PREVALENCE OF *FUSARIUM* AND *ASPERGILLUS* SPECIES IN MAIZE GRAIN FROM KITUI, MACHAKOS AND MERU AND USE OF NEAR INFRA-RED LIGHT SORTING TO REMOVE FUMONISINS AND AFLATOXIN CONTAMINATED GRAIN IN KENYA

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DECLARATION

I declare that this thesis is my original work and has not been presented for the award of a degree in any other university

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DEDICATION

Dedicated to my parents Martha and Murithi, my wife Terry and my daughter Gatwiri

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ACRONYMS

AEZ	Agro Ecological Zones
BGYF	Bright Greenish Yellow Fluorescence
BOF	Bright Orange Fluorescence
CFU	Colony Forming Units
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
HPLC	High Performance Liquid Chromatography
LED	Light Emitting Diode
LH	Lower Highland
LM	Lower Midland
MDG	Millennium Development Goals
MDRB	Modified Dichloran Rose Bengal media
MoA	Ministry of Agriculture
MoH	Ministry of Health
NEMA	National Environmental Management Authority
NIR	Near Infra Red
OD	Optical Density
PC	Personal computer
PDA	Potato Dextrose Agar
U.V	Ultra Violet
UH	Upper Highland
UM	Upper Midland

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ABSTRACT

Mycotoxin poisoning in maize has been a frequent occurrence in Kenya with the most significant outbreak being that of 2004 when 317 patients and 125 deaths were recorded. Mycotoxin contaminated grains cannot be obviously identified using naked eyes. This project sought to establish the spread of fumonisins and aflatoxin producing fungi and their associated mycotoxins in Machakos, Meru and Kitui counties of Kenya. The project further tested the efficiency of visible and near infra red light maize sorting machine in decontaminating maize from market samples of Machakos, Meru and Kitui.

Maize samples were collected from 30 markets in diverse agro-ecological zones of Meru, Machakos and Kitui counties during the 2013 harvest crop. *Fusarium* and *Aspergillus* were isolated from maize samples and mycotoxin producing *Fusarium* and *Aspergillus* species identified based on cultural and morphological characteristics. Fumonisins and aflatoxin were extracted from the samples and the mycotoxins analysed by enzyme-linked immunosorbent assay (ELISA). Based on the results of mycotoxins analysis the maize samples were categorised into high fumonisins, high aflatoxin, medium fumonisins, medium aflatoxin and low mycotoxins. Nine samples from each category were sorted using near infra-red single kernel sorting machine into accept and reject fractions. The sorted fractions were subjected to microbiological and ELISA analysis to determine the population of *Fusarium* and *Aspergillus* species and their respective mycotoxin concentrations. The percentage accept / reject kernels was correlated to the population of the mycotoxigenic fungal species and level of respective mycotoxins.

Aspergillus flavus S and L strains and *A. parasiticus* were found to contaminate the maize samples in high levels while *F. verticillioides, F. proliferatum* and *F. oxysporum* were the fumonisins-producing fungi isolated. Other *Aspergillus* species isolated were *A. niger, A.*

ochraceous, A. candudus and *A. tamarii*. The mean aflatoxin levels detected in Meru, Kitui and Machakos were beyond the acceptable limits of 10ng/kg. Kitui had the highest levels of aflatoxin detected while Machakos had the least. Fumonisins was detected in levels above the acceptable limits in Meru and though detected in Kitui and Machakos the levels were within the acceptable limits. The fumonisins levels did not match the population of *F. verticillioides* and this could be attributed to differences in environmental conditions as well as the pathotype.

The near infra-red single kernel sorting machine was effective in removing aflatoxin and fumonisin-contaminated kernels from the samples, with an accuracy of up to 97.8% for aflatoxins and 60.8% for fumonisins. The accepted fractions had statistically lower aflatoxin and fumonisin levels than rejected maize from the same bulk sample while rejecting only 0-15% of the sample. Market maize samples with toxin positive samples had reject rates of 0 to 25% and toxin negative samples having reject rates of 0-1%. These rejection rate data suggest the near infra-red single kernel sorting machine rejects kernels in a dose-dependent manner. There was a positive relationship between percentage accepted grain fractions and fumonisins and aflatoxin levels while total *Fusarium* species did not seem to have any relationship with the percentage accepted grain.

The study established that there are high risks of exposure to mycotoxin poisoning from consuming maize available in local market stores and that optical sorting technique is effective in removing mycotoxin-contaminated grain. The near infra-red single kernel sorting machine detected aflatoxin and fumonisin mycotoxins consistently but did not consistently detect the fungal contamination. The optical sorting technology can be further improved into a high throughput machine and offer potential for commercialization for use by local grain stores and millers to reduce mycotoxin contamination in maize.

CHAPTER ONE: INTRODUCTION

1.1 Background

Maize is one of the primary staple crop in Kenya and much of Eastern and Southern Africa, and is mostly cultivated by smallholder farmers in the regions, even in areas with low maize potential (Hoffmann and Gatobu 2011). Fungi can affect yield, quality, and nutritional content of maize grain, and also contaminating it with mycotoxins of deleterious health effects in animals and humans (Richard and Payne, 2003). Aflatoxin produced by *Aspergillus* spp and fumonisins produced by *Fusarium* species, are the major mycotoxins associated with post-harvest loses in maize (*Zea mays* L.). No maize hybrid is able to resist mycotoxins contamination when grown in environments conducive for outbreaks of aflatoxin or fumonisins (Wicklow and Pearson, 2006).

Usual breeding programmes have produced maize hybrids with substantial resistance to *Fusarium graminearum* Schwabe, which produces deoxynivalenol and zearalenone, but efforts to produce hybrids with adequate resistance to *A. flavus* and *F. verticillioides* have proven more difficult, and effective practical control practices are lacking (Matumba *et al,* 2009). Standard post-harvest cleaning operations have not been shown to be entirely effective for reducing aflatoxin or fumonisins levels in commercially harvested corn (Pearson *et al,* 2004). Aflatoxin outbreaks commonly occur together with outbreaks in fumonisins (Mubatanhema, *et al,* 2002) and both can be present at unacceptable levels in the same grain samples (Martinez, 2000, Ono, *et al*, 2001).

