wetable powder

ABSTRACT

Contamination of maize by fungi represents significant health and economic problems in developing countries as well as the developed world. Fungicides are used to control plant fungal infections in fruits and vegetables but can be useful in controlling fungal infestation of maize in the field. This study was carried out to determine the *in vitro* efficacy of four fungicides on fungi isolated from mycotoxin contaminated maize from different regions in Kenya. A total of 138 maize samples which were part of the study KEMRI-SSC 2151 that tested positive for aflatoxin and fumonisins were subjected to mycological analysis in this study. The samples were collected from Machakos, Nairobi, Mombasa, Kitale and Kisumu. The fungicides Milraz, Antracol, Mistress and Victory were prepared according to the manufacturer's instructions and applied on four maize kernels from each sample before direct plating on Sabourauds dextrose agar (SDA). Untreated maize kernels from each sample were also inoculated on SDA concurrently as controls. The plates were incubated at 30^{o}C for 72 hours after which fungal growth on the kernels was examined and scored in the range of 0 to 100% infestation. Percentage germination of maize kernels on the culture plates was also scored in the range of 0 to 100%. Fungi growing on the maize kernels were identified using macroscopic and microscopic features. The activity of the test fungicides on pure fungal isolates was determined by disk diffusion method. Twenty microlitres of each diluted fungicide was impregnated on 6 mm disks and placed aseptically at the centre of the culture in three replicates before incubation at 30^{o}C for 72 hours. Zone of inhibition on the disks was measured to the nearest millimeter. Pearson correlation analysis was used to analyze the effect of the test fungicides on maize germination.

The mycotoxin positive maize samples were significantly infested by fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium*. There was a significant difference ($p<0.05$) in fungal infestation per region where Mombasa had the lowest infestation of 72.5% while those from Nairobi had the highest infestation of 99.1%. There was a significant inhibition [$p<0.05$ (0.00)] of fungal growth on the treated maize kernels compared to the untreated. Twenty six percent and 34% of mycotoxin contaminated maize samples treated with Mistress and Victory fungicides respectively were not

infested, while those treated with Milraz and Antracol were 10% and 14%respectively. Thirty one isolates were found to be resistant to more than one of the test fungicides. There was a significant positive correlation [(R^2=0.054, $p<0.05$)] between germination and fungicide treated maize kernels. This work has demonstrated the potential use of the test fungicides for the control potentially toxigenic fungi affecting maize. The study also underscores the diversity and existence of fungicide resistant fungi in mycotoxin contaminated maize. Field experiments should be conducted to ascertain the field efficacy of the four fungicides as well as the extent of resistance to other fungicides that could impact the use of related antifungal drugs used clinical settings for treatment of fungal infections.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Fungi are often found as contaminants in agricultural products before or after harvest as well as during transportation and storage. Mycotoxins are secondary metabolites of certain species of fungi which when ingested causes adverse effects on humans and animals resulting in illnesses and economic losses (Hussein & Jeffrey, 2001). Aflatoxins, trichothecenes, ochratoxins, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance (Richard *et al.,* 2003). Some fungi have the ability to produce more than one mycotoxin and some mycotoxins are produced by more than one fungal species (Hussein & Jeffrey, 2001).

Fungal growth and mycotoxin production in food grains is influenced by various factors. Climatic conditions, especially temperature and humidity, play a very important role in this process. High moisture content in cereal grains during harvest and storage encourages the growth of mycotoxin producing fungi (Niaz, Dawar, & Sitara, 2011). Fungi growing in maize grains could be grouped in general as field and storage fungi. The genera *Aspergillus* and *Penicillium* are typical storage fungi but their occurrence in maize during harvest is also possible. *Fusarium* species represent field fungi and their higher amounts in cereals are connected with a higher humidity and colder weather (Senbeta & Gure 2014).

More than 100, 000 species of fungi producing over 300 metabolites which have a toxic potential for humans and animals have been identified (Čonková *et al.,* 2006). It is known that fungi of the genera *Aspergillus* and *Penicillium* produce carcinogenic mycotoxins i.e aflatoxins and ochratoxins respectively. The main mycotoxins produced by *Fusarium graminearum* are deoxynivalenol, nivalenol and

zearalenone, while *Fusarium verticillioides* produces primarily fumonisins B1, B2 and moniliformin (Marasas, 1996). Animals affected by fumonisin toxicity may have reduced weight gain and productivity as well as immunological impairment (Wu & Munkvold, 2008). Recent epidemiologic studies have associated exposure to fumonisins in Africa with an increased susceptibility to HIV infection as a result of immunosuppression (Williams *et al.,* 2010). Exposure to mycotoxins and the symptoms depend on the type of mycotoxin, the concentration and length of exposure as well as age, health status, and the gender of the exposed individual. Various mycotoxins in food and feed are nephrotoxic, hepatotoxic, immunosuppressive, or carcinogenic.Other potentially toxigenic fungi include *Alternaria* spp, *Pithomyces* spp, *Trichithecium* spp, *Rhizoctonian* spp and *Myrothecium* spp (Smith & Moss, 1985*)*.

Fungicides are mainly chemical compounds, live organisms antagonistic to pathogenic species and natural products such plant extracts used to eradicate or inhibit fungi and their spores (Wise & Mueller*,* 2011). Conventionally, the control of fungi has been achieved using chemical fungicides, variation in cultural practices, and development of resistant cultivars. Additionally, postharvest sorting of contaminated yields, such as maize, wheat, peanuts and tree nuts, have been used to reduce mycotoxin content in these commodities (Schatzki & Haddon, 2002; Campbell, Molyneux,, & Schatzki, 2003; Pearson, Wicklow & Pasikatan, 2004). Biological control methods have also been investigated for controlling seed borne fungi affecting maize and several bacterial and fungal antagonists have been developed with the aim of producing biological controls against potentially toxigenic fungi (Dorner, 2004; Cotty, 2006). This study tested the efficacy of four commercial foliar fungicides; Antracol (propineb), Milraz (propineb 700g/kg and Cymoxanil 60 g/kg), Mistress (Cymoxanil 8% and Mancozeb 64%) and Victory (Metalaxy 80g/kg and Mancozeb 640g/kg) on potentially toxigenic fungi isolated from maize grains that were positive for fumonisins and aflatoxins. This was part of a larger study

KEMRI SSC 2151 which tested maize from different regions in Kenya for the presence of mycotoxins.

1.2 Statement of the Problem

Mycological contamination of maize and the consequent production of mycotoxins have continued to create solemn health and economic problems throughout the world. Fungal infestation of maize in Kenya by potentially toxigenic fungi poses a major threat to the food security and the lives of many Kenyans. In the year 2004, one of the largest aflatoxicosis outbreak occurred in the rural Eastern province in Kenya. This outbreak was as a result of maize contamination by the mycotoxin which caused 317 cases and 125 deaths. Potential toxigenic fungi affecting maize are mainly of the genera *Aspergillus, Fusarium and Penicicillium*. These fungi are also major plant pathogens that cause food spoilage. The fungi are also seed borne and they affect maize at early stages of growth. Some species are associated with the production of several distinct mycotoxins. Effects of mycotoxins occur on both humans and animals with varied health effects including carcinogenic potential. Mycotoxin contaminated products also create a problem in their disposal since they can contaminate the environment and water sources. Fungicides are made to protect plants from destructive fungi before and after germination. However, fungicide resistant plant fungal pathogens have been identified in many parts of the world. On the other hand, use of fungicides in Agriculture has been linked to an emerging resistance to antifungal drugs in clinical settings.

1.3 Justification

Maize is a staple food in Kenya consumed by 90% of the population with a yearly production of 2.6 million tones. Contamination of maize by potentially toxigenic fungi results in spoilage of the product and detrimental health effects occur in humans and animals due to mycotoxin production. Fungicides are used in the control of various plant fungal diseases including fungi affecting food grains such as

maize. The test fungicides Antracol, Milraz, Mistress and Victory fungicides are indicated for use in vegetables, tomatoes, potatoes, onions, cucurbits and ornamental plants. Therefore this study tested the *in vitro* efficacy of the fungicides against fungi isolated from mycotoxin contaminated maize from different regions in Kenya. The fungicides could be used to control potentially toxigenic fungi affecting maize in Kenya to ensure food safety. Data from this study also gives an insight on fungal resistance to selected fungicides and also opens an avenue for further research on the emergence of resistance to antifungal agents used in clinical settings which has been associated to the use of certain groups of fungicides in agriculture.

1.4 Research Questions

I. What is the incidence of fungal infestation on mycotoxin contaminated maize from different regions?
II. Are commercially available fungicides effective against potentially toxigenic fungi isolated from mycotoxin contaminated maize?
III. What is the distribution and incidence of potentially toxigenic fungi in mycotoxin contaminated maize?
IV. What is the effect of selected fungicides on maize germination?

1.5 Objectives

1.5.1 General objective

To determine the efficacy of selected fungicides on fungi isolated from mycotoxin contaminated maize in Kenya.

1.5.2 Specific Objectives

1. To isolate and identify fungi from mycotoxin contaminated maize from Machakos, Mombasa, Nairobi, Kitale and Kisumu.

2. To determine the antifungal activity of Antracol, Mistress, Milraz and Victory fungicides on fungi isolated from mycotoxin contaminated maize.
3. To compare the distribution and incidence of fungal genera from mycotoxin contaminated maize in the five selected sites.
4. To determine the effect of the test fungicides on maize germination

CHAPTER TWO

LITERATURE REVIEW

2.1 Fungicides and their mode of action

Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue, and they only protect the plant at the site where it is deposited. When sprayed, translaminar fungicides redistribute from the upper, sprayed leaf surface to the lower, unsprayed surface. Systemic fungicides are absorbed and distributed through the xylem vessels to the upper parts of a plant which then protects new leaf growth for a short period (Wise & Mueller, 2011). Chemical agents as well as biological fungicides are used for the control of fungal diseases in plants. Different fungal and bacterial antagonists of phytopathogenic fungi have been evaluated for their efficacy in inhibiting fungal pathogens (Palumbo *et al.,* 2008).

Different chemical fungicides target various fungal physiological processes such as nucleic acid biosynthesis, mitosis and cell division, respiration, amino acids and protein synthesis, signal transduction, lipids and membrane synthesis, sterol biosynthesis in membranes, and cell wall biosynthesis (Hall, 2006). Fungicides are grouped by similarities in chemical structure and mode of action. Site-specific fungicides disrupt single metabolic processes or structural sites of the target fungus such as nucleic acid (DNA and or RNA) synthesis (Table 2.1). The activity of site-specific fungicides may be reduced by single or multiple-gene mutations. The benzimidazole, phenylamide, and strobilurin groups are subject to single-gene resistance and carry a high risk of resistance problems (FRAC, 2011). Azoxystrobin an active ingredient in fungicides used for maize seed treatment has been shown to have the greatest activity against *Rhizoctonia* spp, *Fusarium* spp, and *Penicillium* spp. These fungi cause seedling disease as well as mycotoxin contamination of maize at a later stage.

Table 2.1: Target site and Chemical groups of fungicides

Mode of action and target site		Fungicide chemical group	Common name
Lipid		Aromatic hydrocarbons	Dicloran Etridiazole Triadimefon Triticonazole
	Sterol	Triazoles	
Lipid, sterol, and other membrane components		Tebuconazole Cinnamic acid amide Triazole Morpholine Triazole	Dimethomorph Hexaconazole Fenpropimorph Propiconazole
	Intracellular membrane components	Hydrochloride	Acriflavine
Amino acid and protein synthesis		Glucopyranosyl Antibiotic Tetracycline antibiotic	Streptomycin Oxytetracycline
Signal transduction		Phenylpyrroles Dicarboximides	Fludioxonil Iprodione Vinclozolin
Respiration	NADH oxido-reductase (Complex I) Inhibitors	Pyrimidinamines	Diflumetorim
	Succinate dehydrogenase (Complex II) Inhibitors	Boscalid Flutolanil Carboxin	Boscalid Flutolanil Carboxin
	Oxidative phosphorylation uncouplers	2,6-dinitroanilines Dinitrophenyl carbarmate	Fluazinam Dinocap
Mitosis and cell division	Inhibitor of spindle microtubules assembly	Methyl benzimidazole carbamate Phenylurea	Benomyl Carbendazim Pencycuron
Nucleic acid synthesis	RNA polymerase I inhibitors	Acylalanines	Metalaxy
	Adenosin-deaminase inhibitors	Oxazolidinones Hydroxypyrimidines	Oxadixyl Ethirimol
Multisite activity	Phthalonitrile Dithiocarbamate Phthalimide Dithiocarbamate Antraquinone Copper	Chlorothalonil Mancozeb Captan Thiram Dithianon Copper sulphate	

Source: (Yang, Hamel, Vujanovic & Gan, 2011)

Other fungicide groups with site-specific modes of action include dicarboximides and sterol demethylation inhibitors (DMIs), but resistance to these fungicides appears to involve slower shifts toward insensitivity because of multiple-gene involvement. Many of the site-specific fungicides also have systemic mobility. However, systemic mobility is not necessary for resistance development. Resistance problems have developed in the dicarboximide group and with dodine, which are protectant fungicides.

Multi-site fungicides interfere with many metabolic processes of the fungus and are usually protectant fungicides. Once taken up by fungal cells, multisite inhibitors act on processes such as general enzyme activity that disrupt numerous cell functions. Numerous mutations affecting many sites in the fungus would be necessary for resistance to develop. Typically, these fungicides inhibit spore germination and must be applied before infection occurs. Multi-site fungicides form a chemical barrier between the plant and fungus. The risk of resistance to these fungicides is low. And currently, fungicides are mainly classified by mode of action (Table 2.1).

2.2 Description of test fungicides

2.2.1 Antracol WP 70

This is a broad spectrum protective fungicide effective against early and late blight on potatoes and tomatoes, various fungal diseases on vegetables, fruits and ornamentals. The active ingredient of antracol WP 70 propineb, belongs to a chemical class of dithiocarbamates. Propineb is a fungicide with multisite activity and kills conidia or germinating conidia by contact. The product has a very good distribution and adhesion on the crop plant but it does not penetrate the plant tissues. It is used across the world for the control of various fungi, especially *Oomycetes, Ascomycetes, Basidiomycetes* and Fungi imperfecti. The fungicide is manufactured by Bayer Crop Science.

2.2.2 Mistress 72 WP

This is a systemic and contact fungicide for the control of early and late blight on tomatoes and potatoes. The fungicide is manufactured by Osho chemical industries. The fungicide has Cymoxanil 8% and mancozeb 64%, cymoxanil has preventive and local systemic activity and also inhibits blight. Mancozeb has protective antifungicidal activity and works through translaminar action.

2.2.3 Milraz WP 76

This is a broad spectrum preventive fungicide for the control of fungal diseases in fruits, potatoes, tomatoes, cucurbits and onions (*Phytophthora infestans* and *Plasmopara viticola*). The active ingredients are propineb700g/kg a dithiocarbamate and Cymoxanil which is an ethyl urea 60g/kg. This fungicide is manufactured by Bayer Crop Science.

2.2.4 Victory WP 72

This is a systemic and contact foliar fungicide for the control of foliar and root diseases of potatoes and tomatoes and downy mildew of ornamental crops. The active ingredients in this fungicide are Metalaxy 80g/kg and Mancozeb 640g/kg.

2.2.5 Safety of test fungicides

The four test fungicides and their respective ingredients are registered for use in Kenya by the Pest Control Products Board (PCPB) established under the Pest Control Products Act, Cap 346 of the laws Kenya. The Board regulates the importation, exportation, manufacture, distribution and use of Pest Control Products. The products have been evaluated by the Board for safety, efficacy, quality and economic value (PCPB, 2010). Based on the manufacturer's instructions, these chemicals are poisonous if consumed or inhaled directly hence they are only

recommended for use in the field while observing appropriate safety measures for storage, general handling, disposal, and transportation of chemical substances.

2.3 Resistance to Fungicides

There are different active ingredients in fungicides and, several trade products are available as single or in combination. Most of these active ingredients have the same mode of action, however fungal pathogens may be cross resistant or multi resistant to one or more (Hall, 2006). A plant pathogen is said to be cross-resistant when it is not inhibited by fungicides that target the same growth process. One instance of cross-resistance in a plant pathogen is the resistance to fungicides in the chemical groups of triazoles and pyrimidines. Both triazoles and pyrimidines are demethylation inhibitors that distrupt sterol biosynthesis. Multiple-resistance is of greater concern where a pathogen is not inhibited by fungicides that affect diverse plant-growth processes (Hall, 2006). A fungus would be labeled as having multiple-resistance if the pathogen is resistant to fungicides that inhibit both mitosis and protein synthesis, which are two different fungal growth processes.

In addition to the development of fungicide resistance, there are other effects of widespread fungicide use in corn. Under certain conditions that are not well understood, the use of pesticides can exacerbate insect or mite pressure (Latteur,and Jansen, 2002). Specifically, the use of fungicides in corn can reduce populations of entomopathogenic fungi, leaving the crop more at risk for insect or mite outbreaks (Smitley *et al.,* 1986). Recently, fungicide use has been tied to aphid flare-ups in several cropping systems (Abney *et al.,* 2008; Koch *et al.,* 2010*;* Lagnaoui *et al.,* 1998; Nielsen *et al.,* 2005). This consequence of fungicide use could impact crop production by eventually facilitating the need to manage insects that were not problematic prior to the widespread use of fungicides.

The recent increase in the use of foliar fungicides is raising environmental concerns in corn production areas. QoI fungicides, while defined as reduced-risk (Bartlett *et*

al., 2002), are toxic to several aquatic species. For instance, commonly used QoI fungicides have demonstrated acute toxicity to frog tadpoles (Belden *et al.,* 2010; Johansson *et al.,* 2007), freshwater mussels (Bringolf *et al.,* 2007), freshwater algae and water fleas (Ochoa *et al.,* 2009) in laboratory studies.

2.4 Impact of fungicides on crop yields and mycotoxin production

Fungi that cause leaf diseases in maize are not Mycotoxin producers, however the control of these organisms in maize plants using fungicides could improve the health of the crop making the other plant parts less predisposed to infection by toxin producing fungal species (Rankin & Grau, 2002).

Various manufacturers suggest that these fungicides are able to increase yields even when there is no disease. For instance, benefits such as enhanced stalk strength and vigor of maize are deemed to result from quinone outside inhibitor fungicide (QoI) use. These claims are attractive to producers seeking to retain the standing ability of corn at harvest. As a result, many fungicide applications across the U.S. Corn Belt are being made with the expectation of these apparent benefits instead of use in the event a of disease threat (Wise & Mueller, 2011) .

There has been a growing interest in the use of foliar fungicides for corn and soybean production in the U.S. which has expanded considerably in the past few years (Hershman *et al.,* 2011). Foliar fungicides for maize were previously reserved for fields used for seed production to protect their quality in certain conditions or for special crops. Applications for the purpose of protecting crop yield were rarely economical. However, a current trend in Kentucky, as well as many other corn soybean producing states, is aimed at increased use of foliar fungicides on these crops as a means of maximizing crop yields (Hershman *et al.,* 2011).

The application of fungicides have potential consequences, not only from the economic perspective (use of additional inputs in corn production), but from the

biological impact on fungal populations. QoI fungicides are classified by the Fungicide Resistance Action Committee (FRAC) as high-risk for resistance development. Applications of these fungicides may increase selection pressure that could lead to changes in fungal sensitivity to QoI fungicides. There are currently over 40 fungal pathogens with resistance to this fungicide class worldwide, and fungicide resistance has been detected within a pathogen as quickly as two years after widespread use of the QoI fungicides (FRAC, 2011). Besides, QoI resistance has recently been confirmed for the frogeye leaf spot pathogen, *Cercospora sojina* after just a few years of QoI fungicide use on soybean (Bradley, 2010; Bradley, 2011). There are different modes of resistance to fungicides, these includes altered target in the pathogen, target site overproduction and efflux systems that pump the the chemical agent out of the fungal cell, therefore preventing accumulation to toxic levels (Table 2.2).

Table 2.2 Mechanisms of fungicide resistance

Fungicide or fungicide class	Mechanism of acquired resistance
Aromatic hydrocarbons	**Unknown, cross-resistance with dicarboximides**
Organo-mercurials	**Detoxification by binding substances**
Dodine	**Unknown**
Benzimidazoles	**Altered target site (ß-tubulin)**
2-Amino-pyrimidines	**Unknown**
Kasugamycin	**Altered target site (ribosomes)**
Phosphorothiolates	**Metabolic detoxification**
Triphenyltins Unknown	**Altered target site (RNA polymerase)**
Phenylamides	**Unknown, cross-resistance with aromatic hydrocarbons**
Dicarboximides	**Increased efflux; altered target site.**
DMIs	**target-site product; target-site over-production**

Carboxanilides	**Altered target site (succinate-ubiquinoneoxidoreductase)**

Source: FRAC, 2011

2.5 Mycotoxin contamination of agricultural products in the World

Various genera and species of fungi produce mycotoxins that have significant agricultural, epidemiological and economic impact (Palumbo *et al.,* 2008). The *Aspergillus*, *Fusarium*, and *Penicillium* species are attributed to the majority of agricultural mycotoxin contamination. These mycotoxigenic fungi are part of the microbial flora associated with many agronomic crops, including maize, peanuts, tree nuts, grapes, barley, coffee, cotton, wheat and other cereal grains (Prasad *et al.,* 1987). Depending on the crop plant affected and the fungal species, mycotoxigenic fungi may cause plant disease, such as *Aspergillus* fruit rot of grapes, maize ear rots caused by *Aspergillus* and *Fusarium* species, and *Fusarium* head blight as well as seedling blight diseases on cereal crops (Palumbo *et al.,* 2008) .

Fumonisins are a group of mycotoxin contaminants found in food and feed products. Fumonisin B1 primarily is of international, agroeconomic, and food safety concern. High doses of fumonisin B1-infested corn feed have been shown to cause pulmonary edema in swine, while lower doses lead to hepatic disease (Haschek *et al.,* 1992). Mycotoxins are produced predominantly by toxigenic strains of *Fusarium verticillioides*. The fungus commonly proliferates in maize, causing stalk and ear rot diseases, in addition to mycotoxin contamination.

Fumonisins have been found to be weakly carcinogenic among diverse rodent species (Gelderblom *et al.,* 1993; Voss *et al.,* 1995) and are possible human carcinogens, associated with increased incidence of esophageal cancers in South Africa and China (IARC, 1993). Fumonisin consumption is also a risk factor in neural tube and related birth defects (Marasas*,* 1996). Fumonisins are known to

disrupt sphingolipid synthesis and levels in plasma, which may explain the different modes of fumonisin toxicity in farm animals, investigational animals, and humans (Merrill *et al.,* 1996). Fumonisin content in the US corn was relatively high between 1988 and 1991, but has been regulated to <0.5 μg/g in recent years. Most commercial foods, however, contain 500 ng/g or less due to low fumonisin levels in corn and quality control of ingredients (Shephard *et al.,* 1996).

Aflatoxins are another group of mycotoxins which affect different food grains including maize, maize based food and peanuts. The most common aflatoxins are B1, B2, G1 and G2 which are potential carcinogens produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Senbeta & Gure 2014). These fungi also cause *Aspergillus* ear rot in corn. Aflatoxin contamination is generally a considerable problem within tropical or sub-tropical areas. It occurs when the crop is either damaged by insects or other factors and when crops are stressed by heat and drought (Bennett & Klich, 2003). In the Mediterranean region, pistachios, peanuts and maize are the major crops contaminated with aflatoxins. Outbreaks have occurred in this area, such as in 2003 due to unfavorable environmental conditions (hot and dry for several months) during maize cultivation in northern Italy which resulted to aflatoxin levels 5–70 times higher than during the years 1995–2000 (Tsitsigiannis *et al.,* 2012). Contamination of milk by AFM1 also occurred in this outbreak produced by dairy cattle that consumed contaminated maize and led to the discarding of thousands of tons of contaminated milk. Continuous global climate change to hotter and drier increases the possibility that all of Southern Europe may face a constant threat of aflatoxin contamination (Logrieco and Moretti, 2008).

2.6 Mycotoxin Contamination of maize in Kenya

Mycotoxin contamination of Kenyan maize is not new and incidences of aflatoxicosis were reported in 1982 in Makueni District of Eastern Province. The height of human aflatoxicoses occurred in the same region in 2004 where 317 cases and 125 deaths were reported resulting from acute hepatotoxicity (Gieseker, &

Centers for Disease Control and Prevention, 2004). Fifty five percent (55%) of maize samples tested in districts associated with this outbreak had Aflatoxin B1 (AFB1) levels >20 ppb, (Kenya's regulatory limit), depending on the district, some had >1000 ppb while some as much as 8000 ppb AFB1. Other mycotoxins are likely to occur in the maize products from this region due to the prevailing favorable conditions which encourage the growth of various fungal species, including Fumonisin producing *Fusarium verticilloides.*

2.7 Strategies employed in the control of potentially toxigenic fungi

2.7.1 Biocontrol agents

In maize, isolation of antifungal metabolites from *Acremonium zeae*, a fungal endophyte of maize has been reported (Wicklow *et al.,* 1988). This has been shown to limit *A. flavus* colonization and aflatoxin contamination of maize. These metabolites were determined to be pyrrocidines A and B, which were revealed to be produced by a number of *A. zeae* isolates. Besides the inhibition *A. flavus* growth, these compounds also inhibit growth of *F. verticillioides*. Both *F. verticillioides* and *A. zeae* are endophytes of maize and hence interactions between the two may offer interesting insights into competitive traits that function in their endophytic lifestyles (Wicklow *et al.,* 1988).

A number of yeast species also have been shown to inhibit *A. flavus*and *A. parasiticus* growth and aflatoxin production in vitro. Six isolates of *Kluyveromyces* spp. were shown to inhibit *Aspergillus* spore germination, germ tube elongation, and hyphal growthrates on maize meal extract media at different water activity (aw) levels (La & Etcheverry, 2006). At higher *a*w (0.994 and 0.982), all *Kluyveromyces* isolates completely inhibited aflatoxin productionin the tested *Aspergillus* strains. On the other hand, at lower *a*w (0.955and 0.937), certain combinations of *Kluyveromyces* and *Aspergillus* resulted in increased aflatoxin levels relative to control levels. The use of these yeasts as biocontrol agents would be potentially

unfavorable under drought stress conditions given that aflatoxin production isassociated with drought stress in maize (Payne, 1998).

Several reports have focused on the interactions between *F. verticillioides* and possible bacterial antagonists from maize environments. Studies to select bacterial antagonists of *F. verticillioides* for use as biocontrol agents have been performed (Cavaglieri *et al.,* 2004). In one study correlations of niche overlap index (NOI) i.e index of dominance, antibiosis, inhibition of fumonisin production, and effects on fungal growth rate and lag phase between combinations of 11 bacterial strains and 13 *F. verticillioides* strains isolated from the maize rhizosphere were determined (Cavaglieri *et al.,*2004). The NOI is a measure of competitiveness related to nutrient source utilization common between organisms (Wilson & Lindow, 1994). Higher NOI values, hence, indicate greater potential for nutrient competition against the mycotoxigenic fungi.

Greenhouse experiments showed that, one of these bacterial strains, an isolate of *Azotobacter armeniacus* had a positive niche overlap index (NOI) and antibiosis phenotypes and significantly controlled *Fusarium verticillioides* relative to maize root colonization in native soil (Cavaglieri *et al.,*2004).

Some studies indicate that biocontrol bacteria obtained from maize are potential alternatives to chemical control agents. Surveys on maize soil and rhizosphere bacteria for antagonism against *F. verticillioides*aimed at isolating bacterial strains for simultaneous control of *F. verticillioides* and *A. flavus* have been performed (Palumbo *et al.,* 2007).

Co-culture experiments in maize kernel extract medium, with strains of *Bacillus*, *Burkholderia*, and *Pseudomonas* showed comparable inhibitory activity against both fungi (Palumbo *et al.,* 2007). Moreover, these strains appreciably increased plant growth as result of maize seed treatment, protected maize plants from growth inhibition caused by *F. verticillioides*, and reduced disease incidence by 60% to 86%.

The implication of maize seed treatment with individual strains of *B. amylolique faciens* and *M. oleovorans* was analysed in relation to resident fungal and bacterial populations in the maize rhizosphere (Pereira *et al.,* 2007). The two bacterial treatments successfully reduced *F. verticillioides* populations and fumonisin B1 levels in maize grain samples recovered from plants grown from treated seed. It is suggested that some bacterial treatments may selectively target unwanted phytopathogens including mycotoxigenic fungi causing no harm to native innocuous and beneficial microorganisms in the treated system (Palumbo *et al.,* 2007).

Apart from bacteria and yeasts, atoxigenic *Aspergillus* strains and chemical control agents (i.e. fungicides) have been shown to inhibit the growth of aflatoxigenic fungi and the resultant aflatoxin biosynthesis. The application of competitive, atoxigenic strains of *A. flavus* or *A. parasiticus* has been shown to reduce aflatoxin contamination of agricultural products such as peanuts (Dorner, 2004), rice, pistachios, maize (Dorner *et al.,* 1999), and cottonseed (Cotty, 1994). The effect is thought to be mediated through competition for substrate and the potential production of inhibitory metabolites. It is well-known that aflatoxin contamination of crops can be reduced by atoxigenic biocontrol strains. The application of non-aflatoxigenic strains of *A. parasiticus* in field soils has been demonstrated to result in lesser aflatoxin levels in peanut crops, with levels falling from 531, 96 and 241 µg kg-1 in untreated soils to levels of 11, 1 and 40 µg kg-1 for treated soils during three successive years (1987, 1988, and 1989), respectively (Dorner *et al.,* 1999). Another study found that, a non-aflatoxigenic strain of *A. flavus* reduced aflatoxin contamination by 80 to 95% in maize (Brown *et al.,* 1991).

There are two strains of A *flavu*s that have been reported i.e. S and L found worldwide in maize fields (Cotty, 1997) The S strain is known to produce more aflatoxins and sclerotia (dormant body of fungus) but fewer conidia (asexual spores). A high population of S strain has been correlated with frequent outbreaks of aflatoxin contamination An atoxigenic strain of A.*flavus* is introduced into the crop

environment where it competes with the toxigenic strains The NRRL 21882 strain an active ingredient in Afla-Guard is one of the atoxigenic strains that have been applied in the fields (Cotty, 1997) Aflasafe^{TM} is currently being tested in the field in various countries including Kenya, Nigeria, Zambia and Senegal where Aflasafe^{TM} is formulated to consist a combination of local atoxigenic strains (Monda & Alakonya 2016). A reduction of 80-95% in aflatoxin contamination has been demonstrated by other studies due to competitive exclusion and displacement of toxigenic strain by atoxigenic strain. Maize was co-inoculated with toxigenic and atoxigenic strain (AF13) (Monda & Alakonya, 2016)

The German government funded the first biocontrol research actitivities carried out by IITA in Africa in the Republic of Benin in the late 1990s. In 2003, the biocontrol effort was initiated in Nigeria with continued funding from the German government and in collaboration with USDA-ARS and University of Ibadan. Originally, more than 4200 *Aspergillus* strains isolated from maize and soil samples in Nigeria were tested and around 20 atoxigenic genetic groups were selected (Dorner *et al.,* 2004).

2.7.2 Genetic engineering by RNA interference (RNAi)

Genetic engineering approaches have been explored to control phytopathogenic fungi. One of the key strategies is the interference of Ribonucleic Acid (RNAi) of vital fungal gene (s) expression through the host. The genetic manipulation advances are now known to be effective in the control of *Fusarium verticillioides, F. graminearum, Puccinia striiformis* f.sp. *tritici* and *Aspergillus flavus* (Yin, Jurgeson & Hulbert 2011). A number of studies have suggested that there exists a micro RNA (miRNA) or small interfering RNAs (siRNAs) trafficking channel between plant hosts and their fungal pathogens. The channel could, consequently, create a platform for the management of economically important fungal pathogens including aflatoxigenic *A. flavus*. A sequenced genome of *A. flavus* is now available as well as a complete elucidation of the aflatoxin biosynthetic pathway; there exist possible targets in *A. flavus* that could be altered to either limit fungal growth or aflatoxin

biosynthesis (Koch *et al.,* 2013; Yu, 2012). Numerous gene function studies on the aflatoxin biosynthetic pathway have identified the transcription factor *aflR* as a potential *in planta* target against aflatoxin build up (Reverberi *et al.,* 2012).

A study conducted in Kenya demonstrated the transformation of *aflR* hairpin constructs into a susceptible tropical maize line resulting in a marked (14-fold) reduction of aflatoxin levels. While these results were creditable, the transgenic maize had an altered plant phenotype perhaps due to HIGS mis-targeting by *aflR*si RNAs (Masanga *et al.,* 2015). Thus, the study creates a platform from which the effects of silencing other aflatoxin biosynthetic genes on aflatoxin levels could be evaluated.

Three major challenges of transgenic approaches in the control of mycotoxins have been highlighted these include (i) poor farmers find it difficult to adopt new seed technologies, (ii) Stringent regulations on the approval of biotechnology crops and (iii) distrust of transgenics among consumers. (Monda & Alakonya 2016)

2.7.3 Chemical control agents

Several chemical control agents have been developed among these are Quinone-outside inhibitor fungicides (QoI) which were first labeled for use on maize in mid-2000. These fungicides are commonly referred to as strobilurin fungicides. They are now extensively marketed in corn production for management of both biotic and abiotic stresses (Wise & Mueller, 2011). This group of fungicides is applied preventively or as early as possible in a disease cycle. They are effective against fungal spore germination and early mycelium growth but this has less or no effect when the fungus is already established (Wise & Mueller, 2011).

Quinone-outside inhibitor (QoI) fungicides are chemical compounds which act at the quinol outer binding site of the cytochrome bc1 complex. This then inhibits fungal mitochondrial respiration that stops energy production in the fungus leading to even-

tual death. These fungicides are effective against a broad spectrum of fungi (Wise & Mueller, 2011).

2.7.4 Natural products

Several plant derived products have been evaluated for their ability to inhibit mycotoxigenic fungi and Mycotoxin production. Inhibitory effects of neem plant extracts on biosynthesis of aflatoxins (group B and G) by *Aspergilus flavus* and *A. parasiticus* have been demonstrated (Cotty *et al.,* 2006). From another study, production of aflatoxins by *A. parasiticus* was suppressed according to the concentration of plant aqueous extract that were added to culture media at the time of spore inoculation. Aflatoxin production in fungal mycelia grown in culture media having 50% neem leaf and seed extracts was inhibited by 90 and 65%, respectively (Razzaghi *et al.,* 2005).

An investigation of *Syzgium aromaticum* (clove) against *A. flavus*and aflatoxin production showed complete inhibition of mycelia growth (Reddy *et al.,* 2007). More than 280 plant species have been studied for their inhibitory potential against toxigenic *Aspergilli* of which about 100 of these plants had some activity on growth or toxin production by the fungi (Montes & Carvajal, 1998).

Studies conducted on ochratoxigenic fungal species *Aspergillus ochraceus, Penicilliumverucosum and P. brevicompactum* showed the ability of neem plant extracts to inhibit fungal growth and not ochratoxin production by these species (Mossini *et al.,* 2009). The inhibitory effect of neem extracts against fumonisin producing *Fusarium verticilloides* has also been reported including its possible use as a control for this fungus in maize (Anjorin *et al.,* 2008)

In a study conducted to test the activity of Garlic juice against toxigenic fungi, it was shown that garlic juice effectively inhibited fungal growth at all concentrations and its activity improved as the concentration increased. In addition, all tested isolates responded to garlic juice regardless of the concentration used (Yassin *et al.,* 2012). *A. flavus* and *P. oxalicum* corn isolates were the most sensitive to all concentrations of garlic juice tested. Both showed significant inhibitory effects of 63.70 and 75.56%,

respectively, at a concentration of 1.25%. On the other hand, popcorn isolate of *A. fumigatus* mainly sensitive to all concentrations as indicated by significant inhibition of 62.22-88.89% (Mohamed *et al.,* 2012).

2.7.5 Clinical significance of fungicides

Pathogenic fungi which are resistant to fungicides have been increasingly reported in clinical environments (White *et al.,* 2002 and Cowen *et al.,* 2000). Azole fungicides, which act as sterol biosynthesis inhibitors by blocking C14-demethylation activity, are used in both agricultural and clinical environments, thus concerns have been raised that fungal species living in both environments may acquire resistance to agricultural azoles in the field, infect humans and may subsequently be difficult to control by medical azole antifungal drugs (Hof, 2001). Certainly, cross-resistance to fungicides of the same fungicide class is likely to occur *Aspergillus fumigates*, a widespread filamentous fungus in the environment and can therefore be exposed to antifungals used in medical and agricultural environments.

In a drug susceptibility study that included 400 clinical and 150 agricultural *A. fumigatus* isolates, 10 clinical and 36 environmental isolates with resistance to itraconazole were detected (Meneau & Sanglard, 2005). Several cases of infection of humans by plant pathogenic fungi such as *Alternaria alternata or F. oxysporum* have been reported in recent years (Kebabcı *et al.,* 2004; Ortoneda *et al.,* 2004; Sasama *et al.,* 2005). In addition, five *Colletotrichum* species have been classified as clinically relevant, since they have been reported to infect humans (Cano *et al.,* 2004; Castro *et al.,* 2001; Guaro *et al.,* 1998; O'Quinn *et al.,* 2001; Shukla *et al.,* 1983.). These pathogens are likely to get in contact with fungicides and especially azoles in agricultural environments. However infections by *Fusarium*, *Alternaria* or *Colletotrichum* species occur rarely. In the case of *Colletotrichum*, specific requirements must be met before an infection can take place. The risk is increased in immunocompromised patients and in diabetics. Additionally, *Colletotrichum* species require wounded skin in order to infect. On non-wounded skin, germination rates are

low, but as soon as trauma occurs, conidia germinate, immediately become invasive and rapidly colonize the human tissue. Case reports of *Colletotrichum* infections of man have been published, and in some cases fungicide resistance of individual isolates have been determined (Guarro *et al.,* 1998 & O'Quinn *et al.,* 2001. Guarro *et al.,* (1998) analyzed isolates of *C.dematium*, *C.coccodes*, and *C. gloeosporioides* from diverse sources for their susceptibility to antifungal drugs (amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, and miconazole) which belong to different chemical classes and differ in their modes of action. Majority of the isolates displayed a major degree of resistance to one or few fungicides. One isolate of *C. coccodes* was clearly resistant to all of the drugs tested.

In another study which investigated whether *Colletotrichum* species could adapt to azole fungicide stress and develop a method allowing efficient adaptation to the agricultural azole tebuconazole, adapted strains were able to grow efficiently on agar plates containing fungicide concentrations of 30 ppm and above (Deising *et al.,* 2008). Fungicide sensitivity assays performed with *C. graminicola* in vitro showed that adaptation to an azole fungicide used in agricultural environments also resulted in resistance to azoles used in clinical environments (Deising., *et al.,* 2008). Fungicide sensitivity determined by radial growth assays *in vitro* correlated well with viability staining of fungal hyphae and infection assays with excised human skin (Serfling *et al.,* 2007).

In a study conducted in Tanzania, a total of 30 soil and woody debris samples from the surroundings of Kilimanjaro Christian Medical Centre, Moshi, Tanzania, were processed for detection of azole resistant *Aspergillus fumigatus* isolates tested for susceptibility to itraconazole, voriconazole, posaconazole and isavuconazole. The study reported the isolation of resistant *A. fumigatus* strains harbouring TR46/Y121F/T289A mutation from Africa. It was concluded that the Recovery of TR46/Y121F/T289A from the environment is worrisome and effective surveillance

of clinical and environmental sources to detect azole resistance in *A. fumigat*es should be enhanced (Chowdhary *et al.,* 2014).

At present, the question of whether or not extensive application of azole fungicides in agriculture could lead to increased azole resistance and thus cause failure in disease control in clinical environs is debated at the scientific and political level (Deising *et al.,* 2008).

Studies by Deising *et al also* showed that fungicide application in agriculture did not correlate with the occurrence of fungicide-resistant fungal strains in clinical environments. It was concluded that the lack of correlation suggests that fungicide application in agriculture does not represent a driving force for the development of fungicide resistance in medicine. Statistical studies rather suggest that extensive use of azoles in medicine caused fungicide resistance in fungi attacking humans, and that infections with azole-resistant strains predominantly occur in clinical environments (report of the Scientific Steering Committee of the European Commission). Research on fungicides with the aim of developing strategies to reduce the risk of fungicide resistance is urgently needed in agriculture and in medicine. Mechanisms providing the basis for fungicide resistance are likely to be conserved among pathogens of plants and man. Consequently, plant pathologists and medical microbiologists should develop strategies counteracting the development of fungicide resistance in a concerted action (Deising *et al.,* 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This was a laboratory based cross sectional study carried out at the Mycology Laboratory, Center for Microbiology Research, Kenya Medical Research Institute (KEMRI). This was part of a larger study KEMRI SCC number 2151. The aim of the study (SSC 2151) was to determine the presence of mycotoxins (aflatoxin and fumonsin) in maize collected from different regions in Kenya and whether the aflatoxin producing fungi *Aspergillus flavus*were super toxigenic at the genetic level. This research was conducted under the public mycotoxicological food safety program.

3.2 Study Samples

A total of 138 maize samples that tested positive for fumonisins and aflatoxins in assays carried out as per the study KEMRI-SSC-2151 were included in this study. The maize samples were tested for the presence of fumonisin and aflatoxin using quick scan method (Appendix 12 and 13 respectively). The bulk samples were collected from five sites in Kenya namely Machakos, Nairobi, Mombasa, Kitale and Kisumu. Five hundred grams of maize kernels were obtained from the collection point and packaged in sterile polythene bags after which they were transported to the Mycology Laboratory in brown sugar paper Samples from Nairobi were collected from open markets and posho mills while those from Kitale, Mombasa, Kisumu and Machakos were collected from household stores and the field

3.3 Sampling and Sample size Determination

One hundred and thirty eight samples were used in this study. The minimum sample size was 70 as determined by Fischer's exact formula (Fischer *et al.,* 2000).The confidence interval used was 95%.