When Ultra-violet light is directed on aflatoxin infected kernels a characteristic bright greenish yellow fluorescence (BGYF) in whole kernels is roughly indicative of aflatoxin levels in maize (Shotwell and Hesseltine, 1981). Bright orange fluorescence (BOF) is roughly indicative of fumonisins levels (Pearson *et al,* 2010). Both BGYF and BOF have

been effective at detecting aflatoxin and fumonisins contaminated maize kernels, but the sensitivity of these technologies under various fungal infection sites, endosperm, germ, or pericarp, has not been well characterized (Willock and Pearson 2006). This research seeks to determine the efficacy of near infra-red and ultra violet light sorting in reducing aflatoxin and fumonisins contamination in maize grain. Optical sorters that can simultaneously eliminate both aflatoxin- and fumonisins contaminated kernels in a single pass, have been developed by Moore and Pearson (2013). Some sorting machines are able to measure two spectral bands with two way sorts which can process corn at rates of approximately 7,000 kg/hr (Willock and Pearson, 2006).

1.2 Problem statement and justification

Contamination of maize with mycotoxins is a serious problem in many Sub-Saharan African countries. The traditional maize-producing grain basket regions of Rift valley of Kenya can no longer meet the national grain demand, hence the shift towards increased grain production in marginal areas of Eastern and Coastal regions. However due to the prevailing warm climate and higher humidity, grain produced in these regions is prone to aflatoxin contamination. In 2010, three million bags of maize from Eastern and Coastal areas was contaminated and destroyed; some of this grain was from irrigation schemes like Bura and from maize seed distributed by government (Kang'ethe, 2011), hence curtailing the Governments effort of achieving food security and vision 2030. Several outbreaks of acute aflatoxicosis have been periodically reported in Kenya, in Machakos, Makueni and Kitui Districts in 1982, 2004 and 2005 (Afla Control, 2010, Lauren, 2004, Kang'ethe, 2011, Ngindu *et al,* 1982). In 2004, during the worst known outbreak of aflatoxicosis in Kenya, 317 cases were reported and 125 people died (Afla Control, 2010 and Kang'ethe, 2011). The last line of defence to eliminate maize grains contaminated with mycotoxins is postharvest

cleaning and sorting. It is well known that cleaning and sorting of raw maize grains can significantly reduce the contamination level of aflatoxin and fumonisins in maize (Komen *et al,* 2008). Grains highly contaminated by aflatoxin and fumonisins are not evenly distributed in a grain lot and may be concentrated in a very small percentage of the product (Kimatu *et al,* 2012). Therefore, removing a small percentage of contaminated kernels, instead of discarding the entire lot is a reasonable approach for reducing aflatoxin or fumonisins contamination to satisfy required levels.

Optical sorting techniques have been used to remove contaminated kernels in developed countries before (Willock and Pearson, 2006). Optical sorting machines have been developed for sorting maize contaminated with aflatoxin and fumonisins simultaneously (Willock and Pearson, 2006). This technology if introduced in Kenya will help save farmers as well as traders from post-harvest losses due to aflatoxin and fumonisins contamination. It is therefore imperative to calibrate the sorting machine using local samples and make sure that the technology works before introducing it to farmers. This is more valuable if done with a clear understanding of prevalence of these specific mycotoxins within the areas where aflatoxicosis has been reported before. Among these areas include Kitui, Meru and Machakos counties (Kang'ethe, 2011). This project addressed these gaps in two specific objectives: on prevalence of maize mycotoxins and efficiency of optical sorting.

1.3 Objectives

The general objective of this study was to determine the prevalence of *Fusarium* and *Aspergillus* species in market samples of lower Eastern and evaluate the effectiveness of use of near infra red light sorting machine to manage aflatoxin and fumonisins of maize in Kenya**.**

The specific objectives of the study were:

1. To determine the prevalence of *Aspergillus* and *Fusarium species* and their associated mycotoxins in market samples of maize from Kitui, Meru and Machakos.
2. To evaluate the effectiveness of using Near Infra Red sorting technology in reducing aflatoxin and fumonisins in maize grain.

1.3.3 Hypothesis

1. Aflatoxin and Fumonisin are highly prevalent in Lower Eastern province of Kenya.
2. The optical sorting machine can effectively reduce aflatoxin and fumonisins contamination in maize.

CHAPTER TWO: LITERATURE REVIEW

2.1 Maize production in Kenya

Maize production is one of the major farming activities within small holder farmers in Kenya mainly due to its dominance in eating habits (Mantel and Van Engelen, 1994). Small holder farmers contribute about 75% of the overall production, with the remaining 25% being contributed by the large-scale farmers (Njenga, 2013). A panel survey of farming households collected by the Tegemeo Institute, Egerton University in three sites representing low, medium and high agricultural potential from 1997 to 2007 showed that almost 100 percent of sample households grew maize (Brooks *et al,* 2009). Maize is estimated to account for more than 20% of total agricultural production in Kenya (Muasya and Diallo, 2001). According to FAO statistics (2005-2007), maize contributes about 68% of daily per capita cereal consumption, 35% of total dietary energy consumption and 32% of total protein consumption (FAOSTAT, 2010). The crop is mostly produced under rainfed conditions in agroecological zones that support this activity (Schroeder *et al,* 2013). Kenya has 1.6 million hectares of maize area and there is limited room for further expansion (Makokha, 2001 and Kibaara, 2005), this means management of post-harvest loses is key in ensuring food secure country as envisioned in the Millennium Development Goals and Kenya vision 2030.

2.2 Postharvest loses in maize associated with fungal and mycotoxins contamination

Post-harvest losses of maize are mainly due to poor storage and harvesting. Grain damage by pests and mycotoxins contamination is the major types of losses incurred in maize value chain (Kimatu *et al,* 2012 and Kang'ethe, 2011). Grain contamination by aflatoxin resulted in widespread outbreak of aflatoxin contamination in Eastern Kenya in the year 2008 (MoA,

2009) this was due to wet weather during the harvesting period of the short rains crop planted in October/November 2009 mainly in Eastern, Central and Coast Provinces (Songa and Irungu, 2010). In all these cases grains were contaminated beyond 10ppb with aflatoxin and were rendered unfit for human consumption and hence not marketable (Songa and Irungu 2010, MoA, 2009).

During harvesting, dropping of dehusked cobs on the bare ground increases the chances of mycotoxins contamination. This practice exposes maize to fungal spores which are mainly found in the soil (Kang'ethe, 2011). A survey conducted by the Ministry of Agriculture in 2007 indicated that over 90% of small scale farmers in Eastern province placed maize cobs on the ground during harvesting (Nyaga, 2010). Maize is stored in granaries after harvesting and in most cases poorly ventilated.