Sample size; $n \geq Z^2\alpha/2\ (p\ (1-p))/d2$

$$n \geq 1.96^2\ (0.1(1-0.1)/0.05^2 = 70$$

Where n is the sample size, $z^2_{\alpha/2}$ is the corresponding value to the 95% confidence interval (1.96),p= 10%estimated prevalence of mycotoxin contamination of maize in Kenya (International Food Policy Research Institute, 2010). The confidence interval is 95% and d= Absolute precision (0.05). The 138 samples were distributed among the sites based on the estimated regional prevalence.

Region	Estimated prevalence (IFPRI, 2010)	Number of samples
Kitale	25%	25/113*138=31
Machakos	36%	36/113*138=44
Mombasa	27%	27/113*138=33
Kisumu	10%	10/113*138=12
Nairobi	15%	15/113*138=18
Total	113	138

3.4 Test Fungicides

Fungicides tested in this study are commonly used as foliar fungicides in Kenya .The active ingredients are registered by the pest control products board (PCPB, 2010). The fungicides were prepared according to the manufacturer's instructions and appropriate concentrations were made. Four fungicides namely; Antracol, Mistress, Milraz and Victory fungicides were tested in this study for their activity against fungi isolated from mycotoxin contaminated maize. The respective dilutions were as follows;

I. Milraz WP 76 -40g per 20 litres
II. Mistress WP 72 -30g per 20 litres
III. Victory WP -50g per 20 litres
IV. Antracol WP 70- 50g per 20 litres

3.5. Isolation of fungi from maize samples

Four maize kernels were surface sterilized for 1 min in 2.5% NaOCl, washed in three changes of sterile distilled water and then washed with the test concentration of each fungicide independently and inoculated on Sabourauds dextrose agar (SDA) supplemented with 10mg/L chloramphenicol to suppress bacterial growth. Each SDA plate was inoculated with four maize kernels prepared in triplicates for each sample (Muthomi & Mutitu 2003). Control maize kernels from the samples were washed concurrently with sterile distilled water and inoculated on SDA plates simultaneous with the treated samples in triplicates. The culture plates were incubated at 30^{o}C for 72 hours for fungal growth. Growth of fungi on the maize grains treated with the fungicides was scored to determine the percentage infestation and compared to the untreated kernels. If all the four maize kernels had visible growth of fungi this was scored as 100% infestation and if only one grain out of the four plated kernels was infested with visible growth of fungi, the score was 25% infestation. Where two out of the four kernels were infested, this was scored as 50% infestation while three infested

kernels was scored as 75% and where all the four kernels were infested, this was scored as 100%.

Isolation frequencies (Fq) of each fungal genus from the five regions were calculated according to Gonzalez *et al*. (1999). This was used to determine the distribution and incidence of different fungal genera in the five selected sites.

$$Fq\ (\%) = \frac{\text{Number of isolates of a genus x 100}}{\text{Total number of fungal genus per region}}$$

3.6 Determination of germination

The effect of the test fungicides on germination of the maize grains could be assessed concurrently with the fungal infestations in the range of 0 to 100%.Untreated maize kernels washed with sterile distilled water were used as controls for germination of fungicide treated maize kernels. Where all the four maize kernels germinated, this was scored as 100% while the germination of 3 kernels was scored as 75% and germination 2 and 1 kernel in the plate was scored as 50% and 25%respectively.

3.7 Identification of Isolated fungi

Fungi growing on the maize kernels were sub-cultured and identified by morphological characteristics using both microscopic and macroscopic features. Cultural characterization was based on presence of aerial mycelium, colour of aerial mycelium as well as colour on the obverse and reverse of the colony. Microscopic identification was based on spore and conidiophore morphology. This was done according to Larone, (1995) identification scheme. *Aspergillus* colonies were identified to the species level while *Fusarium* and *Penicillium* colonies were sub-cultured on SDA and identified to genus level (Larone, 2002).

3.8 Preparation of tease mount (Lactophenol cotton blue staining)

Lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi. The preparation has three components: phenol which kills any live organisms, lactic acid which preserves fungal structures and cotton blue which stains chitin in fungal cell walls. Fungal elements are stained intensely blue (Larone, 2002).

A drop of LPCB was placed on a clean glass slide and a small portion of fungal colony on agar surface was picked using a sterile rigid needle. A portion of fungal culture was placed on the drop of LPCB and the mycelial mass teased apart gently using two dissecting needles. A cover slip was placed gently on to the slide avoiding bubbles. The preparation was left for 5 minutes to stain and then initial examination was done using a low power (X10) objective lens and then at higher power (X40) objective for more detailed examination of spores and other fungal structures (Larone, 1995).

3.9 Bioactivity of the test Fungicides

The activity of the four fungicides onfungal isolates was determined by disk diffusion technique. Absorbent Watman filter paper disks of 6 mm were impregnated with 20µl of the diluted fungicides independently. Saborauds dextrose agar (SDA) plates were inoculated with 0.5McFarland suspensions prepared from pure cultures of each fungal isolate that grew on maize kernels. The inoculum was then spread evenly on the culture plate using a sterile swab Fungicide impregnated disks were placed aseptically at the centre of inoculated SDA plates using a sterile forceps. The plates were incubated at 30^{o}C for 72 hours and thereafter, the zones of inhibition around the disks were measured in millimeters (mm) after 48 hours. Fluconazole antifungal disk was also set for each of the isolates as a reference for susceptibility or

resistance to the four fungicides and interpreted according to CLSI guidelines for susceptibility testing of filamentous moulds by disk diffusion (Espinel-Ingroff, Cantón & Pemán, 2012).

Data generated in this study was entered onto a laboratory log book and later transferred onto Excel spreadsheets. Data was stored on the computer drive and flash disks. The data was later exported onto SPSS version 17 for analysis.

3.6.1 Data Analysis

Data generated in this study was analysed using statistical software SPSS version 17. Statistical analysis to compare the susceptibility of different fungal isolates to the fungicides was done by Analysis of Variance (ANOVA). ANOVA was also used to compare the percentage infestation on mycotoxin positive maize kernels treated with four fungicides to the controls washed with sterile distilled water. Spearman correlation analysis was used to determine the effect of the test fungicides on maize germination.

3.7 Scientific and ethical approval

This study was reviewed and approved by the KEMRI Scientific Steering Committee (SSC number 2602) (Appendix 2), Ethical Review Committee (ERC) (Appendix 3) and KEMRI publications committee (Appendix4).

CHAPTER FOUR

RESULTS

4.1 Microscopic and Macroscopic features of fungal isolates

Fungi belonging to the Genera *Aspergillus*, *Penicillium, Fusarium, Rhizopus* and *Cladosporium* were isolated from the mycotoxin contaminated maize samples. The fungi were identified by their distinct macroscopic and microscopic features. Macroscopic characteristics used for identification included colour on the obverse and reverse of the colony, presence or absence of exudates, shape and elevation (Table 4.1).

Table 4.1: Macroscopic characteristics of fungi isolated from mycotoxin positive maize

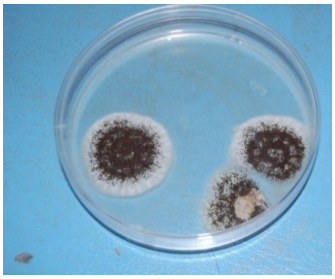
Fungal species	Colour on obverse	Reverse	Exudates	shape	Elevation	Results
Aspergillus niger	Dark-black with Black conidia	White–yellow	Nil	filamentous	raised	**Plate 4.1 b**
Aspergillus flavus	Greenish with white fluffy mycelia	pale yellow	Nil	filamentous	flat	**Plate 4.2 b**
Aspergillus vesicolar	bluish–green with white border	Yellow	present	Folded-rugaled	raised	**Plate 4.3 b**
Fusarium spp	Deep rose coloured center with pink periphery	Light coloured	Nil	circular	flat	**Plate 4.4 b**
Penicillium spp	bluish–green with white border	Brown	Nil	flat	flat	**Plate 4.5 b**
Cladosporium spp	Black-grayish	Black	Nil	folded	raised	**Plate 4.6 b**
Rhizopus spp	White-to dark gray	White	Nil	rhizoidal	flat	**Plate 4.7 a**

Microscopic features used in identification of the fungal isolates from mycotoxin positive maize were recorded. The isolates were distinguished by the structure of phialides, formation of conidia, seriation and the structure of vesicles. Microscopic photographs captured are also referenced (Table 4.2)

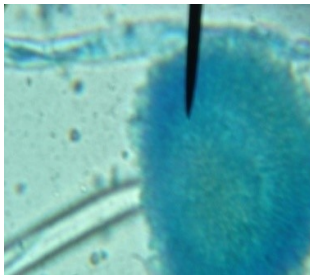
Table 4.2: Description of microscopic characteristics of fungi isolated from mycotoxin contaminated maize (Larone, 2002)

Type of fungi	Fungal structures	Results
Aspergillus niger	Biseriate, phialides, covering entire vesicle, radiate head	Plate 4.1 c
Aspergillus flavus	Biseriate/uniseriate phialides covering entire vesicle pointing in all directions, with smoothly roughened conidia.	Plate 4.2 c
Aspergillus vesicolar	Biseriate phialides, losely radiate covering most of the vesicle	Plate 4.3 c
***Fusarium* spp**	Sickle-shaped macroconidia produced from phialides on, ubranched conidiophores.	Plate 4.4 c
***Penicillium* spp**	Branched conidiophores, flask shaped phialides bearing unbrached chains of smooth conidia	Plate 4.5 c
***Cladosporium* spp**	Oval short branching conidia	Plate 4.6 c
***Rhizopus* spp**	Round sporangia with unbranched sporangiophores	Plate 4.7 b

Plate 4.1: (a) *Aspergillus niger* (obverse)



(b) White colour on the reverse of colony



(c) Radiate head covering entire vesicle X 40 Mg

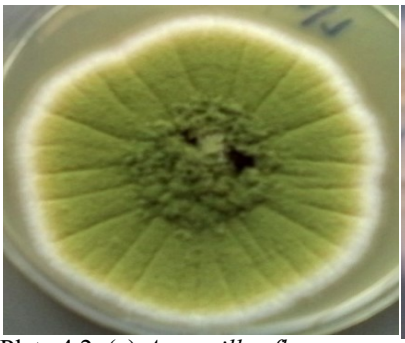
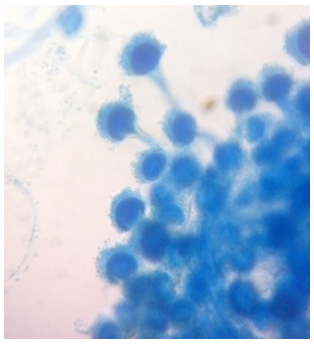


Plate 4.2: (a) *Aspergillus flavus* with greenish colony surface (obverse)

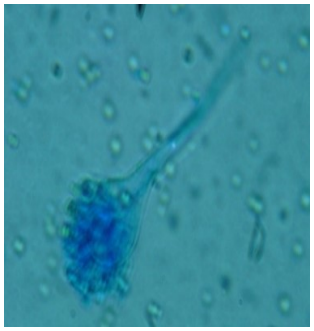
(b) Pale yellow colour on the reverse



(c) Conidial heads with phialides pointing in all directions X 40 Mg

Plate 4.3: (a) *Aspergillus vesicolar* bluish green surface with exudates (obverse)

(b) Yellow colour on the ·everse



(c) Biseriate phialides on conidial heads, loosely radiate X 40 Mg

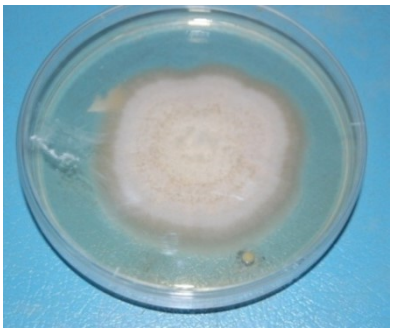
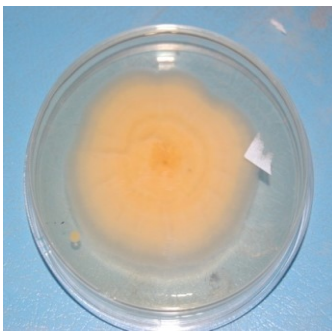
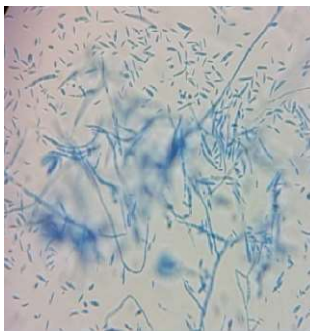


Plate 4.4 (a) *Fusarium* spp with pink colour on the surface of the colony spp (obverse)



(b) Yellow colour on the reverse



(c) Sickle shape macroconidia X 40 Mg

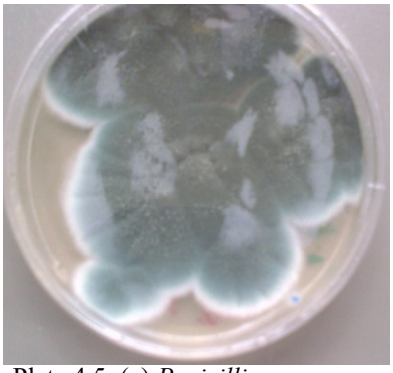
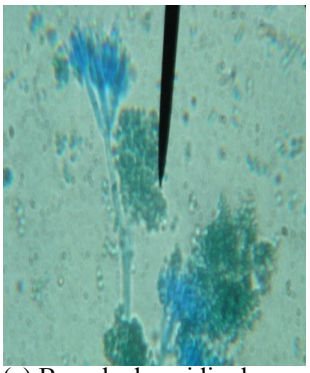


Plate 4.5: (a) *Penicillium* spp with bluish green surface and a white border (obverse)

(b) White colour on the reverse of colony



(c) Branched conidiophores, flask shaped phialides X 40 Mg

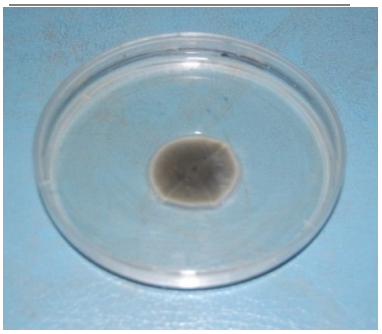
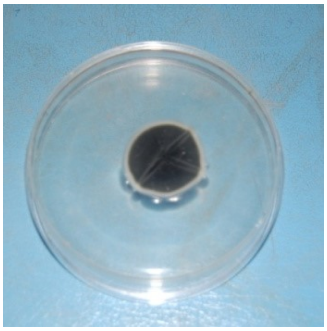
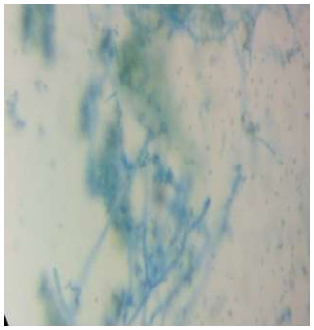


Plate 4.6: (a) Cladosporium spp with characteristic black colour on the obverse



(b) black colour on the reverse



(c) Oval short branching conidia X 40 Mg

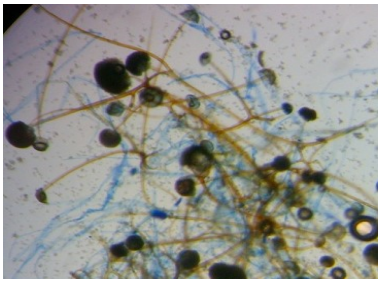


Plate 4.7: (a) *Rhizopus* spp with gray colour on the obverse (b) Round sporangia with unbranched sporangiophores

4.2 Culture plates of different fungi isolated from mycotoxin contaminated maize

4.3 Fungal infestation on mycotoxin contaminated maize following treatment with fungicides

Varied levels of infestation on fungicide treated maize kernels were recorded in the five regions. A large number of fungicide treated maize kernels had less infestation by fungi compared to the untreated maize kernels (Figure, 4.1). Maize kernels treated with Mistress had the lowest infestation among the four fungicides where the lowest infestation of 28% was recorded in mycotoxin positive maize from Mombasa (Figure 4.1). Untreated maize samples from Mombasa also had the lowest infestation of 72.5% among the five regions. On the other hand, untreated maize kernels obtained from Nairobi and Kitale had the highest fungal infestation of 99.1% and 98.2% respectively (Figure, 4.1).

Maize samples treated with Milraz had a slightly high infestation compared to the other three fungicides tested. In Kitale and Kisumu, infestation on maize treated with Milraz was highest with 76.8% and 81% respectively. This was also recorded in Mombasa where the infestation was high (59.4%) in maize treated with Milraz compared to Mistress (28%), Victory (37.5%) and Antracol (50%) (Figure, 4.1). Nevertheless, maize samples from Nairobi treated with Antracol had the highest infestation of 92% while those from Kisumu treated with Milraz were also more

infested (81%) compared to all the fungicide treated maize kernels from all the regions.

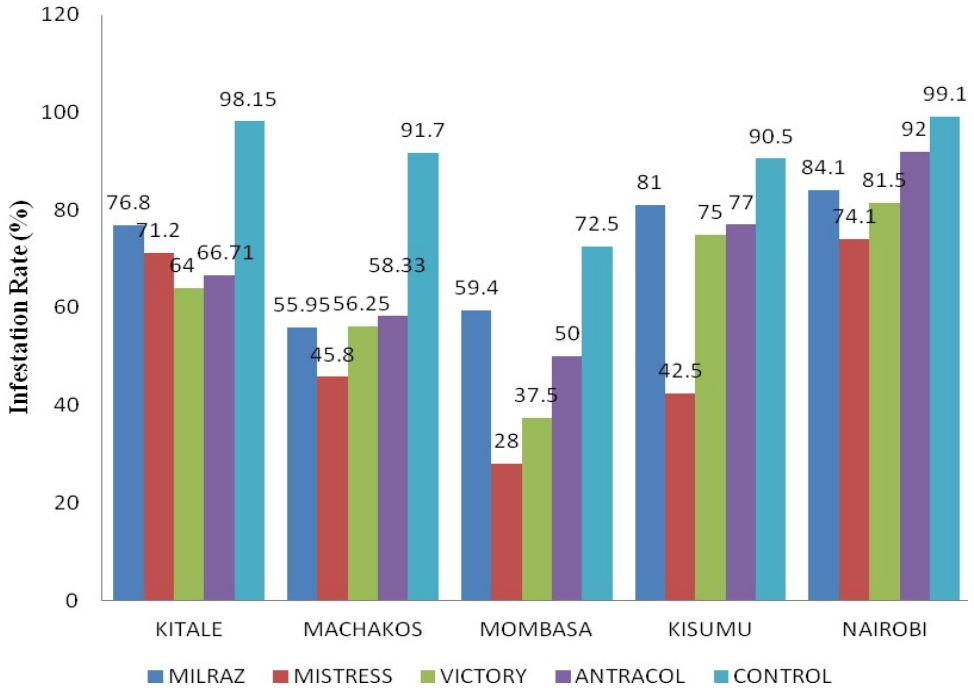


Figure 4.1: Fungal infestation on mycotoxin positive maize samples from different regions in Kenya following treatment with four fungicides

Fungal Infestation on maize samples from the five regions compared by ANOVA revealed that the general mean difference in the infestation among the five sites was significant ($p< 0.05$) (Table, 4.4). However, there was no significant difference in the infestation rates between Kitale and Nairobi $p>0.05$ ($p=0.66$) as well as Kitale and Kisumu $p>0.05$ ($p=0.189$). The same case occurs in the infestation rates between Machakos and Mombasa where $p>0.05$ ($p=0.621$). Apart from the above similarities, the differences in fungal infestation between Machakos, Kitale, Nairobi and Kisumu were highly significant {$p<0.05$ ($p= 0.00$)} (Table 4.4).

Table 4.3: Statistical comparison of fungal infestation per region

Comparison of regional infestation on mycotoxin positive maize						
(I) Region	(J) Region	Mean Difference (I-J)	Std. Error	Sig (p).	95% Confidence Interval	
					Lower Bound	Upper Bound
Kitale	Nairobi	-1.916	4.359	0.66	-10.48	6.65
	Machakos	35.677*	4.359	0	27.11	44.24
	Kisumu	7.769	5.903	0.189	-3.83	19.37
	Mombasa	32.769*	5.903	0	21.17	44.37
Nairobi	Kitale	1.916	4.359	0.66	-6.65	10.48
	Machakos	37.593*	4.318	0	29.11	46.08
	Kisumu	9.685	5.873	0.1	-1.85	21.22
	Mombasa	34.685*	5.873	0	23.15	46.22
Machakos	Kitale	-35.677*	4.359	0	-44.24	-27.11
	Nairobi	-37.593*	4.318	0	-46.08	-29.11
	Kisumu	-27.907*	5.873	0	-39.45	-16.37
	Mombasa	-2.907	5.873	0.621	-14.45	8.63
Kisumu	Kitale	-7.769	5.903	0.189	-19.37	3.83
	Nairobi	-9.685	5.873	0.1	-21.22	1.85
	Machakos	27.907*	5.873	0	16.37	39.45
	Mombasa	25.000*	7.095	0	11.06	38.94
Mombasa	Kitale	-32.769*	5.903	0	-44.37	-21.17
	Nairobi	-34.685*	5.873	0	-46.22	-23.15
	Machakos	2.907	5.873	0.621	-8.63	14.45
	Kisumu	-25.000*	7.095	0	-38.94	-11.06

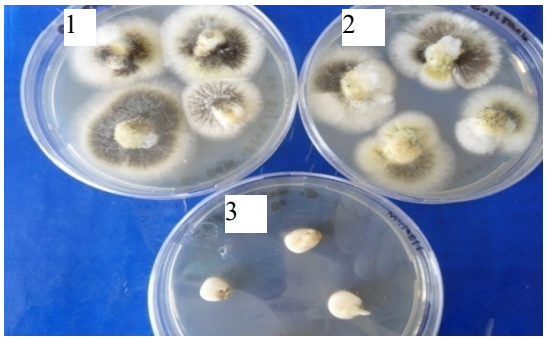
The mean general mean difference in the infestation per region is significant at the 0.05 level. (Fisher's Significant Difference Test) However the infestation between some regions was no statistically significant.

The distribution of fungicide treated and untreated maize samples in each category of infestation was recorded (Table 4.5). Most of the control samples were heavily infested by fungi having 75% (n=11) and 100% (n=115) infestation compared to the treated samples. All the untreated samples were infested by different fungal genera. Many samples treated with Antracol and Millraz had 100% infestation (n=54 and n=55 respectively) compared to those treated with Mistress and Victory which were n=30 and n=29 respectively. On the other hand, a large number of mycotoxin positive maize samples treated with Mistress and Victory fungicide had no infestation (n=35 and n=47 respectively) compared to those treated with Milraz and Antracol (n=14 and n=19 respectively) (Table 4.5).

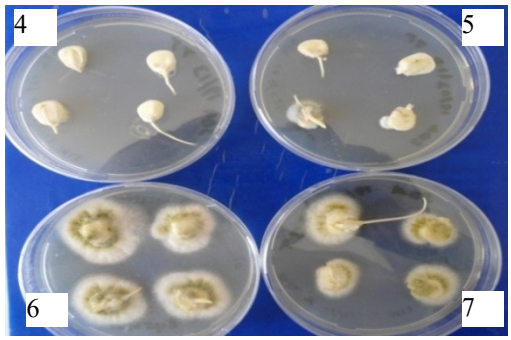
Table 4.4: Fungal Infestations in fungicide treated and untreated maize samples

Percentage infestation Categories		**Type of fungicide used**				
		MILRAZ	MISTRESS	VICTORY	ANTRACOL	CONTROL
0	% within type of fungicide used	10%	26%	34%	14%	
		n=14	n=35	n=47	n=19	
25	% within type of fungicide used	21%	27%	19%	24%	4%
		n=29	n=37	n=26	n=33	n=5
50	% within type of fungicide used	12%	12%	18%	15%	5%
		n=17	n=17	n=25	n=21	n=7
75	% within type of fungicide used	17%	14%	8%	8%	8%
		n=23	n=19	n=11	n=11	n=11
100	% within type of fungicide used	40%	22%	21%	39%	83%
		n=55	n=30	n=29	n=54	n=115
Total	% within type of fungicide used	100%	100%	100%	100%	100%
		n=138	n=138	n=138	n=138	n=138

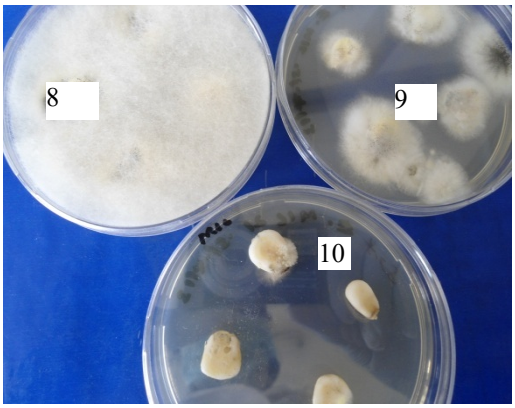
Fungal growth on maize kernels treated with the test fungicides as well as the untreated controls was recorded. The fungicide treated kernels exhibited a significant reduction in mycoflora while the untreated kernels were heavily infested (Palate 4.8).



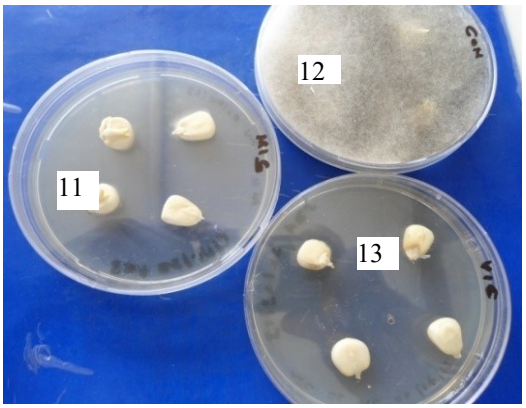
a Untreated maize kernels infested by *Aspergllus niger* (1&2) and kernels treated with Antracol (3)



b. Maize kernels treated with Mistress (4) and Victory (5) fungicide having low infestation and kernels infested by *Aspergillus flavus* (6 & 7).



c. Maize kernels infested by *Fusarium* spp (8) and mixed infestation of *Aspergillus falvus and A. niger* (9) and maize kernels treated with Milraz ((10)



d. Untreated maize kernels heavily infested by *Rhizopus* spp (12) and maize kernels treated with Milraz (11) and Victory (12) fungicides

Plate 4.8: Fungal cultures on fungicide t[illegible]

4.4 Disc Diffusion Tests

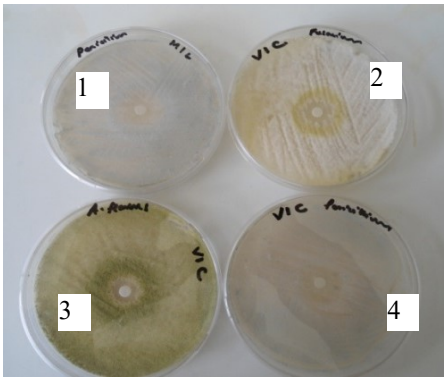
Thirty one isolates from the five regions exhibited resistance to at least one of the test fungicides (Table 6). *Aspergillus flavus* isolates from Kisumu were more resistant to Milraz compared to the other fungicides and exhibited no zone of inhibition on Saborauds dextrose agar plates. Among the resistant fungi, *A. flavus* and *Fusarium* spp isolates were more resistant than *Penicillium* spp and *Rhizopus* spp. On the other

hand, most of these isolates were susceptible to Mistress and Victory while being resistant to one or the other fungicides.

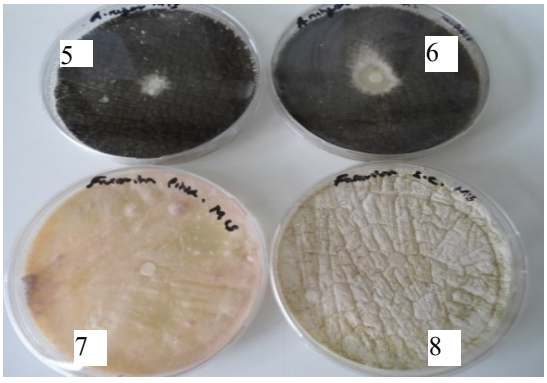
Table 4.5: Fungal isolates that exhibited resistance to at least one of the test fungicides

Region and fungal isolate number	**Average zone diameter (mm) for resistant fungal isolates**				
Kitale	**Milraz**	**Mistress**	**Antracol**	**Victory**	**Fluconazole**
Aspergillus flavus kt 4	18 I	19 S	11R	19 S	23 S
Fusarium spp kt10	0 R	16 I	11R	20 S	28 S
Cladosporium spp kt22	14 S	13 R	25 S	35 S	30 S
Penicillium spp kt11	0 R	23 S	10 R	21 S	22 S
Aspergillus niger kt18	0 R	18 I	0 R	14 S	21 S
Kisumu	**Milraz**	**Mistress**	**Antracol**	**Victory**	**Fluconazole**
A. flavus ks2	9 R	13 I	11 R	10 R	19 I
A. flavus ks4	0 R	13 I	10 R	8 R	24 S
A. flavus ks5	0 R	11 R	8 R	9 R	28 S
A. niger ks*7*	9 R	22 S	10 R	13 I	26 S
Penicillium spp ks8	19 S	21 S	21 S	11 R	21 S
*Penicillium*spp ks 3	11 R	20 S	19 S	10 R	25 S
Fusarium spp ks5	16 I	43 S	40 S	35 S	34 S
Fusarium spp ks11	17 I	29 S	25 S	15 S	31 S
*Rhizopus*spp ks10	20 S	0 R	22 S	0 R	-
Mombasa	**Milraz**	**Mistress**	**Antracol**	**Victory**	**Fluconazole**
Penicillium spp mbs 7	25 S	20 R	25 S	25 S	23 S
Fusarium spp mbs 3	11 R	19 I	19I	13 R	26 S
Fusarium spp mbs 11	12 R	31 S	30 S	25 S	24 S
A. flavus mbs 16	8 R	13 R	10 R	0 R	20 S
Cladosporium spp mbs 28	0 R	13 R	10 R	0 R	18 S
Machakos	**Milraz**	**Mistress**	**Antracol**	**Victory**	**Fluconazole**
A. flavus mc16	0 R	13 R	10 R	10 R	21 S
Fusarium spp mc19	24 S	31 S	27 S	17 I	24 S
Rhizopus spp mc 2	35 S	39 S	40 S	31 S	29 S
Penicillium spp mc 17	18 I	23 S	27 S	17 I	24 S
A. niger mc 36	8 R	10 R	12 R	0 R	17 I
Nairobi	**Milraz**	**Mistress**	**Antracol**	**Victory**	**Fluconazole**
A. flavus nrb2	13 R	17 I	13 I	10 I	18 S
A. flavus nrb 6	18 I	20 S	18 I	13 R	23 S
A. niger nrb 9	14 I	18 I	10 R	0 R	16 I
Fusarium spp nrb 7	17 I	24 S	20 S	11 R	21 S
Rhizopus spp nrb 14	24 S	35 S	40 S	29 S	28 S
Penicillium spp nrb 6	17 I	23 S	22 S	10 R	25 S
Fusarium spp nrb16	20 S	22 S	14 S	10 R	27 S

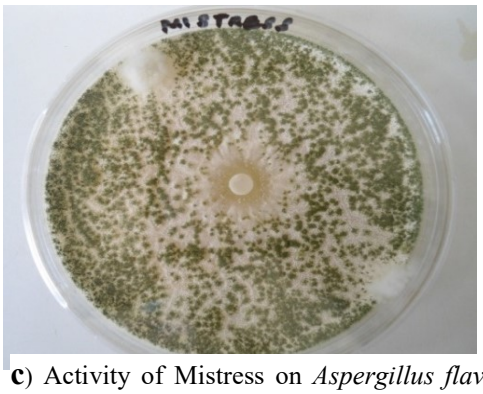
Key: **S**-susceptible (≥20 mm), **I**-intermediate(12-19 mm), **R**-resistant (≤11 mm) (0.5 McFarland inoculum) (Espinel *et al.,* 2012)



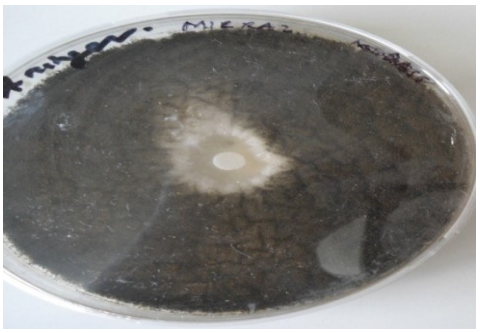
a) Activity of Milraz on *Penicillium* spp (1), Victory on (*Fusarium* spp (2), Victory on *Aspergillus* flavus (3) and *Penicillium* spp(4)



b) Activity of Mistress on *Aspergillus niger* (5), *Fusarium* spp (7), *A. flavus* (8) and Victory fungicide on *Aspergilus niger* (6)



c) Activity of Mistress on *Aspergillus flavus* (growth inhibition with formation of a clear zone around fungicide impregnated disk)



d) Activity of Milraz on *Asperillus niger* (growth inhibition with formation of a clear zone around fungicide impregnated disk)

Plate 4.9: Activity of test fungicides on fungal isolates from mycotoxin contaminated maize

The incidence of *Aspergillus* spp*, Penicillium* spp, and *Fusarium* spp respectively in the five study sites according to the frequency of isolation from the mycotoxin positive maize was variable. Fungi of the genera *Aspergillus* and *Fusarium* were frequently isolated from contaminated maize from Kitale with 38% and 42% respectively. The genera *Penicilllium* were frequently isolated (29%) from mycotoxin positive maize samples obtained from Mombasa. Mycotoxin contaminated maize from Machakos and Mombasa had the lowest infestation by the genera *Penicilllium* (5%) and *Fusarium* (4%) respectively.

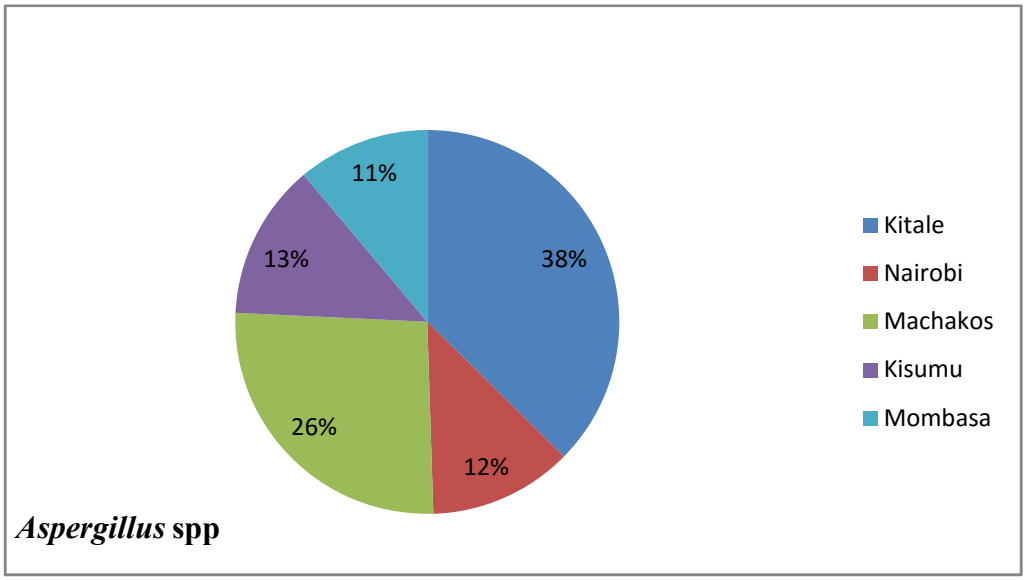


Figure 4.2: Incidence of *Aspergillus* spp isolated from mycotoxin positive maize from the five regions

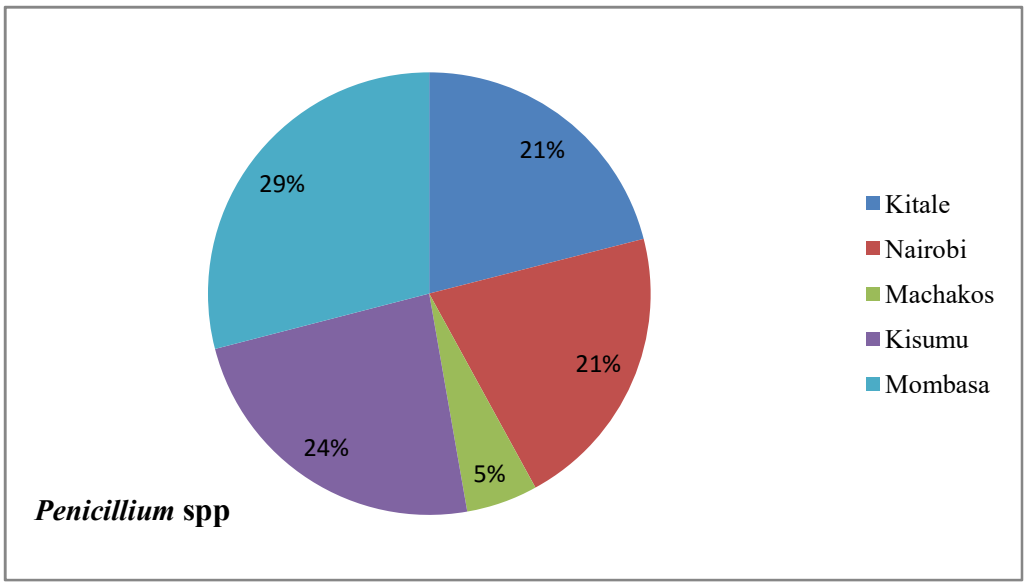


Figure 4.3: Incidence of *Penicillium* spp isolated from mycotoxin positive maize from the five regions

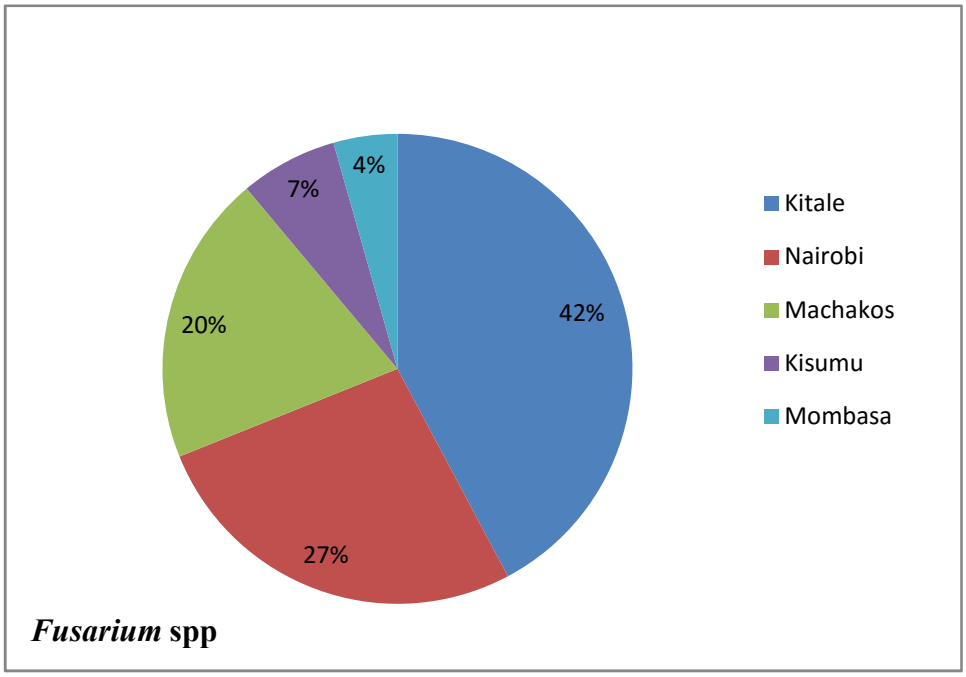


Figure 4.4: Incidence of *Fusarium* spp isolated from mycotoxin positive maize from the five regions

4.5 Effect of test fungicides on germination of maize Kernels

The four test fungicides exhibited negligible inhibition of maize germination. A large number of maize kernels treated with the four fungicides germinated beyond 50% in the five categories of 0, 25, 50, 75, and 100% (Figure 4.4). Out of 138 samples, only 3 treated with Victory, 4 treated with Mistress, 9 treated with Milraz, and 8 treated with Antracol were did not germinate. There was a significant positive correlation (R^2=0.054, $p<0.05$) between the germination of fungicide treated and untreated maize kernels. Positive correlation shows that the fungicides can enhance germination. A greater percentage of treated maize kernels germinated to 75% and 100% as well as the untreated maize kernels Figure 4.4).

Photographs of maize kernels treated with the four fungicides that germinated on culture were captured after 72 hours (Plate 4.9). Germination occurred at varying levels.