2.3 Production of aflatoxin by *Aspergillus* section *flavi* species

Aflatoxins are toxic fungi metabolites produced by *Aspergillus flavus* and *A. parasiticus* Spear (Robert *et al,* 1993). Among 18 different types of aflatoxin identified, major members are aflatoxin B1, B2, G1 and G2. Aflatoxin B1 is normally predominant in amount in infected maize (Reddy and Farid, 2000) and the most toxic of the four (Kang'ethe, 2011). *Aspergillus flavus* infects the maize crop before harvest or during harvesting and remain even after harvest. *Aspergillus flavus* has two morphotypes, the S strain and the L strain, that differ in aflatoxin-producing ability and other characteristics (Rao *et al,* 1997). Fungal communities on maize dominated by the S strain of *A. flavus* have repeatedly been associated with acute aflatoxin poisonings in Kenya (Probst *et al,* 2011). The conditions for fungal growth are different from those of mycotoxin production (Price *et al,* 2005) and therefore presence of mould on the kernels does not necessarily indicate presence of aflatoxin. In areas prone to

drought conditions like Kitui and Machakos maize plants are stressed and rendered susceptible to contamination by *Aspergillus* sp. (Wilson and Payne, 1994).

2.4 Aflatoxin producing *Aspergillus*

Aspergillus flavus is one of several known species that produces aflatoxins, including, *A. parasiticus, A. nomius, A. bombycis, A. pseudotamarii,* and *A. ochraceoroseus* (Moore et al 2011; Yu and Ehrlich, 2011). The major aflatoxin producers are *A. flavus* and *A. parasiticus* that are closely related and grow as a saprophyte on plant debris of many crop plants left on and in the soil (Rao *et al,* 1997 and Probst *et al,* 2011). *A. flavus* as a species contains two morphotypes that differ in sclerotial size and in their ability to produce aflatoxins (Moore et al 2011; Probst et al 2011; Cotty, 1989). Large (L) and small (S) sclerotial strains are often found in soils from both agricultural fields (Cotty and Bayman, 1994; Horn 2007) and non-agricultural areas (Ehrlich and Kobbeman, 2007). S - strain, which is capable of producing much higher concentrations of aflatoxins than the L - strain, is a more important source of aflatoxin contamination in maize (Zhang and Cotty 2007). Beyond sclerotial size, another difference between the L - and S - strain is colony morphology, since S - strain isolates produce many more sclerotia and, in the dark, fewer conidia (Cotty and Bayman, 1994). Members of the genus *Aspergillus* are characterized by the production of non-septate conidiophores (Robert *et al,* 1993), which are quite distinct from hyphae and which are swollen at the top to form a vesicle on which numerous specialized spore-producing cells, known as phialides or sterigmata are borne either directly (uniseriate) or on short outgrowths known as metulae (biseriate) (Rao *et al,* 1997). The colonies usually have biseriate sterigmata; reddish-brown sclerotia are often present, conidia are finely roughened, variable in size and oval to spherical in shape (Gathumbi, 2001). Colonies of *A. parasiticus* are dark green on Czepak's agar, remain green with age. Sterigmata are uniseriate, sclerotia are

usually absent; conidia are coarsely echinulate, uniform in shape, size and echinulation. *A. flavus* has different strains, strain S and L. Strain S readily produces aflatoxin (Rao *et al,* 1997) compared to L strain.

2.5 Production of Fumonisins by *Fusarium* species

There are several plant pathogenic species of *Fusarium* that are found to be associated with maize including *F. verticillioides*, *F. proliferatum, F. graminearum* and *F. anthophilum* (Scott, 1993; Munkvold and Desjardins, 1997). Among them, *F. verticillioides* is the most common species isolated worldwide from diseased maize (Munkvold and Desjardins, 1997). Fumonisins are produced by several *Fusarium* species (Marasas, 2001) including: *F. verticillioides, F. proliferatum, F. nygamai, F. anthophilum, F. dlamini, F. napiforme, F. thapsinum,* and *F. globosum* (Fandohan et al 2003). Amongst these, *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers (Shephard et al., 1996).

Although a high percentage of the grains may be colonized by these fungi, fumonisins are not always produced (Fandohan et al 2003). Many factors, including environmental conditions and host susceptibility, determine the incidence and severity of grain mould and subsequent mycotoxin contamination (Bii *et al,* 2012). Conditions favouring production of fumonisins by *Fusarium* moulds have not been clearly defined; however, periods of drought followed by cool, wet conditions during pollination and kernel development may favour production ((Fandohan et al 2003; Bii *et al,* 2012). Significant fumonisin accumulation in maize occurs when weather conditions favour *Fusarium* kernel rot (Marasas, 2001). The relatively warm tropical highlands of western Kenya thus appear to provide suitable conditions for the production of fumonisins in maize. This fungus often produces a symptom on the corn kernels referred to as "starburst," or a white streaking of the kernel (Fandohan, *et al,* 2003). Dry weather early in the season, followed by wet weather during silking of the corn plant,

and insect infestation increase the amount of fungal infection of corn kernels (Shephard, *et al,* 1996; Fandohan, *et al,* 2003). Typically, infection by *F. verticillioides* does not greatly affect the yield of corn (Bii *et al,* 2012 and Fandohan, *et al,* 2003).

2.6 Problems associated with aflatoxin and fumonisin contamination of maize in Kenya

Kenya's Ministry of Health (MOH) and partners identified 331 cases of acute hepatic failure in Eastern, Central Kenya and Kitui in 2004; 125 cases occurred in persons who subsequently died during the illness (Eduardo *et al,* 2005, and Lewis, 2005; Muture and Ogana, 2005). Onsongo (2004) reported that sampled maize from the affected area had concentrations of aflatoxin B_1 as high as 4,400 ppb, which is 220 times greater than the 20 ppb limit for food suggested by Kenyan authorities. Aflatoxicosis outbreaks had occurred previously in 1981 within the same geographical area, resulting in 12 deaths (Ngindu *et al,* 1982 and Kang'ethe 2011). In 2006, 20 cases of aflatoxicosis were reported in Machakos, Makueni and Kitui 10 of which resulted in deaths (Nyaga, 2010). While in 2007 and 2008 nine cases were documented from Kibwezi, Kajiado, Mutomo and Makueni four of which were deaths (Muthomi *et al,*2009, Wagacha and Muthomi, 2008). No fatal cases were reported in 2009 but 31,000 bags of maize were condemned in Mbeere and 1,213 bags of maize condemned in Bura irrigation scheme while in 2010 there was a widespread detection of aflatoxin in maize in Eastern and coast provinces (Nyaga, 2010).

Interest in fumonisins developed primarily due to the discovery that they can inhibit biosynthesis of sphingolipid and that they can impair animal health (Nelson *et al,* 1993; Marliére, *et al,* 2009). Fumonisins consumption can cause leucoencephalomalacia in horses (Kellerman *et al,* 1990), pulmonary oedema in swine as well as oesophageal cancer in humans (Marasas, 2001; Visconti, *et al,* 1999; Marliére, *et al,* 2009). The International

Agency for Research on Cancer (IARC) categorized fumonisins as carcinogens of group 2B of possible carcinogens for humans based on sufficient evidence of oesophageal and hepatic studies (Visconti, *et al*, 1999; Marliére, *et al*, 2009). In Kenya F. verticillioides is most common in warmer highlands such as Mt. Kenya region and western Kenya (Bii, *et al*, 2012).