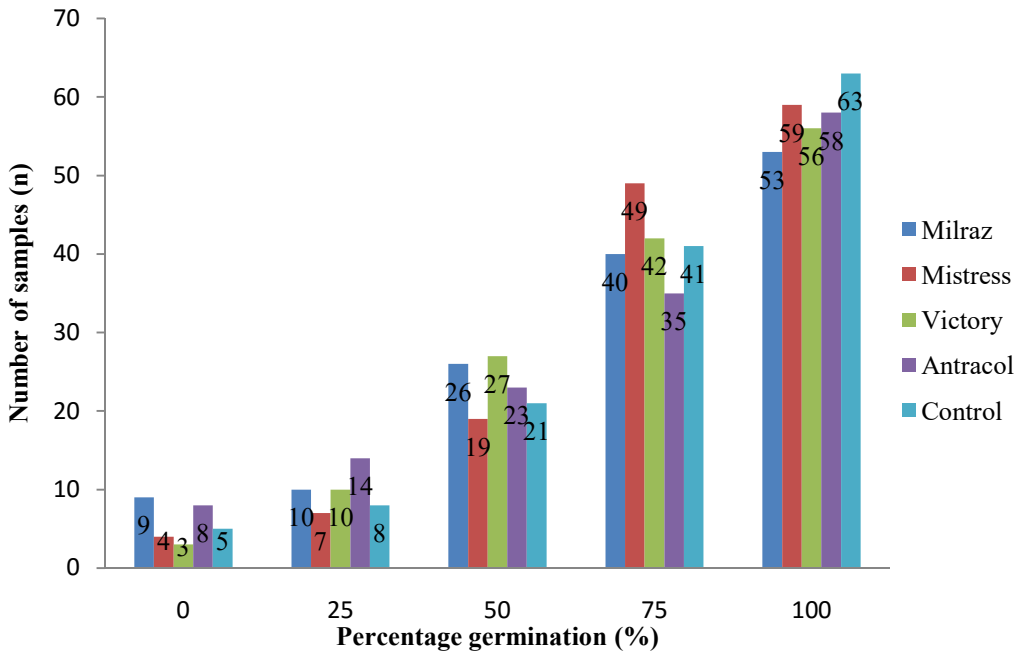
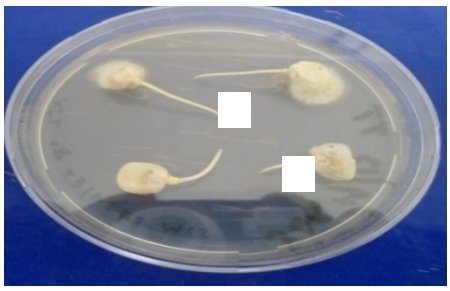


Figure 4.5: Number of maize samples in each category of germination following treatment with fungicides



a). Maize kernels treated with Milraz having 100% germination

b). Maize kernels treated with Mistress with 50% germination

d) Maize kernels treated with Victory fungicide

d) Maize kernels treated with Victory fungicide

Plate 4.10: Germination of fungicide treated maize kernels

CHAPTER FIVE

DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5. 1 Discussion

This study highlights the potential use of four test fungicides for control of potentially toxigenic fungi affecting maize in Kenya. The fungi were isolated from mycotoxin contaminated maize from different regions in Kenya. Mycotoxin positive maize samples from the five regions showed significant ($p>0.05$) infestation by different fungal genera mainly *Aspergillus* spp, *Fusarium* spp, *Penicillium* spp and *Rhizopus* spp. The percentage infestation per region on untreated mycotoxin positive maize kernels was high with 99.1% and 98.15% for Nairobi and Kitale respectively.

A variety of agricultural produce are contaminated by various mycotoxin producing fungi such as the *Aspergillus flavus* which was frequently isolated from maize samples analyzed in this study. Though the presence of the *Aspergillus* mould does not essentially designate aflatoxin contamination, there is definitely an increased risk for the production of these toxins (Robertson, 2005**)**. Other studies have shown that the presence of these fungi on grains may cause spoilage and affect its quality (Candlish *et al.,* 2001; Rasooli & Abyaneh, 2004).

Fungicides have been used to control fungi affecting several plant products, synthetic chemicals have been tested for their efficacy against mycotoxigenic fungi (Ajorin *et al.,* 2013). It has been demonstrated that natural fungicide application on seeds would result in more efficient control of toxigenic fungi and may lead to reduction of mycotoxins in stored products (Ajorin *et al.,* 2013). However some fungicides are known to be hazardous to beneficial non-target organisms and humans; while few could even support the growth of pathogenic fungi (Yang *et al.,* 2011).

In consideration of the zones of inhibition, a few isolates were able to grow even to the point of covering the disks impregnated with the test fungicides. However most

of the isolates were responsive to the fungicides with varying zone diameters. Some isolates of *Aspergillus niger* and *A. flavus* were resistant to the fungicides and there was an overgrowth of the fungi on the plates covering disks impregnated with the test fungicides. This phenomenon shows the possible occurrence of some fungal species that are inherently resistant to the antifungals. Resistance to the fungicides could be intrinsic or may have been acquired in the environment as a result of overexposure to fungicides used in Agriculture.

Definitive susceptibility profiles for the fungi need to be ascertained using other techniques such as minimum inhibitory concentration method (Rodriguez *et al.*, 2008). *Rhizopus* spp was considerably susceptible to all the test fungicides. However, it was not completely eliminated by these agents since it contributed to the infestation observed in fungicide treated maize kernels in the five regions. *Rhizopus* spp is known to be a beneficial fungal species that contributes to degradative activities in the environment availing nutrients to other organisms (Yang *et al.,* 2011). The results suggest that the test fungicides may inhibit a population of beneficial fungal species such as the *Rhizopus* spp. Nonetheless, this effect is not very significant since a substantial population will remain and proliferate to replace the lost population. The effect on these beneficial degrading organisms is an indicator of the environmental effects that could occur due to the use of these chemical substances in the control seedborne fungi affecting maize that are also capable of producing mycotoxins.

All the untreated mycotoxin positive maize kernels were infested by different fungal genera; therefore none of these control samples had no infestaion. At 0% infestation, Mistress and Victory had the highest percentage of uninfested maize samples 26% and 34% respectively. The two fungicides exhibited a significant fungicidal activity on the mycoflora of maize kernels to which they were applied. Among the toxin positive samples treated with Milraz and Antracol, 40% and 39% respectively were absolutely infested compared to those treated with Victory and Mistress 21% and

22% respectively). These results are an evidence that some fungal species were resistant to Milraz and Antracol and hence the large number of infested maize samples. In comparison with the untreated maize kernels that had 100% infestation (n=115), the four test fungicides exhibited significant fungicidal activity on the resident mycoflora. A few samples (n=29) treated with Victory fungicide had 100% infestation while those treated with Milraz were the highest (n=55) in this category. Though a large number samples (n=55) treated with Milraz had absolute infestation, this was however low by half compared to that of the untreated control samples (n=115). These findings however demonstrate that a significant percentage of the mycoflora could still be inhibited by Milraz.

Milraz, Mistress and Victory fungicides are composed a combination two fungicidal compounds which seem to act synergistically while of Antracol contains a single compound probineb which also exhibits fungicidal effect. The presence of mancozeb in Victory and Mistress appear exhibit a higher fungicidal activity evidenced by the lowest infestation recorded in maize kernels treated with the two fungicides.

A number of bioagents and different fungicides have been found to reduce mycoflora on seeds and enhance germination as well as vigour Index of various grains such as cowpea (Mogle & Maske, 2012). In a study which tested the efficacy of Benomyl, Dithane M-45 75% WP (Manganese ethylene bis dithio carbamate plus zinc) and Bavistin 50% WP (Methyl-H-benzimidazole-2ylcarbamate) on seed mycoflora of cowpea, variations in their infestation rate was observed depending on the respective treatments (Mongle & Maske, 2012). The most prevailing fungal pathogens observed on treated and untreated seeds of cowpea included, *Aspergillus flavus, Rhizoctonia solani, Aspergillus niger, Cladosporium*spp, *Penicillium* spp, *Fusarium oxysporum, Fusarium solani*among others.

In another study that tested different concentrations of fungicides against various pathogens, Dithane M-45 and Bavistin were found to be effective in reducing seed-borne infection of *Fusarium* spp. on maize seeds (Kumar & Dubey 2012). *In vitro*

comparative studies have also been done with some fungicides such as Captan, Dithane M-45, Bavistin, and Vitavax for their efficacy in controlling *Fusarium* species on cereal grains (Singh *et al.,* 2002).) A reduction of wilt disease following the application Mancozeb M-45, Bavistin and Vitavax has also been reported and, germination of fungicide treated seeds was enhanced compared to controls (De & Chaudhary 1999). Untreated seeds had the highest seed mycoflora infestation of 68% and lowest seed germination of 80%. High infestation of untreated maize kernels was also seen in the current study and a positive correlation was observed between fungicide treatment and germination of treated maize kernels. The results obtained in this study demonstrate that treatment of maize kernels with the test fungicides could enhance germination and significantly reduce the population of potentially toxigenic fungi.

During the years of commercial use of a fungicide, there can arise populations of the target pathogen that are no longer sufficiently sensitive to be controlled adequately (Ball, 1988). They appear as a response to repeated use of the fungicide, or to repeated use of another fungicide related to it chemically and/or by biochemical mechanism of antifungal action. Some isolates of *Aspergillus flavus*, *Fusarium* spp and *Penicillium* spp exhibited resistance to one or more of the test fungicides.

Fungicides tested in this study inhibited the growth of different fungal species from the treated maize kernels. The chemical agents therefore exhibited a broad spectrum activity inhibiting distinct fungal genera. Victory and Mistress fungicides showed a higher fungicidal activity on the mycoflora of toxin positive maize kernels. The two fungicides were effective against *Asperillus flavus A. niger* and *Rhizopus* spp. Culture plates with maize kernels treated with the two fungicides appeared clearer compared to the heavily infested untreated kernels from the same sample pack. The increased efficacy of Victory and Mistress could be attributed to the combination of two chemical compounds in the fungicides. Mistress WP 72 is composed of Cymoxanil 8% and Mancozeb 64% while Victory WP is a combination Metalaxy

80g/kg and Mancozeb 640g/kg. The compounds act synergistically against the fungal cell therefore exhibiting a high fungicidal effect. Metalaxy targets nucleic acid synthesis while Mancozeb has a multisite activity (Yang, *et al.,* 2011).

Several methods for controlling potentially toxigenic seed borne fungi affecting maize have been established, these include physical, chemical and biological methods mainly aimed at preventing the growth of fungi on the maize yield (Dorner, 2004). The use of chemical inhibitors which suppress spore germination of fungi, and the development of the fungal mycelium is one of the most effective ways which has been used successfully (Moreno *et al.,* 2000). Although several alternatives to chemical fungicides are being sought, an effective biocontrol or natural fungicide that can completely replace chemical fungicides in the market is yet to be found. However, the necessity to develop fungal disease control measures by means of phytochemicals as a substitute to synthetic chemicals has become a priority of researchers worldwide (Reddy *et al.,* 2009).

The significant ($p<0.05$) differences in the incidence of potentially toxigenic fungal genera in the five regions suggests that varied climatic conditions could influence the distribution of and existence of these species. The frequent isolation of *Aspergillus* spp in maize samples from Machakos (29%) signifies the frequent contamination of maize from this region by aflatoxins produce by *Aspergillus*. *flavus and A. parasiticus*. This region is part of Eastern Kenya where several incidences of aflatoxicoses have occurred. The largest outbreak was reported in 2004 where residents consumed aflatoxin contaminated maize resulted in hospitalization, death and loss of tones of maize yields (Gieseker & Centers for Disease Control and Prevention, 2004). Drought conditions are more likely to occur in this region, which stresses plants making them more susceptible to contamination by *Aspergillus* spp. (Holbrook *et al.,* 2004; Robertson, 2005).

Some members of the genera *Fusarium* frequently isolated from toxin positive samples from Kitale (42%) are known to produce Fumonisisns. The high frequency

of these fungal genera indicates a likelihood of high contamination by fumonisins. Kitale is part of Western Kenya and is the leading producer of maize in the country where the crop is grown on large scale (Alakonya *et al.,* 2008). In this region, farmers are known to habitually leave their maize yields in the field upon maturity to allow drying. However, the harvest season repeatedly coincides with second rains which increase rotting and infestation by *Fusarium* fungi (Alakonya *et al.,* 2008). This could be a reason for the frequent occurrence of this mould species in the toxin positive maize samples from Kitale. Previous studies on maize from Western Kenya have isolated a wide variety of potentially toxigenic fungi both of the *Fusarium* spp and *Aspergillus* spp. These include *Fusarium verticillioides*, *Fusarium graminearum, F. subglutinans* as well as *A. flavus* and, *A. parasiticus* known to produce varied toxins (Kedera *et al.,* 1999).

The low infestation of maize samples from Mombasa and Kisumu by *Fusarium* fungi compared to Kitale and Machakos could be as a result of the different maize varieties grown in these regions as well varied climatic conditions that exist which may not be favorable to *Fusarium* mould. On the other hand, the toxin positive maize samples from Machakos had the lowest population of *Penicillium* spp (5%). This indicates that maize from this region are likely to have less contamination by Citrinin, ochratoxin A. and penicillic acid produced by fungi of the genera *Penicillium* such as *Penicillium uiridicatum* (Dowd, 1989).

Post-harvest contamination of maize with aflatoxin is mainly due to infestation with aflatoxigenic *Aspergillus* species at pre-harvest stage, poor management practices and unfavourable conditions at post-harvest. High moisture content in the grains also increases post-harvest aflatoxin contamination. Consequently, it is essential to dry maize to a kernel moisture level of 13% and groundnuts to 7%, as aflatoxin levels increase in food during storage. Some of the factors contributing to aflatoxin contamination in maize are excessive heat, high humidity, lack of aeration in stores and insect and rodent damage (Hell & Mutegi, 2011)

The disparity in resistance to fungal infection and aflatoxin contamination in maize can be linked to genetic charateristics associated with resistance as such alongside other resistance-associated traits for instance, kernel characteristics, stress tolerance and pest resistance, however these genetic resistances are vastly influenced by the environment (Williams *et al.,* 2014).

The findings in this study suggest a significant positive correlation ($p<0.05$, $R^2=0.054$) between the germination of fungicide treated and untreated maize kernels. Therefore it is possible that the fungicides can enhance germination of maize kernels. A greater percentage of fungicide treated maize kernels germinated to 75% and 100%. On the other hand, the untreated control maize samples germinated to 75% and 100%. This trend could imply that that the fungicides have no negative influence on the germination of maize kernels hence this may not be of great concern if the fungicides are used to control potentially toxigenic fungi affecting maize in the field.

5.2 Conclusion

1. The present study indicates that mycotoxin positive maize samples from the five regions were heavily infested by potentially toxigenic fungi of different genera. The results in have demonstrated the *in vitro* antifungal activity of the four test fungicides to significantly inhibit the mycoflora in contaminated maize kernels {$p<0.05$ (0.00)}. Mistress and Victory fungicides exhibited a higher fungicidal activity.
2. A large number of the isolates particularly *Aspergillus* spp*, Fusarium* spp and *Pencillium* spp were susceptible to the test fungicides. However some were resistant to more than one of the test fungicides with no zone of inhibition on fungicide impregnated disks. The four test fungicides are used for controlling fungal diseases in food crops such as vegetables, tomatoes, fruits potatoes and ornamentals in the field whose safety has been guaranteed. Therefore they could be used to control seed borne fungi affecting maize.

3. There was a significant ($p<0.05$, $R^2=0.054$) positive correlation between the germination of fungicide treated and untreated maize kernels. Most of the fungicide treated maize kernels were able to germinate to 75% and 100%. Therefore the data suggests that the fungicides did not inhibit the germination of maize and could be assessed further for use in the control of potentially toxigenic fungi affecting maize.

5.3 Recommendations

1. Field experiments should be conducted to ascertain the field efficacy of the four fungicides tested in this study. Mistress and Victory fungicide which exhibited higher fungicidal activity should be evaluated further for use in controlling potentially toxigenic fungi affecting maize for public food safety.
2. More elaborate studies should also be carried out to determine the extent of fungicide resistance in the environment since this could have far reaching implications in the use of antifungal drugs for treatment of fungal infections in clinical settings particularly to those with similar modes of action.
3. Studies should be carried out to determine the predominant populations of fungal genera in different regions in Kenya as well as the conditions and factors that favor their occurrence and spatial variability.

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APPENDICES

Appendix 1: Scientific Steering Committee (SSC) approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-206901, 0733-460033; Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website:www.kemri.org

ESACIPAC/SSC/101841 **4th July, 2013**

Josephat Tonui

Thro'
Director, CMR
NAIROBI

Forwarded 11/7/13

REF: SSC No. 2602 (Revised) – Susceptility of mycotoxigenic fungi to commercial fungicides potential for mycotoxin control in maize

Thank you for your letter dated 26th June, 2013 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

Encl[s]

In Search of Better Health

Appendix 2: Ethical Review Committee (ERC) approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **November 7, 2013**

TO: **JOSEPHAT TONUI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. WILLIE SANG,**
ACTING DIRECTOR, CMR,
NAIROBI

forwarded on 15th 11/2013

Dear Sir,

RE: **SSC PROTOCOL NO. 2602 - (*RESUBMISSION*): SUSCEPTIBILITY OF MYCOTOXIGENIC FUNGI TO COMMERCIAL FUNGICIDES AS A POTENTIAL FOR MYCOTOXIN CONTROL IN MAIZE**

Reference is made to your letter dated 7th August 2013. The ERC Secretariat acknowledges receipt of the revised study protocol on 6th November 2013.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document and noted that the issues raised at the 217th meeting have been adequately addressed. Consequently, the study is granted approval for continuation effective this **7th day of November 2013.** Please note that authorization to conduct this study will automatically expire on **November 6, 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **September 25, 2014.**

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received. Please note that any unanticipated problems resulting from the conduct of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Yours faithfully,

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 3: Publications committee approval letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax. (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/LIB/9/18/2 21st July, 2014

Josephat Tonui.

Thro'
Director
CMR

KEMRI/PUB/3465 - Susceptibility of mycotoxigenic fungi to commercial fungicides a potential tool for mycotoxin control in maize, Kenya.

This is to inform you that the above manuscript has been approved by the publications committee for publication.

Thank you.

Dr. Cecilia Mbae
for SECRETARY
PUBLICATIONS COMMITTEE

Appendix 4: African Journal of Food Agriculture Nutrition and Development Manuscript acceptance letter

ajfand
AFRICAN JOURNAL OF FOOD, AGRICULTURE, NUTRITION AND DEVELOPMENT
ISSN 1684 5374

P.O. Box 29086 - 00625, Nairobi, KENYA.
Email: oniango@iconnect.co.ke and/or info@ajfand.net
Website: www.ajfand.net

28th November 2014

Mr. Josephat Tonui
Jomo Kenyatta University of Agriculture and Technology
College of Health Sciences
P.O. Box 62000-00200
Nairobi

Dear Mr. Tonui,

Re: Article Acceptance by AJFAND
'Susceptibility of mycotoxigenic fungi to commercial fungicide, a potential tool for mycotoxin control in maize in Kenya'
[SNo. 13985]

This is to certify that the above mentioned article that is authored by Mr. Josephat Tonui and co-authored by C. Kiiyukia and C.B. Christine, has been accepted for publication in AJFAND. The article has already undergone and successfully completed our international peer review cycle and had satisfied the reviewers with its contents.

The article will appear in a volume yet to be determined and shall be accessible online on our website www.ajfand.net

We thank the author[s] for publishing with AJFAND.

Yours sincerely

Hon. Prof. Ruth Oniang'o, PhD, DSM, SS
Founder Editor-in-Chief, AJFAND

Appendix 5: Global Advanced Research Journal of Agricultural Science (GARJAS) acceptance letter.

Global Advanced Research Journal of Agricultural Science
www.garj.org

ACCEPTANCE OF MANUSCRIPT

16th June 2015

Author(s): Josephat, Kiiyukia and Christine
[1*, 2] Jomo Kenyatta University of Agriculture and Technology, College of health Sciences.
[3] Kenya Medical Research Institute (KEMRI)

Dear Mr. Tonui K. Josephat,

RE: GARJAS-15-075

I am pleased to inform you that reviewers have recommended your manuscript "**Mycotoxigenic fungi, distribution and infestation of maize in selected sites- Kenya.**" and it has been accepted for publication in the Global Advanced Research Journal of Agricultural Science (GARJAS) ISSN: 2315-5094; Impact Factor (ISI)= 1.217; Indexed by Scopus, EyeSource, DRJI. It is an excellent paper and we have made only few minor corrections. It will be published in the June 2015 issue of the journal.

The galley proof will be sent to you shortly.

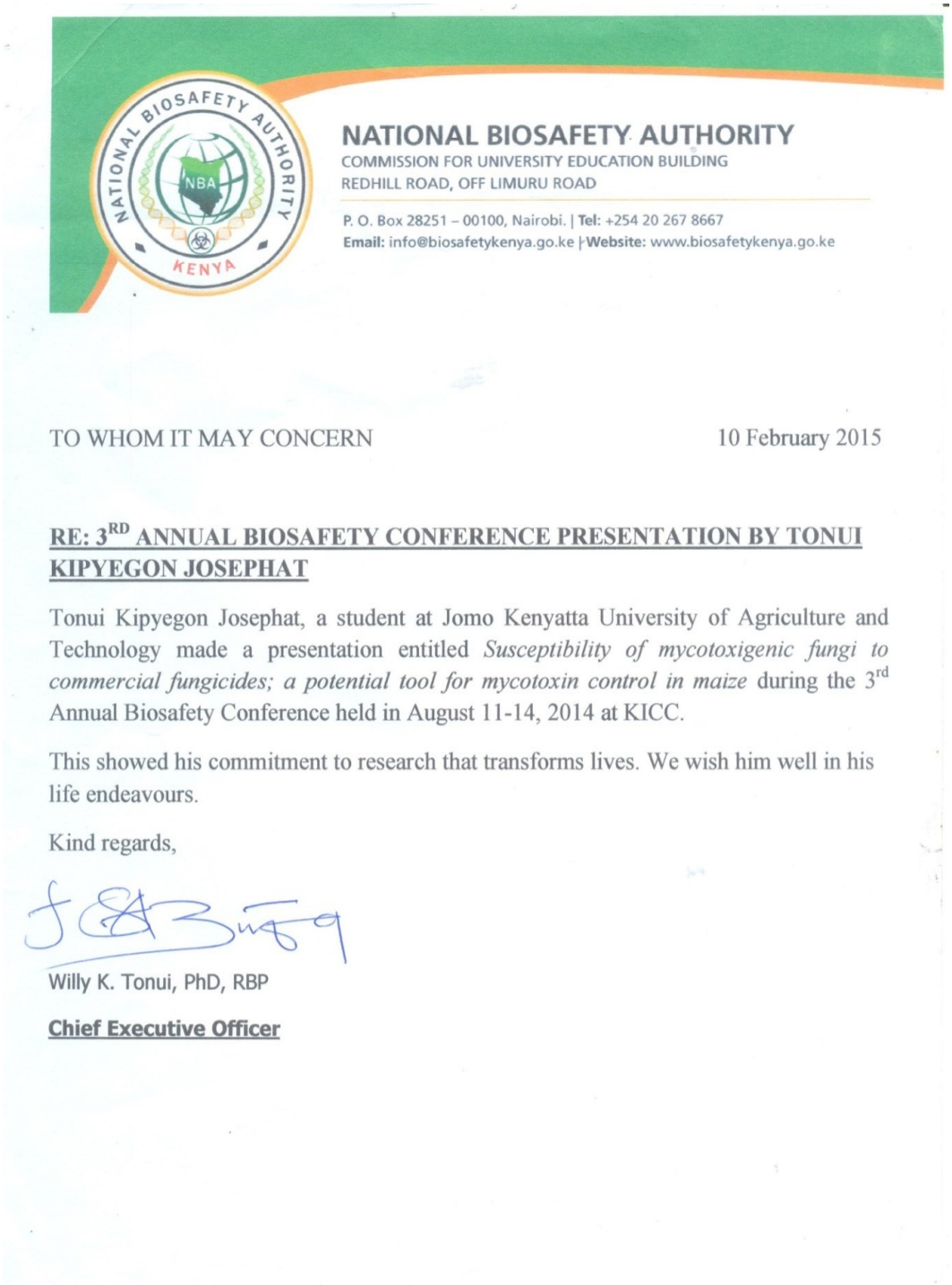
Kindly contact our Accounts Sections at garj.account@garj.org, acc.servicegarjournal@gmail.com for payment of processing fee (if you have not done so) and send confirmation email of payment.