2.7 Management of mycotoxins contamination in maize

Maize drying remains one of the most effective methods of managing aflatoxin in maize value chain. Moisture content in harvested maize is an important aspect of fungal growth. Traditionally, maize is processed by dehulling or pounding using either a stone quern or mortar and pestle (Kang'ethe *et al*, 2011). The aim is to remove the outer covering to soften the maize for cooking. Dry milling was also traditionally carried out using water mills (Kang'ethe, 2011). Traditional processing methods such as dehulling, soaking and cooking maize have been reported to reduce the levels of aflatoxins by 46.6%, 28-72% and 80-93% in maize containing 10.7-270 ng/g of aflatoxin levels in Kenya (Mutungi *et al*, 2008). Exposure to acute aflatoxin levels is minimized during food processing and preparation (Kang'ethe, 2011). Generally, these processing techniques have been traditionally used for increasing the palatability of different food recipes but can also be strategies capable of reducing aflatoxin contamination of grains.

Sorting and selection is done in the field when cobs are being removed from the maize stalks (Kang'ethe, 2011). This selection is not adequate as many cobs that are rotten to various levels may be passed depending on the judgment of those harvesting. Hand sorting of maize kernels reduced the mean total fumonisin contamination by 66% (Hoffmann, et al 2013). The use of physical methods, including cleaning, separation of screenings, washing, aqueous extraction, dehulling and milling, has been shown to be effective, to a certain extent, in reducing mycotoxins in cereals (Fandohan *et al*, 2005 and Shetty and Bhat, 1999). Before

processing maize is sorted using sorting tables made of wire mesh to allow damaged kernels and dust to fall off (Kang'ethe 2011; Hoffmann and Gatobu, 2010). Standards for moisture content exist in the formal maize market in Kenya, but maize traded through informal channels is not subject to these regulations (Hoffmann and Gatobu, 2010).

2.8 Optical sorting of maize contaminated with mycotoxins

Optical sorting machines can measure reflectance over two different spectral bands and use these as a basis for sorting (Pearson, *et al*, 2009). Procedures have been developed to analyze whole visible and near infrared spectra to select optimal filters to distinguish products using commercial sorting machines (Pearson *et al*, 2004 and Haff and Pearson, 2006). Using this procedure, it was found that aflatoxin and fumonisins in yellow corn could be reduced by 81% and 85%, respectively, by optically sorting the corn using filters centred at 750 and 1200 nm (Pearson *et al*, 2004; Haff and Pearson, 2006). Only one pass through the sorter is required, to achieve these levels of mycotoxin reduction. It is hypothesized that a similar procedure developed by Pearson *et al*, (2004) for sorting yellow corn could be applied to removing aflatoxin and fumonisin from white corn produced by commercial hybrids exposed to late-season drought stress.

Aflatoxin fluoresces strongly in UV (ca. 365 nm); aflatoxin B1 and B2 produce a blue fluorescence whereas aflatoxin G1 and G2 produce green fluorescence (Reddy and Farid, 2000). Ultra-violet light has traditionally been used to screen lots of maize that should be further tested for aflatoxin because a characteristic bright greenish yellow fluorescence (BGYF) in whole kernels and ground maize is roughly indicative of aflatoxin levels in maize (Shotwell and Hesseltine, 1981). Bright orange fluorescence (BOF) is roughly indicative of fumonisin levels (Pearson *et al*, 2010a). From analysis of the spectra on individual kernels, it was found that a pair of optical filters centered at 500 and 1200 nm could discriminate

approximately 87% and 93% of kernels having high levels of aflatoxin (greater than 100 ppb) and high levels of fumonisin (greater than 40 ppm), respectively, from kernels having low levels of aflatoxin (greater than 10 ppb) or fumonisins (less than2 ppm) (Pearson *et al*, 2010b). Near Infra Red light was found useful for detecting and sorting fungal infected yellow corn due to endosperm damage (Pearson *et al*, . 2010) and NIR light was found effective for identifying white corn infected by fungi using a laboratory spectrometer with the kernels well oriented and stationary (Shotwell and Hesseltine, 1981). Firrao *et al*, (2010) presented a new approach for early identification of maize contaminated with fumonisins, based on digital images. The authors stated that the method developed produce reliable fumonisins contamination estimation, within a few minutes, requiring minimal equipment, and may be used to assist in selecting lots during maize processing.

CHAPTER THREE: PREVALENCE OF *ASPERGILLUS* AND *FUSARIUM* SPECIES AND THEIR ASSOCIATED MYCOTOXINS IN MARKET SAMPLES OF MAIZE FROM KITUI, MERU AND MACHAKOS COUNTIES

3.1 Introduction

Mycotoxins are secondary metabolites produced by different fungi that contaminate agricultural and non-agricultural products (Martins *et al,* 2014). Mycotoxins that are of the greatest significance in Sub-Saharan Africa are Fumonisins (Kadera *et al,* 1999; Marasas *et al,* 2005; Bii *et al,* 2012 and aflatoxin (WHO, 2006; Nelson 2014) both of which are food-borne. Fumonisins are mycotoxins produced by *Fusarium verticillioides, F. Proliferatum, and F. oxysporum* (Bii *et al,* 2012; Fandohan *et al,* 2003) and aflatoxin are produced by some *Aspergillus* section *Flavi* species (Probst *et al,* 2011; Cotty 1989). The fungus resides in soil and crop debris, infects crops and produces the toxin in the field and in stores (Zuber *et al,* 1986; Muthomi *et al,* 2009; Sanchis *et al,* 1994; Rajamalar and Ravikumar 2014). Aflatoxin contamination in maize has been associated with drought and stress to growing plants combined with high temperatures as well as insect injury, poor harvesting practices and improper storage (Magan and Aldred 2007; AFLASTOP 2013). Aflatoxigenic members of *Aspergillus* section *Flavi* are said to dominate soils of agroecological zones in lower eastern Kenya and result to aflatoxin contamination in those regions when conditions are favourable (Muthomi *et al,* 2009).

Postharvest aflatoxin and fumonisins contamination is a threat to the health maize consumers most of which are small holder households in Kenya (Bandyopadhyay, 2014). Aflatoxins are acutely toxic, immune suppressive, mutagenic and carcinogenic compounds (Rajamalar and Ravikumar, 2014). Aflatoxin is one of the causes of hepatocellular carcinoma, the most often and malignant primary tumor of the liver (Ferlay *et al,* 2010; Liu and Wu 2010; Brankov *et*

al, 2013; Sakuda *et al,* 2014) while fumonisins have been implicated with hepatotoxicity and one of the causes of gastrointestinal cancer (Šegvić 2001; Peraica 1999; NTP, 1999). Fumonisins have been associated with a number of animal diseases such as leucoencephalomachia in equines, haemorrhage in the brain of rabbits (Marasas, 1995) and can also cause hepatotoxicity and nephrotoxicity of many animals (Howard *et al,* 2001).

Aflatoxins mainly occur in maize and groundnuts while fumonisins occur mainly in maize (Wagacha and Muthomi, 2008; Bankole *et al,* 2006). Aflatoxin poisoning has occurred severally in Eastern and Central Kenya with the most fatal aflatoxin poisoning being in 2004 (Kang'ethe 2011; Muthomi *et al,* 2009; Nyaga 2010). Aflatoxicosis outbreaks had occurred previously in 1981 within the same geographical area, resulting in 12 deaths (Ngindu *et al,* 1982; Kang'ethe 2011). Onsongo (2004), reported that sampled maize from the affected area had concentrations of aflatoxin B1 as high as 4,400ng/kg, which is 440 times greater than the 10ng/kg limit for food suggested by Kenyan authorities. In 2006, 20 cases of aflatoxicosis were reported in Machakos, Makueni and Kitui 10 of which resulted in deaths (Muture and Ogana, 2005; Nyaga 2010) while in 2007 and 2008 nine cases were documented from Kibwezi, Kajiado, Mutomo and Makueni four of which were deaths (Muthomi *et al,* 2009; Wagacha and Muthomi, 2008; Nyaga 2010). Although no deaths were reported in 2009 huge economic losses were incurred by farmers who lost 2,790,000 Kgs of maize condemned and 109,170 Kgs condemned in Bura irrigation Scheme (Nyaga 2010; AFLASTOP 2013). Destruction of such huge quantities of condemned maize causes further economic and environmental challenges. In 2010 there was widespread detection of aflatoxin in maize in Eastern (AFLASTOP 2013) and coastal Kenya (Nyaga 2010) although no deaths were recorded. Cases of aflatoxin and fumonisins poisoning have been underreported in Kenya and therefore the effects could be bigger than perceived. It is estimated that 60% of maize in lower Eastern and 85% in upper eastern held by farmers is unsafe for human consumption

(Nyaga 2010). Several measures such as training of farmers and extension service providers (Nyaga 2010), biocontrol measures which includes aflasafe (Mutegi 2013; Atehnkeng *et al*, 2008; Bandyopadhty, 2013) have been put in place by government and other stakeholders to address the problem of mycotoxin contamination in maize. Most of the available measures are towards avoiding contamination. When maize is already contaminated sorting is the only way to reduce exposure. Contamination by both toxins is possible without visible signs of the fungus (Bandyopadhty 2013; Probst, *et al,* 2011; Probst *et al,* 2009) and therefore sensitive technologies that can detect contaminated grains will greatly improve food safety (Pearson *et al,* 2009; Pearson *et al,* 2013). The objective of this study therefore is to determine the prevalence of mycotoxin producing *Aspergillus* and *Fusarium* species and their associated mycotoxins in maize from Eastern Kenya

3.2 Materials and methods

3.2.1 Study site

This study was conducted in Meru, Kitui and Machakos counties of lower Eastern province in Kenya. In Meru County the study was carried out within three sub Counties namely; Imenti South, Meru Central, Imenti North. In Meru rainfall ranges between 300 mm and 2600mm per annum (Jaetzold *et al*, 2006). The long rains come in April/May and short rains in November/December and the hot months are between June and September and January and February. In Kitui County, Kitui Central Sub County was sampled. The district is located between Longitudes 37^{o}, 45" and 39^{o} 0" east and longitudes 0^{o} 37" and 3^{o} 0" south (National Environmental Management Authority (NEMA), 2013). The altitude of the district ranges between 400m and 1800m above the sea level. The central part of the district is characterized by hilly ridges separated by wide low lying areas. The climate of the district is arid and semi-arid with very erratic and unreliable rainfall. The annual rainfall in the County ranges

between 500 -1050m (Jaetzold *et al,* 2006). The long rains come in April/May and short rains in November/December (Jaetzold *et al,* 2006). The high- land areas of Central hills in Kitui and Mutitu in the Eastern parts of the district receive between 500- 760mm of rainfall per year. The county experiences high temperatures throughout the year, which ranges from 16^{o}C to 34^{o} C. The hot months are between June and September and January and February. About 32% of the district falls under AEZ LM4 and LM5 which are Agri-marginal areas. Meanwhile 2% of the district falls under AEZ UM4 and LM3 which is regarded as suitable for agricultural production. Machakos county landscape is largely a plateau that rises from 700m to 1700 m above sea level. It has two rainy seasons, the long and the short rain seasons. The long rains seasons starts at the end of March and continues up to May, while the short rains season starts at the end of October and lasts till December. The annual average rainfall ranges between 500mm to 1300mm. Machakos Central and Mwala Sub-counties receive slightly higher rainfall than the lowland areas. Mean monthly temperatures vary between 180^{o}C and 250^{o}C. The coldest month is July while October and March are the hottest. Machakos District stretches from latitudes 0º45´S to 1º31´S from north to south and from longitudes 36º45´E and 37º45´E from east to west.

3.2.2 Sampling

Maize samples were collected from stores of traders in local markets of Meru, Kitui and Machakos Counties. Ten markets were sampled in each County and at least three traders in each market were sampled (Table 3.1). At least two quarter of a kilogram unique samples were picked from each trader for laboratory analysis. Samples were collected after thoroughly mixing maize in the bag to increase chances of getting the fungi. The samples were stored at temperatures below 4^{o}c to await analysis.

Table 3.1: Number of markets and samples from each agro-ecological zone studied in Meru, Kitui and Machakos counties of Kenya

County	Total samples per county	AEZ	Number of samples per AEZ	Number of Markets	Markets
Meru	62	UM1	16	2	Mikumbune and Mariene
		UM2	18	4	Kanyakine, Ntharene, Gakoromone, Igoji
		UM3	16	2	Chaaria, Ndurumo
		LM3	12	2	Giaki and Mitunguu
Kitui	72	LM4	25	4	Itoleka, Ithiani, Wikilikye, Mulango
		LM5	29	3	Kyangunga, Kamali, Kisasi
		UM4	18	3	Kalundu, Mutune, Morutu
Machakos	70	UM4	21	2	Kaseve and Machakos town
		LM4	23	4	Kaani, General Mulinge, Kithangathini, Kavumbu
		LM3	26	4	Masii, Kyaitha, Kyethivo, Mbaani

AEZ adapted from Farm Management Handbook of Kenya (Jaetzold *et al,* 2006) LM, Lower Midland; UM, Upper Midland zones. AEZ, agroecological zones as defined by using soil moisture availability index and rainfall.