Thanks for publishing with us.

Yours sincerely,

James Omiponle,
Editorial Officer.
Global Advanced Research Journal of Agricultural Science (GARJAS)
Tel:+18593059163; E-mail : submit.garjas@garj.org
editorial.garjas@globaladvacedresearchjournals.com
http://garj.org/garjas/index.htm

Appendix 6: Third National Biosafety Authority Conference presentation acknowledgement letter



NATIONAL BIOSAFETY AUTHORITY
COMMISSION FOR UNIVERSITY EDUCATION BUILDING
REDHILL ROAD, OFF LIMURU ROAD

P. O. Box 28251 – 00100, Nairobi. | **Tel:** +254 20 267 8667
Email: info@biosafetykenya.go.ke | **Website:** www.biosafetykenya.go.ke

TO WHOM IT MAY CONCERN 10 February 2015

RE: 3RD ANNUAL BIOSAFETY CONFERENCE PRESENTATION BY TONUI KIPYEGON JOSEPHAT

Tonui Kipyegon Josephat, a student at Jomo Kenyatta University of Agriculture and Technology made a presentation entitled *Susceptibility of mycotoxigenic fungi to commercial fungicides; a potential tool for mycotoxin control in maize* during the 3rd Annual Biosafety Conference held in August 11-14, 2014 at KICC.

This showed his commitment to research that transforms lives. We wish him well in his life endeavours.

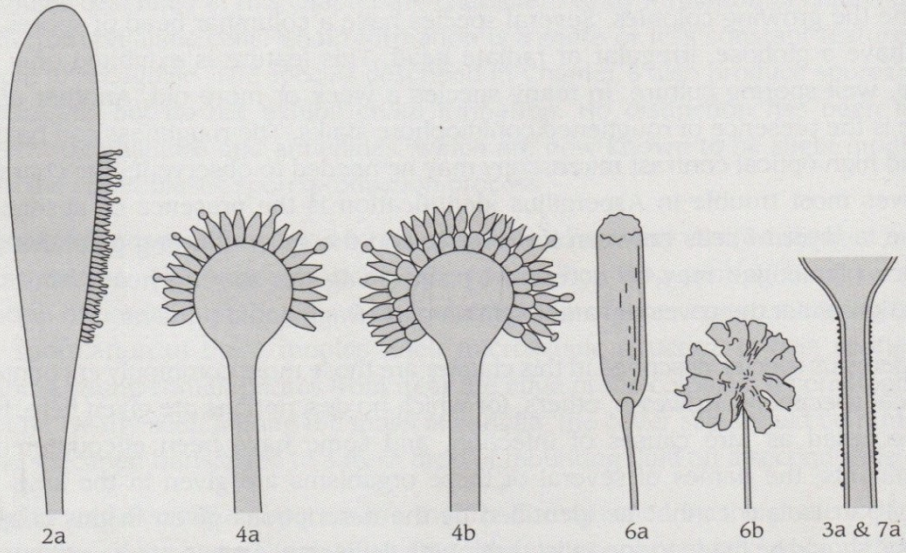
Kind regards,

Willy K. Tonui, PhD, RBP

Chief Executive Officer

Appendix7: Key to *Aspergillus* species (Larone, 1995)

Key to *Aspergillus* species



1a	Colony some shade of green	2
1b	Colony not green	7
2a	Vesicles elongated (> 100 µm long)	*Aspergillus clavatus* (not described)
2b	Vesicles rounded	3

Although this manual does not describe the numerous species of *Penicillium* that laboratories may encounter as contaminants, the following description of the major morphological groups, which are based upon the degree of branching of the conidiophore within the genus, may be useful. In monoverticillate species the stalk is crowned by a number of phialides, i.e. there is a single point of branching. Biverticillate species have two points of branching, and are subdivided into two groups: those with very symmetrical branching systems and elongated phialides, and those with less symmetrical branching and short phialides. The large group of terverticillate *Penicillium* species are characterised by three points of branching. Individual species are identified by such features as shape and size of the various component parts of the conidiophore and conidia. The reader should refer to specialist texts for full speciation.

The Hazard Group 3 pathogen *Penicillium marneffei* is a biverticillate species which

3a	Colony uniformly yellow-green, stalks rough	*Aspergillus flavus*
3b	Colony dark green, sometimes with yellow areas; stalks smooth	4
4a	Metulae absent	5
4b	Metulae present	6
5a	Phialides on upper two thirds of small flattened vesicle	*Aspergillus fumigatus*
5b	Phialides over entire surface of large round vesicle	*Aspergillus glaucus*
6a	Stalks pale brown, heads columnar in old cultures	*Aspergillus nidulans*
6b	Stalks colourless, heads globose or irregular	*Aspergillus versicolor*
7a	Stalks rough, colony orange-brown	*Aspergillus ochraceus* (not described)
7b	Stalks coloured brown or yellow	8
7c	Stalks colourless	9
8a	Colony dull grey to charcoal	*Aspergillus ustus*
8b	Colony yellow to buff	*Aspergillus flavipes* (not described)
9a	Colony black or dark brown	*Aspergillus niger*
9b	Colony cinnamon-brown to sand coloured	*Aspergillus terreus*
9c	Colony white or pale cream	*Aspergillus candidus*

Key to other species

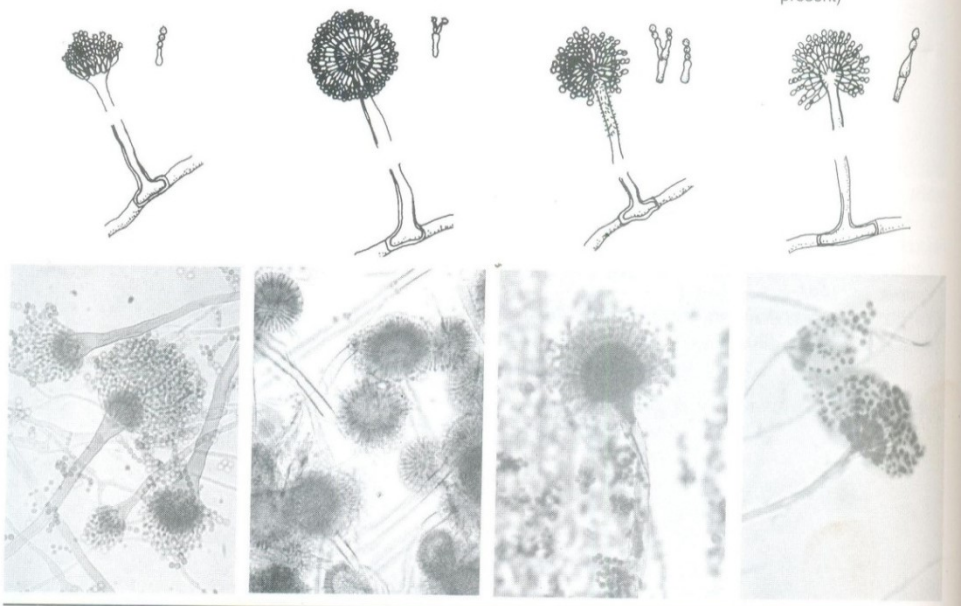
1a	Colony green	*Penicillium* spp. (but see 3b)
1b	Colony not green	2
2a	Conidia large, round, with a flat scar	*Scopulariopsis brevicaulis*
2b	Conidia small, oval; scar minute	3
3a	Colony pale purple	*Paecilomyces lilacinus*
3b	Colony greenish buff	*Paecilomyces variotii*

produces a diffusing, red pigment. It is, however, not the only species of *Penicillium* with this combination of features. Suspect isolates should always be referred to a specialist laboratory.

Appendix 8: Identification of most common *Aspergillus* spp (Larone, 2002)

TABLE 22 Identification of the most common species of *Aspergillus* (see description of genus on p. 268)

A. fumigatus[a]	*A. niger*	*A. flavus*	*A. versicolor*
PATHOGENICITY			
Most common cause of invasive disseminated aspergillosis; frequent agent of sinusitis	Most common in ear infections; frequently in aspergilloma; rarely disseminated	Involved in pulmonary, systemic, sinus, ear, and other infections; produces aflatoxins	Only occasionally involved in nail or invasive infection
MACROSCOPIC MORPHOLOGY[b]			
Velvety or powdery, at first white, then turning dark greenish to gray. Reverse white to tan	Woolly, at first white to yellow, then turning black. Reverse white to yellow	Velvety, yellow to green or brown. Reverse goldish to red-brown	Velvety; at first white, then yellow, orangey, tan, green, or occasionally pinkish. Reverse white; may be yellow, orange, or red
MICROSCOPIC MORPHOLOGY OF CONIDIOPHORES			
Short (<300 μm) Smooth	Long Smooth	Variable length Rough; pitted; spiny	Long Smooth
MICROSCOPIC MORPHOLOGY OF PHIALIDES			
Uniseriate, usually only on upper two-thirds of vesicle, parallel to axis of conidiophore	Biseriate; cover entire vesicle; form "radiate" head	Uniseriate and biseriate; cover entire vesicle; point out in all directions	Biseriate; loosely radiate; cover most of vesicle (Hülle cells may be present)



[a] *A. fumigatus* grows well at 45°C or higher.
[b] Classically studied on Czapek-Dox agar; on Sabouraud dextrose agar, most species of *Aspergillus* grow luxuriantly but not always characteristically.

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Appendix 9: Identification of *Penicillium* spp(Larone, 2002)

Penicillium spp.

PATHOGENICITY: Commonly considered contaminants, but found in a variety of diseases in which their etiologic significance is uncertain. They have been known to cause corneal, cutaneous, external ear, respiratory, and urinary tract infections, as well as endocarditis after insertion of valve prostheses. Disseminated disease has been reported in severely immunocompromised patients. Many strains produce toxins.

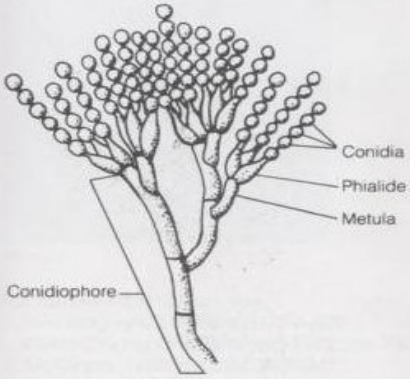
See also *Penicillium marneffei* (p. 156).

RATE OF GROWTH: Rapid; mature within 4 days. Usually no or poor growth at 37°C.

COLONY MORPHOLOGY: Surface at first is white, then becomes very powdery and bluish green with a white border. Some less common species differ in color and texture. Reverse is usually white, but may be red or brown. (Color Plate 143.)

If the isolate produces a red reverse and diffuse pigment in the agar, *P. marneffei* (p. 156) must be considered, and the organism should be tested for thermal dimorphism; this is especially relevant if the patient has recently visited Southeast Asia.

MICROSCOPIC MORPHOLOGY: Hyphae are septate (1.5–5 μm in diameter) with branched or unbranched conidiophores that have secondary branches known as metulae. On the metulae, arranged in whorls, are flask-shaped phialides that bear unbranched chains of smooth or rough, round conidia (2.5–5 μm in diameter). The entire structure forms the characteristic "penicillus" or "brush" appearance.



For further information, see
Barron, 1977, pp. 247–248; de Hoog et al., 2000, pp. 814–845; Kwon-Chung and Bennett, 1992, pp. 750–752, 804–805; McGinnis, 1980, pp. 251–261; Rippon, 1988, pp. 728–730, 754–755

Appendix 10: Identification of *Cladosporuim* spp (Larone, 2002)

Cladosporium spp.

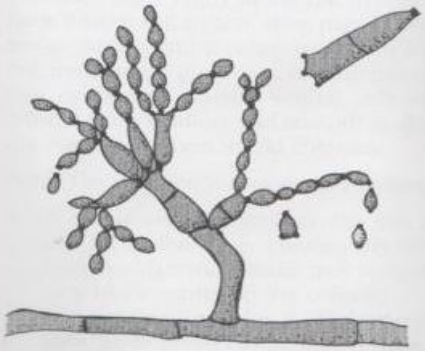
PATHOGENICITY: Commonly considered saprophytic contaminants. They have only occasionally been implicated in infections.

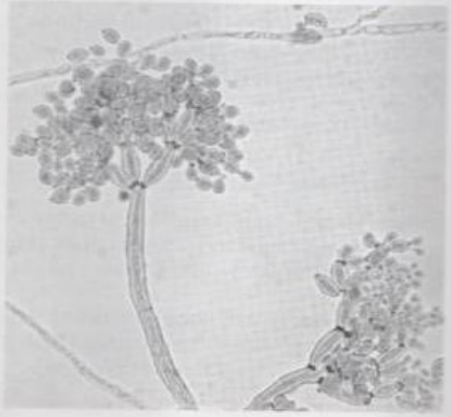
RATE OF GROWTH: Moderately rapid; mature within 7 days at 25°C. Most strains do not grow at 37°C, but some do.

COLONY MORPHOLOGY: Surface is greenish brown or black with grayish velvety nap, becoming heaped and slightly folded. Reverse is black. (Color Plate 81.)

MICROSCOPIC MORPHOLOGY: Hyphae are septate and dark; conidiophores are dark and branched, vary in length, and usually produce 2 or more conidial chains. Conidia are brown, round to oval (3–6 × 4–12 µm), and usually smooth; they form branching treelike chains and are easily dislodged, showing dark spots (hila) at the point where they were attached to the conidiophore or other conidia. The cells bearing the conidial chains are large and sometimes septate, resemble shields, and may be mistaken for macroconidia when seen alone.

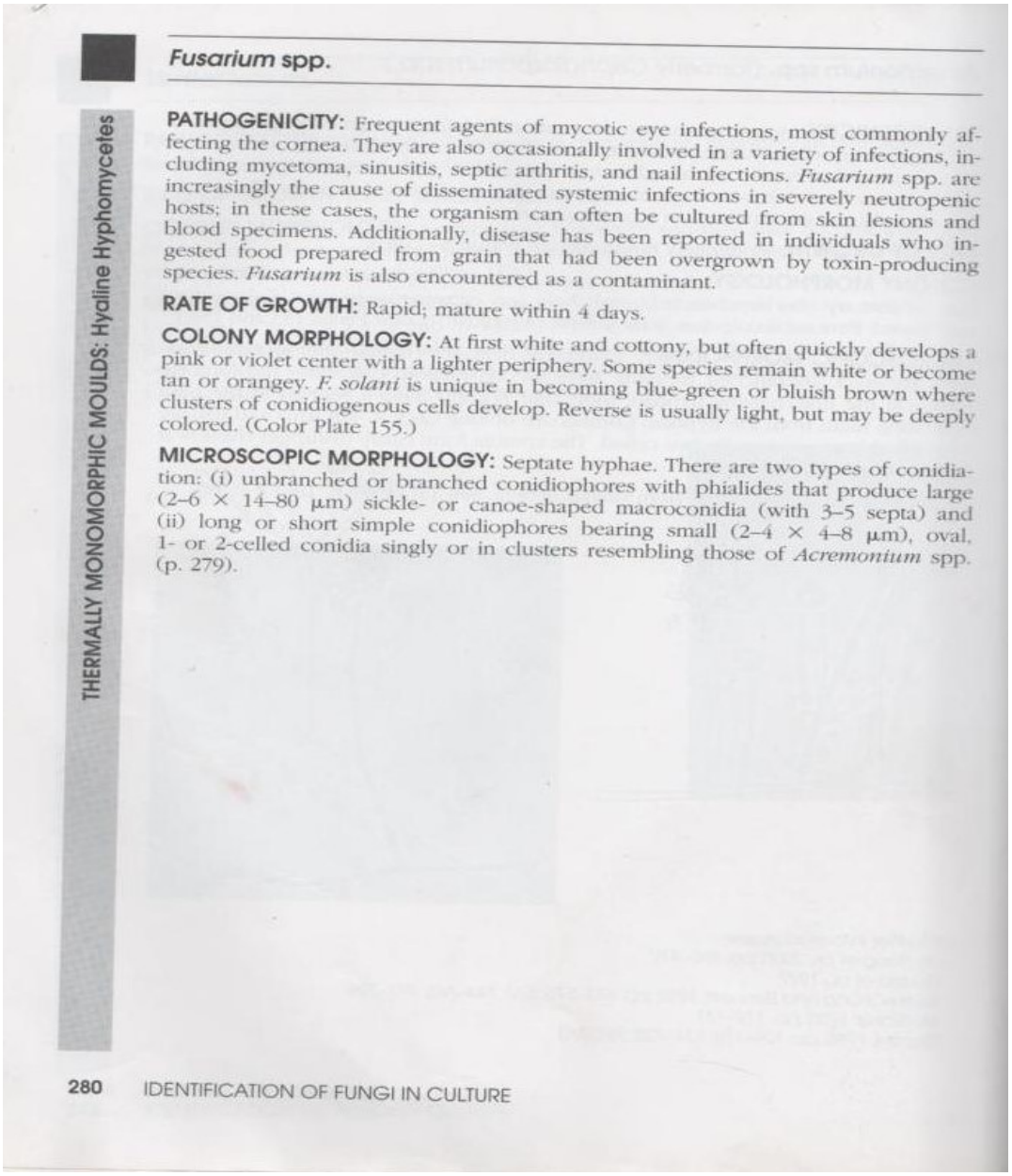
See Table 14 (p. 193) for differentiation from *Cladophialophora* spp.





For further information, see
de Hoog et al., 2000, pp. 582–592
Kwon-Chung and Bennett, 1992, pp. 801–802
McGinnis, 1980, pp. 198–201, 203
Rippon, 1988, pp. 771, 773

Appendix 11: Identification of *Fusarium* spp (Larone, 2002)



Fusarium spp.

THERMALLY MONOMORPHIC MOULDS: Hyaline Hyphomycetes

PATHOGENICITY: Frequent agents of mycotic eye infections, most commonly affecting the cornea. They are also occasionally involved in a variety of infections, including mycetoma, sinusitis, septic arthritis, and nail infections. *Fusarium* spp. are increasingly the cause of disseminated systemic infections in severely neutropenic hosts; in these cases, the organism can often be cultured from skin lesions and blood specimens. Additionally, disease has been reported in individuals who ingested food prepared from grain that had been overgrown by toxin-producing species. *Fusarium* is also encountered as a contaminant.

RATE OF GROWTH: Rapid; mature within 4 days.

COLONY MORPHOLOGY: At first white and cottony, but often quickly develops a pink or violet center with a lighter periphery. Some species remain white or become tan or orangey. *F. solani* is unique in becoming blue-green or bluish brown where clusters of conidiogenous cells develop. Reverse is usually light, but may be deeply colored. (Color Plate 155.)

MICROSCOPIC MORPHOLOGY: Septate hyphae. There are two types of conidiation: (i) unbranched or branched conidiophores with phialides that produce large (2–6 × 14–80 µm) sickle- or canoe-shaped macroconidia (with 3–5 septa) and (ii) long or short simple conidiophores bearing small (2–4 × 4–8 µm), oval, 1- or 2-celled conidia singly or in clusters resembling those of *Acremonium* spp. (p. 279).