3.2.3 Isolation of *Fusarium* and *Aspergillus* species from maize

One of the quarter kilo samples from each trader milled into fine floor using a Laboratory Milling machine. Ten grams of the ground sample was mixed with 100ml sterile water to make a stock solution and serially diluted up to dilution 10^3. The suspension was plated in Potato Dextrose Agar Medium (PDA) (Murray, *et al,* 1985; Acumedia 2011) by mixing 1 ml suspension in molten PDA cooled to 40^{o}C. Isolation media was prepared by weighing 39g of PDA into 1 litre of water. The mixture was autoclaved for 15 minutes at 121^{o}c and 15 PSI pressure. The media was allowed to cool to about 50^{o}c and then amended with 25ng/l of streptomycin and tetracycline (Probst 2009; Navi *et al,* 1999). Petri dishes to be used were labelled appropriately and a millilitre of the diluted sample was poured into a sterile petri dish aseptically and then 18ml of PDA media at 40^{o}c was poured on the same plate and the mixture swirled gently to mix. The mixture was allowed to cool and solidify in the lamina

flow hood and then sealed using parafilm for incubation. The plates were incubated at room temperature for 5 to 7 days.

3.2.4 Identification of *Aspergillus* species

Purification was done by sub culturing *Aspergillus* species identified on PDA and Rose Bengal Medium (10 g glucose, 2.5 g peptone, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 25 mg Rose Bengal, per litre of distilled water) modified with Dichloran fungicide and streptomycin (Mutegi 2010; Mutegi 2012; Probst *et al,* 2011). Sub culturing on PDA was done to differentiate *Aspergillus* section F*lavi* from the other species. Pure cultures of each *Aspergillus* isolated were made on PDA media. *Aspergillus* section F*lavi* species obtained were sub cultured on Rose Bengal Medium. Microscopy on *Aspergillus* was done using modified Riddell mounts (Nissen 2012) to allow detailed study of the conidia. Presence of sclerotia after seventh day of incubation, the pattern of sclerotia growth, colony colour, spore formation on the conidiophores, size of the sclerotia and growth rate was used to differentiate members of the *Aspergillus* section *flavi*. Number of colonies of each species obtained was recorded on the data collection sheet. Data on number of colony forming units for each fungus type was recorded. Number of samples with *A. flavus* strain S and L, *A. parasiticus* and other *Aspergillus* species was recorded. *Aspergillus* Identification Manual by Indratiningsih *et al,* (2013) and Thom and Rapper (1945) were used.

3.2.5 Identification of *Fusarium* species

Fusarium-type colonies were sub-cultured on low strength PDA (Reddy *et al,* 2008) and synthetic nutrient agar (SNA) (SIFIN 2008). Low strength PDA was prepared by weighing 10g agar, 17g PDA, 1g Potassium dihydrogen phosphate (KH_2PO_4), 1g Potassium nitrate (KNO_3), 0.5g Magnesium sulphate anhydrous ($MgSO_4$), 0.5g Potassium chloride (KCl), in 1000ml water (H_2O). The mixture was autoclaved for 15 minutes at 121^{o}c and and 15 PSI

pressure. The media was amended by 25ng/kg of streptomycin and tetracycline. SNA was prepared by weighing 0.2g sucrose, 0.2g glucose, 20g agar, 1g Potassium dihydrogen phosphate (KH_2PO_4), 1g Potassium nitrate (KNO_3), 0.5g Magnesium sulphate anhydrous ($MgSO_4$), 0.5g Potassium chloride (KCl), in 1000ml water (H_2O). The mixture was autoclaved for 15 minutes at 121^{o}c and 15 PSI pressure. The media was allowed to cool to about 50^{o}c and then amended with 25ng/l of streptomycin and tetracycline (Probst 2009; Navi *et al,* 1999). The plates were incubated for 14 days in the dark room to sporulate. Cultures grown on PDA were incubated at 25°C and SNA cultures were incubated at 25ºC under near UV light for two to four weeks. The growth characteristics on the low strength PDA plates were noted and used for identification. Tape and squash mounts (Nissen 2012) were prepared from the SNA plated and observed under ×100 lens a light microscope. The observed features of microconidia, macroconidia, chlamydiospores and phialides were used to identify the *Fusarium* species obtained. The morphological identification of *Fusarium* spp. was made using the criteria described in the *Fusarium* laboratory manual Nelson *et al,* (1983) and Leslie *et al,* (2006).

3.2.6 Determination of levels of aflatoxin and fumonisins

Extraction of aflatoxin was done using 70% methanol as the extraction solvent prepared by adding 30ml of deionised water to 70 ml of 100% methanol. Five grams of ground portion of the sample were added to 25ml of the extraction solvent to make a ratio of 1:5 w/v from a gram of the milled sample for each toxin. The components were mixed using orbital shaker for 2 minutes and allowed to settle. The extract was then filtered through Whatman No. 41 filter paper and diluted 1:10 in phosphate buffered saline containing 500 ml/l Tween-20 and analyzed for aflatoxin contamination using Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA) as described by Waliyar *et al,* (2005). The optical density readings were taken using 450nm filter. A standard curve was constructed by plotting the

mean relative absorbance % obtained from each reference standard against its concentration in ng/mL on a logarithmic curve (figure 3.1 and 3.2). The mean relative absorbance values for each sample were used to determine the corresponding concentrations. The relative absorbance was calculated using the formula:

$$\text{Relative absorbance percentage} = \frac{\text{absorbance standard}}{\text{absorbance zero standard}} \times 100$$