280 IDENTIFICATION OF FUNGI IN CULTURE

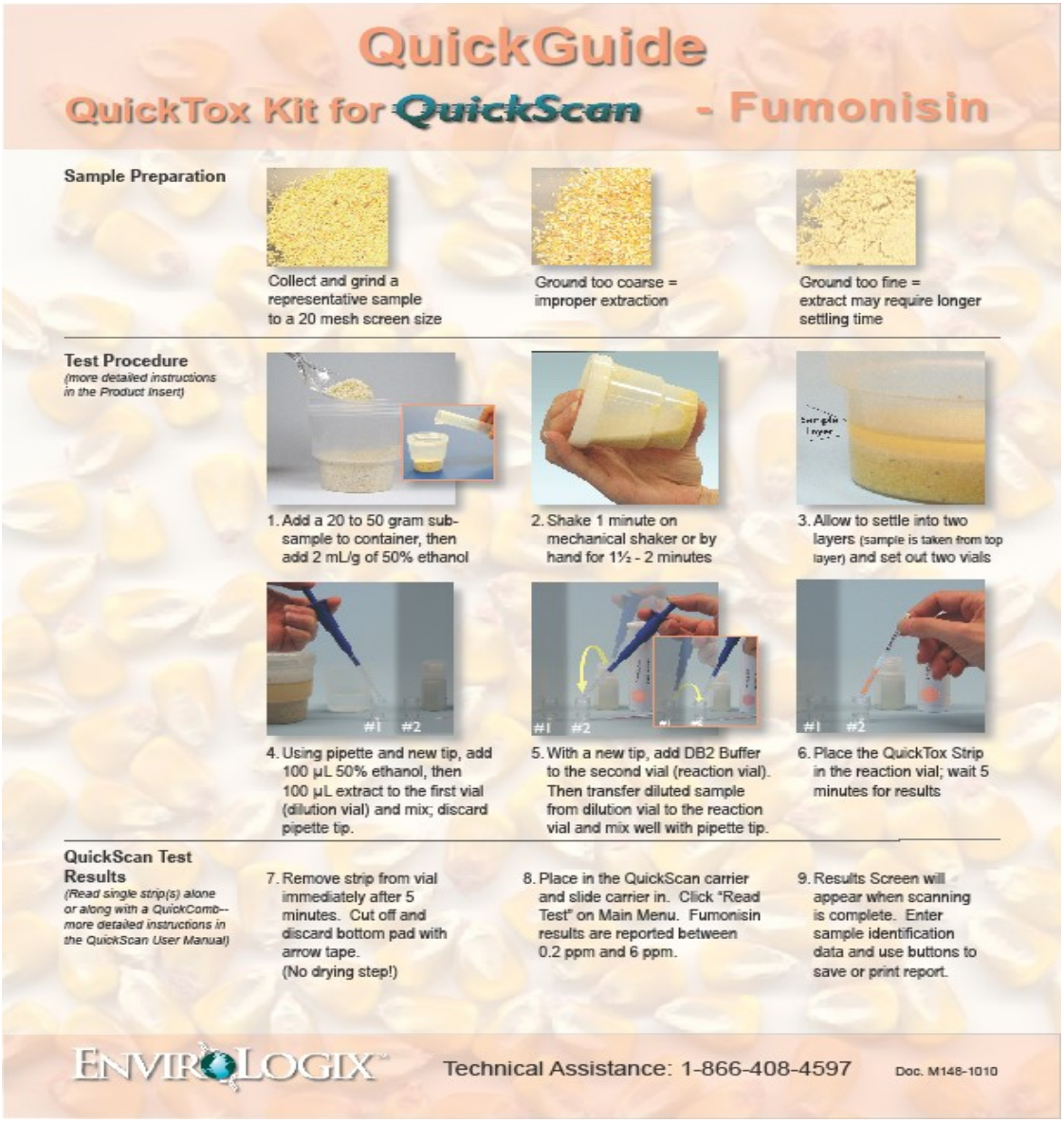
Fusarium spp. (continued)

Macroconidia.

Microconidia.

For further information, see
de Hoog et al., 2000, pp. 681–705
Kwon-Chung and Bennett, 1992, pp. 574–575, 745–752, 802–803
McGinnis, 1980, pp. 218–220, 223
Rippon, 1988, pp. 732–735, 757, 759

Appendix 12: Quick scan test procedure used for the detection of fumonisins in the maize samples (SSC 2151)



Appendix 13: Quick scan test procedure used for the detection of Aflatoxins in the maize samples (SSC 2151).

Appendix 14: African Journal of Food Agriculture Nutrition and Development (AJFAND) published manuscipt

ajfand SCHOLARLY, PEER REVIEWED AFRICAN JOURNAL OF FOOD, AGRICULTURE NUTRITION AND DEVELOPMENT Volume 14 No. 7 December 2014 PUBLISHED BY AFRICAN SCHOLARLY SCIENCE COMMUNICATIONS TRUST ISSN 1684 5374

SUSCEPTIBILITY OF MYCOTOXIGENIC FUNGI TO COMMERCIAL FUNGICIDES, A POTENTIAL TOOL FOR MYCOTOXIN CONTROL IN MAIZE IN KENYA

Tonui JK[1*], Ciira K[2] and CC Bii[3]

Josephat Tonui

*Corresponding author email: kipjoston@gmail.com

[1, 2]Jomo Kenyatta University of Agriculture and Technology, College of Health Sciences. P.O .Box 62000-00200, Nairobi, Kenya.

[3]Kenya Medical Research Institute, Centre for Microbiology Research, Kenya. P.O Box 2632- 00202, Nairobi, Kenya.

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ABSTRACT

Mycotoxin contamination of food grains represents significant health and economic challenges in developing countries as well as the developed world. Mycotoxin-producing fungal species affecting maize mainly belong to the genera *Aspergillus*, *Fusarium*, and *Penicillium*.They pose serious phytopathological and mycotoxicological risks both at pre-harvest and post-harvest stages. Maize in Kenya has been associated with frequent outbreaks of aflatoxin contamination. A number of mycotoxin control strategies both chemical and biological have been developed as potential tools for mycotoxin control. A Laboratory based cross-sectional study was carried out in a Mycology Laboratory at the Center for Microbiology Research in Kenya Medical Research Institute, Nairobi, Kenya. A total of 138 maize samples obtained from Machakos, Nairobi, Mombasa, Kitale and Kisumu were subjected to mycological analysis. The samples were treated with the fungicides; Antracol (propineb), Milraz (propineb700g/kg and Cymoxanil60g/kg), Mistress (Cymoxanil 8% and Mancozeb 64%) and Victory (Metalaxy 80g/kg and Mancozeb 640g/kg.) before inoculation on Sabourauds dextrose agar (SDA). Infestation rates on fungicide- treated and non treated control maize kernels were scored. The susceptibility of the isolates to the four test fungicides was determined by disk diffusion technique. All the maize samples were infested by moulds and there was a significant difference in regional infestation rates ($p<0.05$). Maize from Mombasa had the lowest infestation of 72.5% while Nairobi was the highest with 99.1%. Fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium* were frequently isolated from the five regions. There was a significant reduction {$p<0.05$ (0.00)} of infestation rates on fungicide- treated maize compared to the untreated. Generally, 26% (n=35) and 34% (n=47) of maize samples treated with Mistress and Victory, respectively had 0% infestation while those treated with Milraz and Antracol were 10% (n=14) and 14% (n=19), respectively. Some mycotoxigenic isolates were found to be resistant to more than one of the test fungicides. However, their in- vitro antifungal activity is of great importance and could further be evaluated to determine their field efficacy for mycotoxin control in maize.

Key words: Maize, Mycotoxigenic fungi, Fungicides, Susceptibility

INTRODUCTION

Mycotoxigenic fungi are often found as contaminants in agricultural products before or after harvest as well as during transportation and storage. Mycotoxins are produced by moulds as secondary metabolites which when ingested, adverse effects occur on humans and animals resulting in illnesses and economic losses [1]. Mycotoxigenic fungi are part of the microbial flora associated with many agronomic crops, including maize, peanuts, tree nuts, grapes, barley, coffee, cotton, wheat and other cereal grains [2]. Depending on the crop plant affected and the fungal species, mycotoxigenic moulds may cause plant disease, such as *Aspergillus* fruit rot of grapes, maize ear rots caused by *Aspergillus* and *Fusarium* species, and *Fusarium* head blight as well as seedling blight diseases on cereal crops [3]. Fumonisins are a group of mycotoxin contaminants found in food and feed products produced by *Fusarium* species. Fumonisin B1 primarily is of international, agroeconomic, and food safety concern. High doses of fumonisin B1-infested corn feed have been shown to cause pulmonary edema in swine, while lower doses lead to hepatic disease [4]. The mycotoxins are produced predominantly by toxigenic strains of *Fusarium verticillioides*. The fungus commonly proliferates in maize, causing stalk and ear rot diseases, in addition to mycotoxin contamination. Aflatoxins are another important group of mycotoxins which affect different food grains including maize and maize- based food. The most common aflatoxins are B1, B2, G1 and G2 which are potential carcinogens produced by *Aspergillusflavus* and *Aspergillus parasiticus* [5]. The height of human aflatoxicoses occurred in Eastern Kenya in 2004 where 317 infections and 125 deaths were reported resulting from acute hepatotoxicity [6].

Fungicides are mainly chemical compounds or live organisms used to eradicate or inhibit fungi and their spores [7]. Conventionally, the control of mycotoxigenic fungi was achieved using chemical fungicides, variation in cultural practices, and development of resistant cultivars. Additionally, postharvest sorting of contaminated yields, such as maize, wheat, peanuts and tree nuts, have been developed to reduce mycotoxin content [9, 10, 11]. Biological control methods have also been investigated for controlling mycotoxigenic fungi where several bacterial and fungal antagonists have been developed against the moulds [12, 13]. Several chemical control agents have also been developed. Quinone-outside inhibitor fungicides (qoi) were first labeled for use on maize in the year 2000. These fungicides are commonly referred to as strobilurin fungicides. They are now extensively marketed in corn production for management of both biotic and abiotic stresses [7]. These fungicides are applied as a preventive measure or as early as possible in a disease cycle. They are effective against spore germination and early mycelium growth but this has less or no effect when the fungus is already established [8]. The objective of the current study, therefore, was to determine the susceptibility of mycotoxigenic fungi to some commercial fungicides as a potential for the control of mycotoxin infestation in maize.

MATERIALS AND METHODS

Methods

Study design and sample collection

This was a laboratory based cross-sectional study that involved the collection 138 maize samples distributed equally among five study sites in Kenya. The sites were Machakos, Nairobi, Mombasa, Kitale and Kisumu. These regions represent different agro-ecological zones where various maize varieties are grown. The samples were packaged and transported to Kenya Medical Research Institute, Centre for Microbiology Research, Mycology Laboratory where they were analyzed. Scientific approval for the study was granted by the Kenya Medical Research Institute scientific steering committee.

Test fungicides

Antracol WP 70

This is a broad spectrum protective fungicide effective against early and late blight on potatoes and tomatoes, various fungal diseases on vegetables, fruits and ornamentals. The active ingredient of antracol WP 70 propineb, belongs to a chemical class of dithiocarbamates. Propineb is a fungicide with multisite activity and kills conidia or germinating conidia by contact. It is used across the world for the control of various fungi, especially Oomycetes, Ascomycetes, Basidiomycetes and Fungi imperfecti. The fungicide is manufactured by Bayer Crop Science.

Mistress 72 WP

This is a systemic and contact fungicide for the control of early and late blight on tomatoes and potatoes. The fungicide is manufactured by Osho chemical industries. This fungicide has cymoxanil 8% and mancozeb 64%, cymoxanil has preventive and local systemic activity and also inhibits blight. Mancozeb has protective antifungicidal activity and works through translaminar action.

Milraz WP 76

This is a broad spectrum preventive fungicide. The active ingredients are propineb700g/kg a dithiocarbamate and Cymoxanil which is an ethyl urea 60g/kg. This fungicide is manufactured by Bayer Crop Science.

Victory Fungicide

This is a systemic and contact foliar fungicide for the control of foliar and root diseases of potatoes and tomatoes and downy mildew of ornamental crops. The active ingredients in this fungicide are Metalaxy 80g/kg and Mancozeb 640g/kg. This is manufactured by Victory Chemical Co., Ltd.

Inoculation of Maize samples and Culture of fungi

Maize samples were washed with the recommended concentration of each fungicide independently and inoculated on Sabourauds dextrose agar (SDA). Each SDA plate was inoculated with four maize kernels obtained from each sample for the four fungicides. A control for every sample was washed concurrently with sterile distilled water and inoculated on a different SDA plate. Every single sample from each site was inoculated

in five SDA plates, four for each test fungicide and one untreated control plate for the same sample. The plates were incubated at 30°C for 72 hours to allow for fungal growth. Growth of fungi on the maize grains washed with the fungicides was scored to determine the percentage infestation and compared to their control plates. Where all the four maize kernels had visible growth of fungi in a plate, this was scored as 100% infestation and if three of the four kernels in that plate were infested, this was scored as 75% while two infested kernels was scored as 50% infestation rate. In a case where only one grain out of the four plated kernels was infested, the score was 25% infestation rate.

Identification of Isolates

Fungi growing on the maize after were identified by morphologic characteristics for both microscopic and macroscopic features. The colonial morphology shapes and types of conidia produced by the mould were used to key out the identity of the individual fungi [14].

Bioactivity of the Fungicides

The activity of the fungicides against fungal isolates was determined by disk diffusion technique. Briefly, absorbent Watman filter paper disks of 6 mm were impregnated with 20µl of the test concentration of the fungicides independently. A pure culture of each fungal isolate was inoculated on an SDA plate and the impregnated disks containing the fungicide were placed aseptically onto the SDA plate using a sterile forceps. The plates were incubated at 30°C for 72 hours and thereafter, the zones of inhibition around the disks were measured and expressed in millimeters (mm). A standard azole antifungal drug (fluconazole) was used as a reference for susceptibility or resistance to the four fungicides and interpreted according to CLSI breakpoints.

Data Analysis

Data generated in this study were analysed using the Statistical Software SPSS Version 17. Comparison of infestation rates on fungicide treated maize with controls and infestation per region was done using (ANOVA).

RESULTS

All fungicide-treated maize samples had a reduced infestation by fungi compared to the untreated controls (Figure 1). Maize from all the five regions that were treated with Mistress had low infestation where the lowest value of 28% was from Mombasa (Figure 1). The Infestation rate on untreated control samples was also low in samples from Mombasa with 72.5%. Control samples obtained from Nairobi and Kitale had the highest infestation of 99.1% and 98.2%, respectively. Maize samples treated with Milraz had a slightly higher infestation compared to the other three fungicides used. In Kitale and Kisumu, infestation on Milraz treated maize was highest with 76.8% and 81% respectively. This was also seen in Mombasa where the infestation was high (59.4%) in maize treated with Milraz compared to Mistress (28%), Victory (37.5%) and Antracol (50%). Nevertheless, maize samples from Nairobi treated with Antracol had the highest infestation of 92%, while those from Kisumu treated with Milraz were also more infested (81%) compared to all the fungicide treated maize kernels from all the regions.

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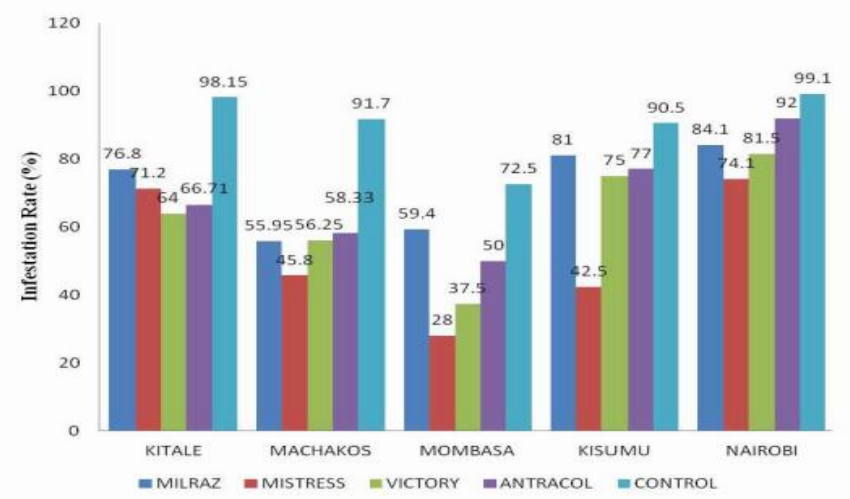

Figure 1: Percentage fungal infestation of maize from different regions in Kenya following treatment of grains with fungicides

Table 1 shows the statistical comparison of infestation rates that occurred in the five regions, the general mean difference is statistically significant $p< 0.05$. However, there was a non statistically significant difference in the infestation rates between Kitale and Nairobi $p>0.05$ ($p=0.66$) as well as Kitale and Kisumu $p>0.05$ ($p=0.189$). The same observation was noted in the infestation rates between Machakos and Mombasa where $p>0.05$ ($p=0.621$). Apart from the above similarities, the differences in the infestation rates between Machakos, Kitale, Nairobi and Kisumu were highly significant {$p<0.05$ ($p= 0.00$)}.

Table 2 shows the distribution of control and fungicide treated maize samples in each category of infestation. From Table 2, most of the control samples were heavily infested by moulds having 75% (n=11) and 100% (n=115) infestation compared to the treated samples. Many samples treated with Antracol and Millraz had 100% infestation (n=54 and n=55, respectively) compared to those treated with Mistress and Victory which were 30 and 29, respectively. In addition, more samples treated with Mistress and Victory had no infestation (n=35 and n=47, respectively) compared to Milraz and Antracol (n=14 and n=35, respectively).

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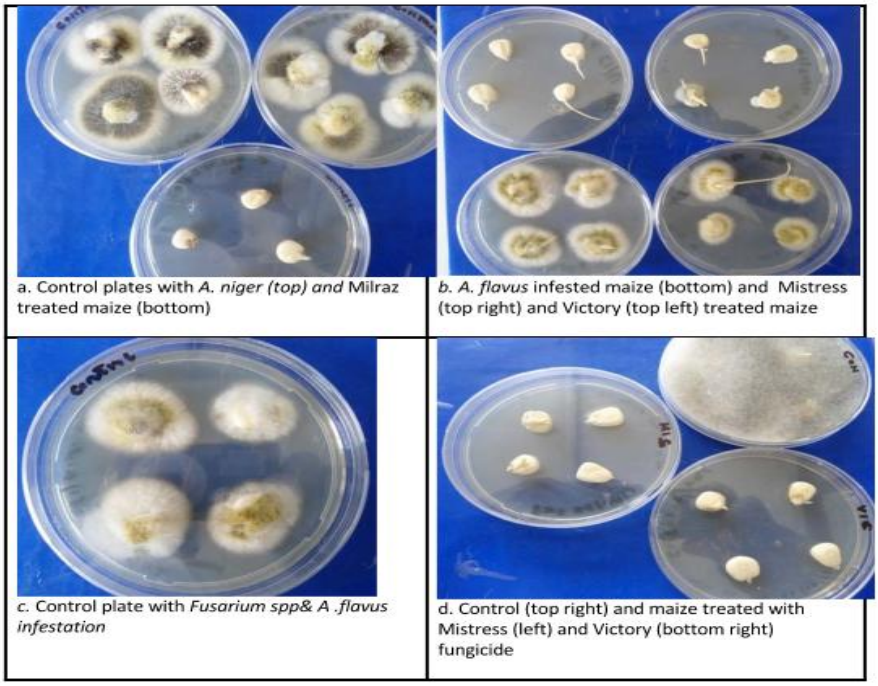

Figure 2: Sabourauds dextrose agar plates showing infestations by different mycotoxigenic fungi and fungicide treated maize

Few isolates from the various regions were resistant to the test fungicides by disk diffusion. *Aspergillus flavus* isolates from Kisumu were more resistant to Milraz compared to the other fungicides where there was no zone of inhibition on SDA plates. Among the resistant fungi, *A. Flavus* and *Fusarium spp* isolates were more resistant than the *Penicillium spp* and *Rhizopus spp.* On the other hand, most of these isolates were susceptible to Mistress and Antracol while being resistant to one or the other fungicides.

e. Disk diffusion of Victory on *Aspergillus spp* (bottom left), Milraz on *Penicillium spp* (top left), Victory on *Fusarium spp* (top right) and *Penicillium spp* (bottom right).

f. Disk Diffusion of Mistress on *A. niger* (top left), *Fusarium spp* (bottom left), *A. flavus* (bottom right) and Victory fungicide on *A. niger* (top right)

Figure 3: Disc diffusion susceptibility testing of mycotoxigenic fungi

DISCUSSION

A variety of agricultural produce are contaminated by various mycotoxin producing moulds. These include the *Aspergillus flavus,* which was frequently isolated from maize samples analysed in this study. Though the presence of the *Aspergillus* mould does not essentially designate aflatoxin contamination, there is definitely an increased risk for the occurrence of these toxins under stressful conditions for the mould [15]. Maize samples from the five regions showed gross contamination with mycotoxin producing moulds of the genera *Aspergillus spp*. *Fusarium spp* and *Penicillium spp*. *Rhizopus spp* was frequently isolated from the maize. The infestation rate per region for the untreated control maize samples was high with 99.1% and 98.15% for Nairobi and Kitale, respectively (Figure. 1). Maize samples from the five regions were also infested by the non mycotoxigenic *Rhizopus* species.

There was a significant difference $p<0.05$ in the activity of the four fungicides as shown on Table 2. All control samples were infested by different fungal species. Generally, a huge percentage of maize treated with Mistress and Victory (n=35) and (n=47), respectively were less infested. The two fungicides were more effective in reducing the mycoflora on the treated maize samples. In general, (n=55) and (n=54) of samples treated with Milraz and Antracol, respectively had 100% infestation rate compared to those treated with Victory and Mistress Fungicides (n=29) and (n=30), respectively. This shows that more fungi could survive in the presence of Milraz and Antracol as opposed to Victory and Mistress. However, the control samples (n=115) had 100% infestation rate which was higher than that of all treated samples. All the four test fungicides reduced the resident mycoflora in the respective treated samples to a great extent. The least number (n=29) of samples with 100% infestation were those treated with Victory fungicide while

the highest number of samples (n=55) with 100% infestation were those treated with Milraz. This indicates that Victory fungicide could prevent heavy infestation of the maize grains compared to the other fungicides.

A number of bioagents and different fungicides have been found to reduce mycoflora on seed while enhancing germination as well as vigour Index of various grains such as cowpea [17]. In a study which tested the efficacy of Benomyl, Dithane M-45 75% WP (Manganese ethylene bis dithio carbamate plus zinc) and Bavistin 50% WP (Methyl-H-benzimidazole-2ylcarbamate) on seed mycoflora of cowpea, variations in their infestation was observed depending on the respective treatments. Dithane M-45 and Bavistin were found to be effective in reducing seed-borne infection of *Fusarium* spp. [17]. This is consistent with the effect of the fungicides tested in this study where the fungicides significantly reduced the infestation on treated maize kernels. In another study that tested different concentrations of fungicides against various pathogens, Dithane M-45 and Bavistin were found to be effective in reducing seed-borne infection of *Fusarium* spp. on maize seeds [18]. *In vitro* studies have found Captan, Dithane M-45, Bavistin, and Vitavax effective in controlling *Fusarium* species on cereal grains [19]. Another study reported the reduction of wilt disease on treatment with Mancozeb M-45, Bavistin and Vitavax. In addition, the germination of fungicide treated seeds was enhanced compared to controls. Untreated seeds had the highest seed mycoflora infestation of 68% and lowest seed germination of 80% [19, 20]. High infestation of untreated maize kernels was also seen in this study.

During the years of commercial use of a fungicide, there can arise populations of the target pathogen that are no longer sufficiently sensitive to be controlled adequately [21]. Some isolates of *A. flavus*, *Fusarium spp* and *Penicillium spp* exhibited resistance to at least one of the test fungicides. This was shown by the overgrowth of the mould on fungicide impregnated disks with no zone of inhibition (plates e and f). This is, therefore, indicative of possible resistance and the ability to thrive and producce mycotoxins in the presence of the test fungicides. However, definitive susceptibility profiles for the moulds need to be ascertained using other techniques such as minimum inhibitory concentration method [16]. The use of chemical inhibitors which suppress spore germination of fungi and the development of the fungal mycelium is one of the most effective ways of controlling the problems caused by aflatoxin contamination in a susceptible product such as maize [24]. Fungicide in agriculture could also lead to resistance of human fungal pathogens to antifungal agents due to similarities in the mode of action of fungicides and antifungal agents. Environmental soil isolates of *Aspergillus fumigatus* from Tanzania were found harboring azole antifungal resistance mutations which are of clinical significance [25].

CONCLUSION AND RECOMENDATIONS

Several methods for mycotoxin control have been investigated and these have included physical, chemical and biological methods [22, 23]. The present study revealed that maize samples collected from five regions of Kenya were heavily infested with mycotoxigenic fungal species. The results in this study have also demonstrated the ability of the four test fungicides to significantly reduce the infestation of maize by mycotoxigenic fungi {$p<0.05$ (0.00)}. A large number of the isolates particularly *Aspergillus* spp, *Fusarium* spp and *Pencillium* spp were susceptible to the test fungicides while few were resistant. Field studies may also be conducted to ascertain their efficacy for use in controlling infestation of maize for public food safety. On the other hand, erroneous use of fungicides for agriculture should be avoided due to the possible development of antifungal resistant mutants which are of great clinical significance.

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Table 1: Multiple comparison of the differences in infestation rates per region

Multiple Comparisons of regional infestation rates

(I) region	(J) region	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
KITALE	NAIROBI	-1.916	4.359	0.66	-10.48	6.65
	MACHAKOS	35.677*	4.359	0	27.11	44.24
	KISUMU	7.769	5.903	0.189	-3.83	19.37
	MOMBASA	32.769*	5.903	0	21.17	44.37
NAIROBI	KITALE	1.916	4.359	0.66	-6.65	10.48
	MACHAKOS	37.593*	4.318	0	29.11	46.08
	KISUMU	9.685	5.873	0.1	-1.85	21.22
	MOMBASA	34.685*	5.873	0	23.15	46.22
MACHAKOS	KITALE	-35.677*	4.359	0	-44.24	-27.11
	NAIROBI	-37.593*	4.318	0	-46.08	-29.11
	KISUMU	-27.907*	5.873	0	-39.45	-16.37
	MOMBASA	-2.907	5.873	0.621	-14.45	8.63
KISUMU	KITALE	-7.769	5.903	0.189	-19.37	3.83
	NAIROBI	-9.685	5.873	0.1	-21.22	1.85
	MACHAKOS	27.907*	5.873	0	16.37	39.45
	MOMBASA	25.000*	7.095	0	11.06	38.94
MOMBASA	KITALE	-32.769*	5.903	0	-44.37	-21.17
	NAIROBI	-34.685*	5.873	0	-46.22	-23.15
	MACHAKOS	2.907	5.873	0.621	-8.63	14.45
	KISUMU	-25.000*	7.095	0	-38.94	-11.06

The mean difference is significant at the 0.05 level (Fisher's Significant Difference Test)

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Table 2: General infestation of fungicide treated maize and controls

Infestation rates on maize	Categories		Type of fungicide used				
			MILRAZ	MISTRESS	VICTORY	ANTRACOL	CONTROL
	0	% within type of fungicide	10% n=14	26%	34%	14%	0%
		used		N=35	N=47	N=19	N=0
	25	% within type of fungicide	21%	27%	19%	24%	4%
		used	N=29	N=37	N=26	N=33	N=5
	50	% within type of fungicide	12%	12%	18%	15%	5%
		used	N=17	N=17	N=25	N=21	N=7
	75	% within type of fungicide	17%	14%	8%	8%	8%
		used	N=23	N=19	N=11	N=11	N=11
	100	% within type of fungicide	40%	22%	21%	39%	83%
		used	N=55	N=30	N=29	N=54	N=115
Total		% within type of fungicide	100%	100%	100%	100%	100%
		used	n=138	N=138	N=138	N=138	n=138

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Appendix 15: Global Advanced Research Journals (GARJAS) published manuscript

Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 4(6) pp. 248-258, June, 2015.
Available online http://garj.org/garjas

Full Length Research Paper

Mycotoxigenicfungi, distribution and infestation of maize in selected sites- Kenya

Josephat[1*] K. T, Kiiyukia C[2] and Christine C .B,[3]

[1*2]Jomo Kenyatta University of Agriculture and Technology, College of health Sciences
[3] Kenya Medical Research Institute, Centre for Microbiology Research, Kenya.

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Mycotoxin producing moulds are of great significance to food safety and food security in the world as well as Kenya. Fungi of the genera *Aspegillus, Fusarium and Penicillium* are of public health importance since they produce potent mycotoxins with adverse health effects to humans and animals. Therefore this study was conducted to determine the distribution and incidence of the mycotoxigenic moulds in maize. A Laboratory based cross-sectional study was carried out at the Mycology Laboratory in Kenya Medical Research Institute, Nairobi, Kenya. A total of 138 maize samples were obtained from farmers in Kitale, Machakos, Nairobi, Mombasa and Kisumu and subjected to mycological analysis. Laboratory analysis of maize samples involved culture on sabourauds dextrose agar (SDA) and incubation at 30oC for 72 hours. Microscopic identification of fungal growth was done by morphological characteristics. Fungal incidence on maize from each region were scored in different categories and compared using ANOVA. Mycotoxigenic fungi of the genera *Aspegillus, Fusarium and Penicillun* were isolated from maize samples obtained from the five regions. Members of the *Aspergillus spp and Fusarium* spp *were* 38% and 42% respectively in Kitale. *While Penicillium* spp was higher in Mombasa (29%) and Kisumu (24%). In Machakos, *Aspergillus* spp contamination was also higher (26%) compared to other regions while contamination by *Penicillium spp* was the lowest at (5%). Maize samples from Mombasa had the lowest infestation of *Fusarium spp* (4%). Generally 47% and 41% of samples from Kitale and Machakos were infested by different fungal species while those from Nairobi had a low infestation rate of 32%. Conclusion: The maize samples tested from the five regions were infested by distinct fungal genera. The varied climatic conditions in the five regions could favor the development of a certain fungal species and hence a specific mycotoxin would be of great significance.

Keywords: Maize, Mycotoxigenic fungi, Infestation and distribution

INTRODUCTION

Mycotoxin contamination in agricultural food products is a threat to food security and food safety in many countries in the world. Mycotoxins are secondary metabolites of fungi mostly found as food contaminants affecting a wide variety of cereals (Reddy et al., 2010). Majority of mycotoxins are produced by species in the genera of *Aspergillus*, *Fusarium* and *Penicilliumare* of most concern due to their effect on humans and animals. These fungiare found in major food crops pre and post-harvest. Mycotoxigenic fungi produce mycotoxins in food grains under favorable conditions. Mycotoxins may also be found in milk and meat

*Corresponding Author's E mail: kipjoston@gmail.com;
Telephone +254 729 683 994

products from animals that have consumed contaminated feed.

Controlled experiments and those conducted in the field environment have revealed that increased environmental temperature contributes to the infection of maize by *Aspergillus flavus* and as a result, high levels of aflatoxins are generated in agricultural products (Jones *et al.,* 1980; Payne *et al.,* 1985). High humidity increases the moisture content in the maize grains therefore encouraging the growth of toxigenic fungal species in the food product.

Mycotoxin contamination of food products is dependent upon climatic conditions. Certainly, the ability of fungi to produce mycotoxins is mainly influenced by temperature, relative humidity, insect attack, and stress conditions of the plants (Miraglia *et al.,* 2009). On the other hand, extreme rainfall and drought events would favour formation of DON and fumonisin, respectively (Miller, 2008).

Fusarium species are ubiquitous and are found mainly in the soils from where they infect the maize crop plants. They are usually considered as field fungi and they affect more than 50% of maize grains before harvest (Reddy *et al.,* 2009). Fumonisins, a group of mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum*, are the major contaminants in maize and maize based food products. Consumption of fumonisin contaminated food has been associated with an increased risk of esophageal cancer in humans in South America, Asia, Africa and the African American populations, South Carolina (Marasas *et al.,* 1996). Fumonisins, are also known to cause equine leukoencephalomalacia and porcine pulmonary oedema including a variety of adverse effects in numerous animal species. Animals affected by fumonisin toxicity may have reduced weight gain and productivity as well as immunological impairment(Wu and Munkvold 2008).In Sub Saharan Africa findings from epidemiologic studies have revealed an association between fumonisin exposure and increased susceptibility to HIV infection(Williams *et al.,* 2010).Exposure to mycotoxins and the symptoms depend on the type of mycotoxin, the concentration and length of exposure as well as age, health status, and gender of the exposed individual. Maize is a critical food product for many countries and in Kenya more 90% of the population is dependent on it. Therefore the aim of the study was to establish the distribution and infestation of maize by mycotoxigenic maize in five agro-ecological zones in Kenya.

MATERIALS AND METHODS

Study design and sample collection

This was a laboratory based cross-sectional study that involved the collection of 138 maize samples distributed among five study sites in Kenya. The sites were Machakos, Nairobi, Mombasa, Kitale and Kisumu. These regions represent different agro-ecological zones where various maize varieties are grown. The samples were packaged and transported to Kenya Medical Research Institute, Centre for Microbiology Research, Mycology Laboratory where they were processed for mycological analysis.

Inoculation of maize samples

Clean maize kernels were picked from each sample, surface sterilized with 70% ethanol and plated on Sabourauds dextrose Agar. Four kernels were plated on each plate and incubated at 30° c andgrowth of fungi on the maize was assessed after 72 hours.Fungal infestation on the culture plates for each region was scored and compared. Where all the four maize kernels had visible growth of fungi, this was scored as 100% infestation and if three of the four kernels in that plate were infested, this was scored as 75% while two infested kernels was scored as 50% infestation. In a case where only one grain out of the four plated kernels was infested, the score was 25%.

Identification of fungal Isolates

Moulds growing on the plates were sub cultured onto SDA and identified according to their morphological characteristics (Larone *et al.,* 1995). Cultural characterization was based on the rate of growth, presence of aerial mycelium, colour of aerial mycelium as well as colour on the obverse and reverse of the plates. Microscopic identification was based on spore and conidiophore morphology.

Data Analysis

The isolation frequency (Fq) of each fungal genus from the five regions was calculated according to the formula by Gonzalez *et al.* 1999. This was used to determine the distribution of the mycotoxigenic fungi in the five regions.

$$Fq\ (\%) = \frac{\text{Number of isolates of a genus} \times 100}{\text{Total number of fungi or genus per region}}$$

Statistical Package for Social Sciences (SPSS) software version 21 was used to compare the infestation rates of maize among the five sites.

RESULTS

The pie charts show the distribution of *Aspergillus* spp, *Penicillium* spp, and *Fusarium* spp isolated from maize in the five regions.

Table 1 shows the general infestation of maize samples in different categories for each region. Maize samples from Kitale had the highest infestation at 100% (n=65) while Nairobi had the lowest number of samples in this category

Table 1. Infestation of maize from selected regions in different categories

Infestation on maize	Categories		Selected Regions Kitale	Machakos	Mombasa	Kisumu	Nairobi
	0	% within region	10% n=14	6% N=8	16% N=22	14% N=19	11% N=15
	25	% within region	14% N=19	14% N=19	19% N=26	8% N=11	22% N=30
	50	% within region	12% N=17	12% N=17	19% N=27	15% N=21	18% N=25
	75	% within region	17% N=23	27% N=37	9% N=12	24% N=33	17% N=23
	100	% within region	47% N=65	41% N=57	37% N=51	39% N=54	32% N=45
Total		% within region	100% n=138	100% N=138	100% N=138	100% N=138	100% n=138

The pie charts show the distribution of *Aspergillus flavus Penicillium spp,* and *Fusarium* spp isolated from maize in the five regions

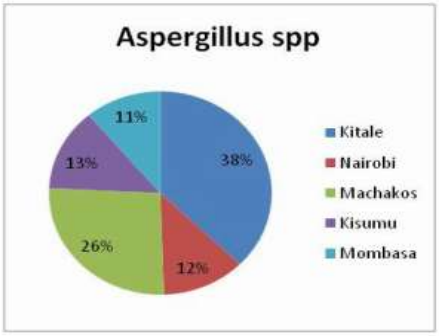

Figure 1. Distribution of *Aspergillus flavus* in maize from the five regions

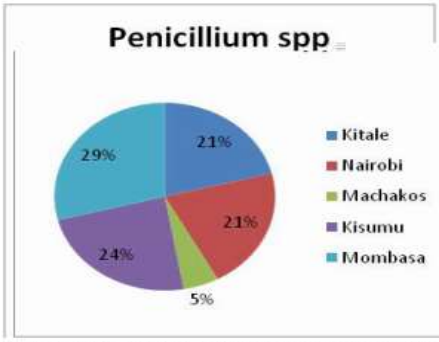

Figure 2. Distribution of *Penicillium spp* in maize from the five regions

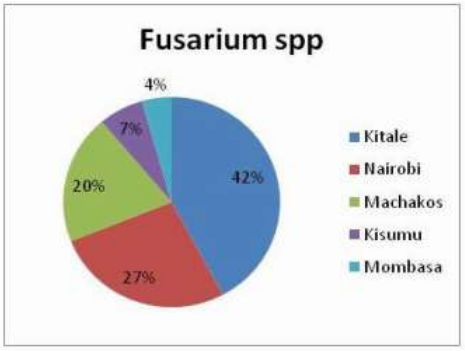

Figure3. Distribution of infested maize samples in the five regions

(n=45). Machakos had the lowest number of samples that were not infested (n=8) while those from Kitale, Kisumu and Nairobi were (n=10, n=19 and n=15) respectively. Mombasa had the highest number in this category (n=22).

DISCUSSION

Contamination of maize by mycotoxin producing fungi is a significant health and economic problem in the world (Miller, 2008). Kenya has experienced serious aflatoxicosis outbreaks associated with maize which has claimed lives as well as maize yield losses. Findings from the five study sites revealed that, potentially mycotoxigenic fungal isolates were found on maize samples. The distribution of the fungi in the five regions was significantly different p=0.54. Maize samples from each region were more infested by a specific fungal genera. Maize grains from Kitale were heavily contaminated by *Aspergillus* spp (38%) **Figure 1** while those collected from Mombasa were frequently contaminated with *Penicillium* spp (29%) **Figure 2**. This possibly shows that the *Penicillium* spp is favoured more than the other fungal isolates in Mombasa due to its high frequency of isolation in this region. The agro-ecological conditions prevailing in each region could favor the development of a certain fungal genera as opposed to another. This concurs with other study findings where it has been reported that, high soil temperature and drought are associated with increased aflatoxin contamination and incidence of aflatoxigenic strains or species (Jaime *et al.,* 2010). Environmental conditions favorable to mycotoxin producing moulds differ, *A. flavus* is known to compete poorly under chilly conditions while their occurrence is higher in warmer environments (above 25°C) than cooler environments (20 -25 °C) (Shearer *et al.,* 1992).

The *Aspergillus* spp were found at high levels in maize samples from Machakos and Kitale at (29%) and (38%) respectively. In Machakos several incidences of aflatoxicoses have occurred (Ngindu *et al.,* 1982). The largest outbreak was reported in 2004 where residents consumed aflatoxin contaminated maize which resulted in hospitalization, death and loss of tons of maize yield (Holbrook *et al.,* 2004). Aflatoxin producing fungi usually thrive in warm arid, semi-arid, and tropical regions with changes in climate resulting in large fluctuations in the quantity of aflatoxin producers (Shearer *et al.,* 1992). Drought conditions are more likely to occur in Machakos, which stresses plants making them more susceptible to contamination by *Aspergillus* spp. (Robertson 2005; Alakonya *et al.,* 2008). In Kenya, Kitale is the leading producer of maize where it is grown on large scale (Alakonya *et al.,* 2008). From the current study, fungi belonging to the genera *Fusarium* were isolated in maize samples from Kitale and Nairobi at 42% and 27% respectively (Figure 3). From the two regions, the frequent isolation of the species shows the possibility of maize contamination by Fumonisins produced by *Fusariumverticilloides*. In Kitale, farmers are known to habitually leave their maize yields in the field upon maturity to allow drying (Alakonya *et al.,* 2008). In addition, the maize harvest coincides with second rains which increase rotting and infestation by moulds. This may be a reason for the frequent occurrence of Fusarium species in these maize samples. Findings from other studies on maize from western Kenya have isolated a wide variety of *Fusarium* spp and *Aspergillus* spp consistent with findings from this study. The mould species included *F. verticillioides*, *F. graminearum*, *F. subglutinans* as well as *A. flavus* and *A. parasiticus* known to produce varied toxins (Kedera *et al.,* 1999).

Plate 1: Maize infested by *A. flavus* **Plate 2:** Maize infested by *A. niger*

Figure: 4 Culture plates showing the infestation on some of the maize samples analysed.

In this study maize samples from Mombasa and Kisumu had the lowest infestation by Fusarium moulds (4% and 7% respectively as shown in Figure 3) compared to Kitale and Machakos thad had (42% and 20%) respectively. This could have been as a result of the different maize varieties grown in these regions as well varied climatic conditions that exist which may not be favorable to the Fusarium mould. From other study findings, the major factors that influence the risk of Fusarium infection and Fumonisn contamination are temperature, insect injury, and drought stress and water activity (Bush *et al.,* 2004).*Fusariums*, such as *F. graminearum*, is predominant in temperate environments, while *F. verticillioides* and *F. proliferatum* and fumonisins are more widely spread in tropical and subtropical environments (Miller, 2001). The most favorable temperature conditions for *F. graminearum* is between 24-28 °C and consequently above this temperature range *F. verticillioides* proliferates more than *F. graminearum* (Miller, 2001; Reid *et al.,* 1999). Rising temperatures within maize growing regions would change the geographical distribution and predominance of *F. verticillioides*, mostly in currently cooler regions where it will replace *F. graminerum*. A shift in *Fusarium* spp may cause a change in mycotoxins from deoxynivalenol and zearalenone (produced by *F. graminierum*) to fumonisin (produced by *F. verticillioides*). The occurrence of *F. verticillioides* and consequent fumonisin contamination due changing weather patterns has been reported in Guatemala, Mexico, Zimbabwe and Kenya(Torres *et al.,* 2007).

CONCLUSION

In conclusion, it was important to determine the distribution and Incidence of fungi that exist in maize from different regions. Climatic factors have different effects on the different mycotoxin-producing fungi.

Therefore, climate change affects the existence of mycotoxigenic fungi in maize and this may affects future food security and health in Kenya. Maize samples from the five regions tested were infested by different mycotoxigenic fungi. Generally, maize from Kitale and Machakos were totally infested by different mould genera. The coexistence of moulds on the maize samples shows the possibility of occurrence of more than one mycotoxin in grains. The current study also highlights the importance of future work that will seek to determine shifts in pathogen populations in different regions.

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