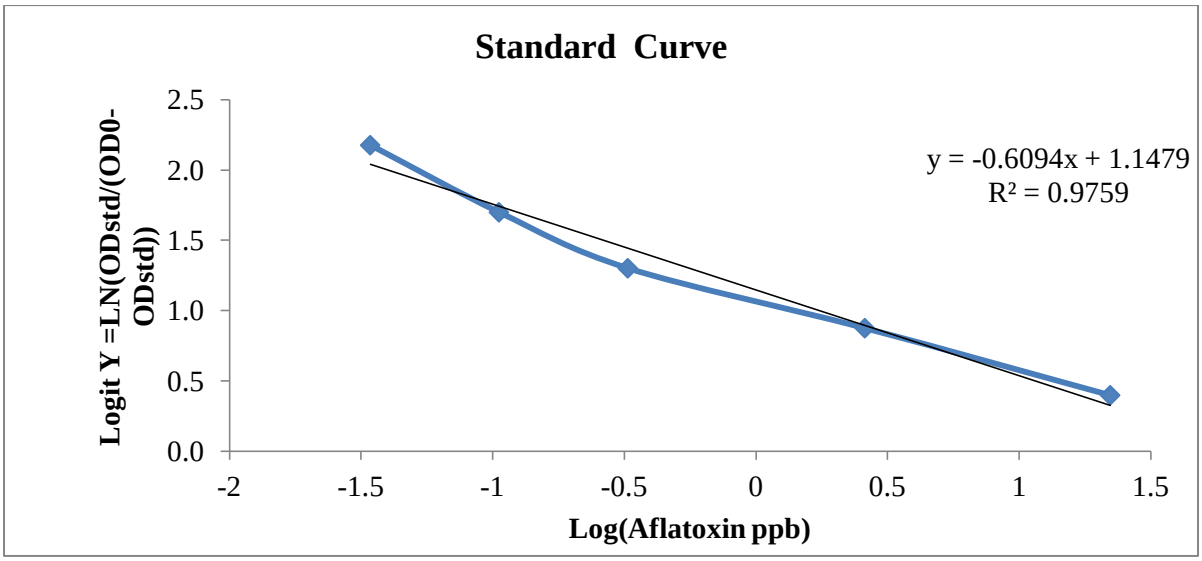


Figure 3.1. A standard curve for aflatoxin constructed by plotting the mean relative absorbance % obtained from each reference standard against its concentration in ng/mL on a logarithmic curve

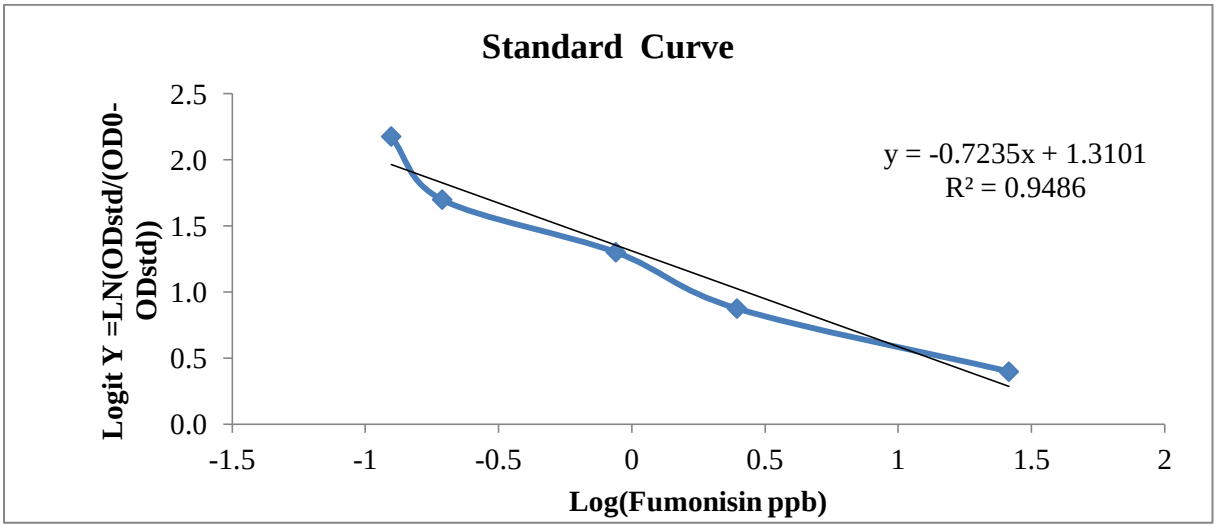


Figure 3.2. A standard curve for fumonisins constructed by plotting the mean relative absorbance % obtained from each reference standard against its concentration in ng/mL on a logarithmic curve

3.3 Results

3.3.1 Total fungi contamination in maize grain samples

The fungi genera isolated from the maize samples in all the regions were *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and others (Table 3.2). *Fusarium* spp. was the most frequently isolated fungus in all regions (Figure 3.3). *Aspergillus* spp. population did not vary significantly within the agroecological zones in Machakos and Kitui, in Meru however, UM3 had the highest *Aspergillus* spp. population while UM1, UM2 and LM3 had statistically similar population. There was a variation among the regions (Figure 3.3). Kitui recorded the highest *Aspergillus* spp. followed by Machakos and Meru came last (Table 3.2).

Fusarium spp. populations were not significantly different in all the AEZs of Meru, while in Machakos and Kitui UM4 which was the highest sampled AEZ in Machakos and Kitui had the highest isolation (Table 3.2). *Fusarium* spp. also varied from one region to another (Figure 3.3). Kitui had the highest isolation of *Fusarium* spp. followed by Meru and Machakos (Table 3.2).

Penicillium spp. population did not vary significantly in AEZs of Meru and Kitui but varied significantly within the AEZ of Machakos with UM4 and LM3 being the highest. There was a variation of *Penicillium* spp. isolated in the three regions (Figure 3.3). Machakos had the highest isolation if *Penicillium* spp. followed by Meru and Kitui (Figure 3.3).

Table 3.2: The population (CFU/g) of fungi contaminating maize grain collected from different agroecological zones in three Counties of Eastern Kenya during the long rains harvest, 2013.

County	AEZ	*Aspergillus*	*Fusarium*	*Penicillium*	Others
Machakos	LM3	1013a	1833a	5718ab	1667a
	LM4	2029a	2928a	1870a	1392a
	UM4	2127a	5966b	7712b	1982a
	Mean	1723	3576	5100	1680
	LSD	1200	2262	5295	1799
	C.V	18	14	4	18
Meru	UM1	500a	7917a	2708a	292a
	UM2	611a	10667a	4981a	37a
	UM3	1854b	13126a	3958a	250a
	LM3	474a	16416a	2033a	28a
	Mean	860	12032	3420	152
	LSD	717	8579	3892	253
	C.V	13	5	3	6
Kitui	LM4	1840a	8534a	533a	187ab
	LM5	2793a	21736b	1149a	448b
	UM4	1136a	25154b	1276a	57a
	Mean	1923	18474	986	231
	LSD	1684	9822	817	320
	C.V	3	10	19	15

AEZ: Agro-Ecological Zones, LM: Lower Midland, UM: Upper Midland, LSD: Least significant difference, C.V: Coefficient of Variation.

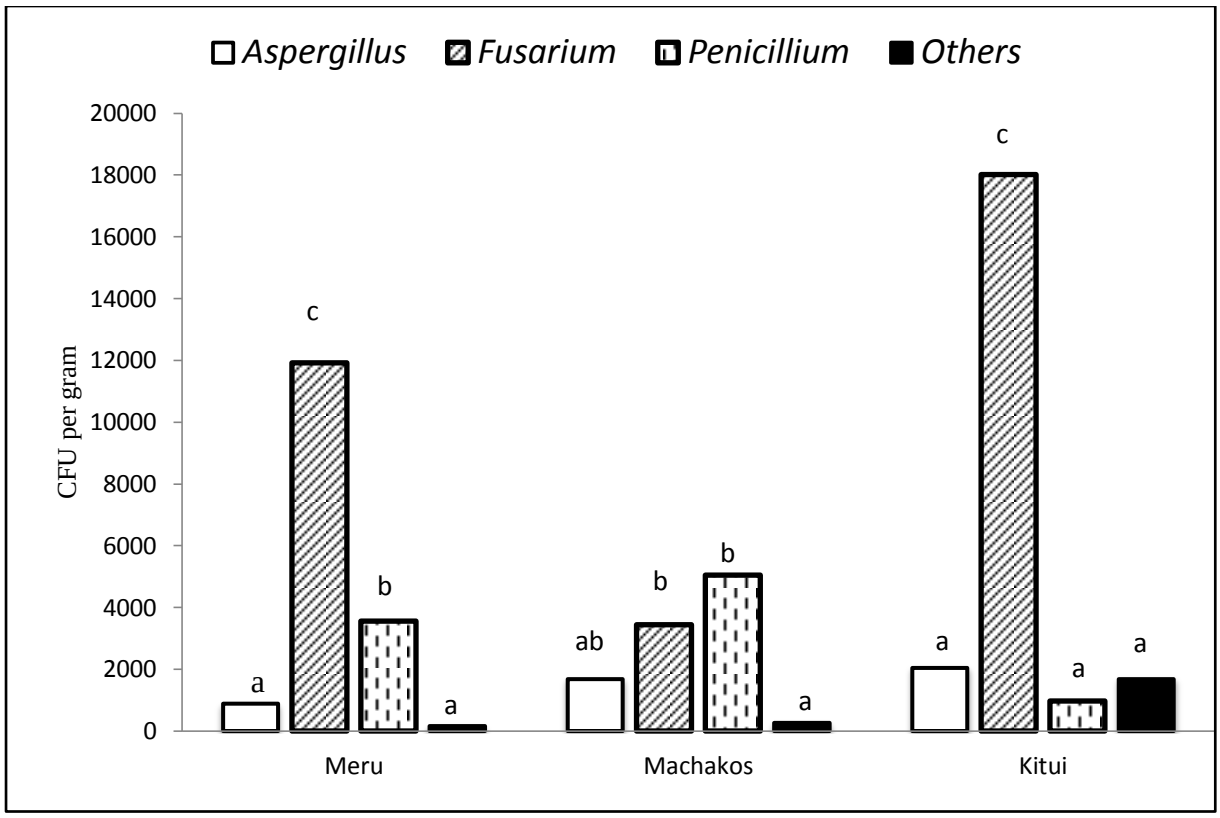


Figure 3.3: The population (CFU/g) of fungi contaminating in maize grain in three Counties of Eastern Kenya during the long rains harvest of 2013.

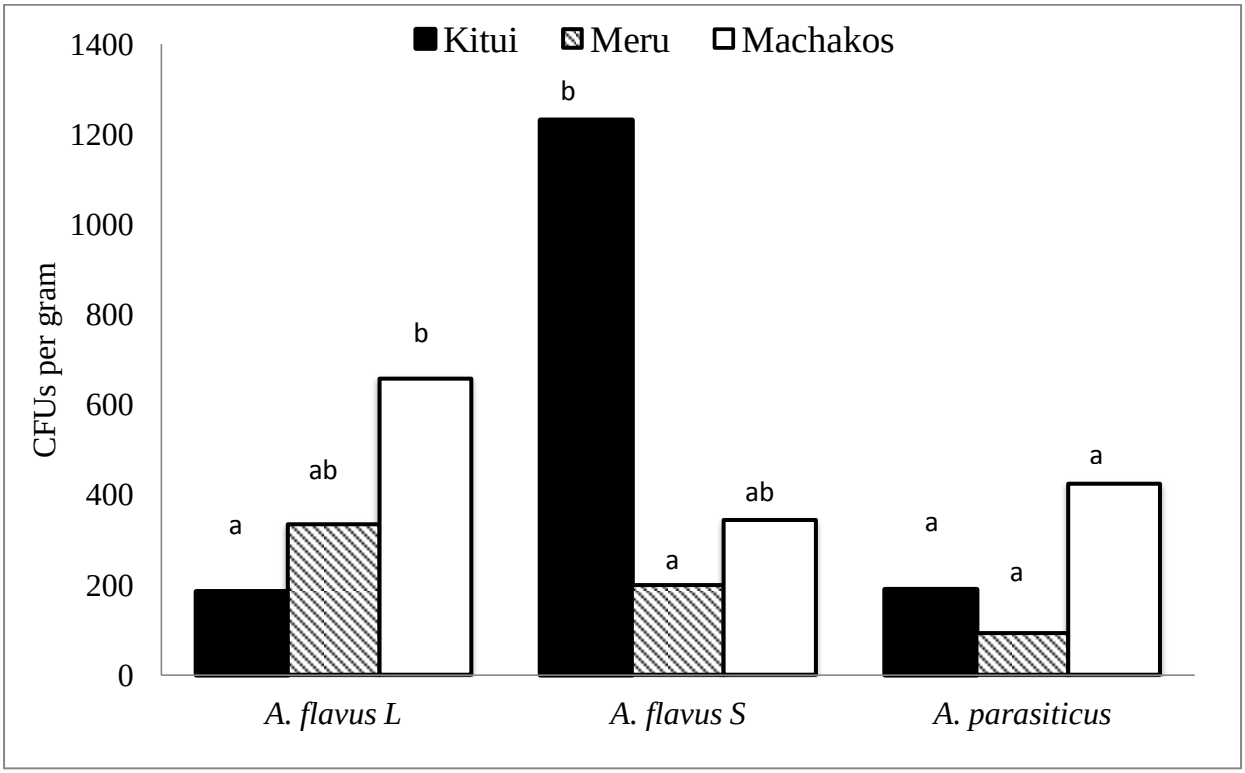


Figure 3.4: The population of (CFUs/g) of aflatoxigenic *Aspergillus* Spp. isolated from maize grain samples collected in the three Counties of Eastern Kenya during the long rains harvest of 2013.

3.3.2 *Aspergillus* species isolated from maize grain samples

The aflatoxigenic *Aspergillus* isolated from Meru, Kitui and Machakos were *A. flavus L* strain, *Aspergillus flavus S* strain and *A. parasiticus* (Figure 3.4). The most frequently isolated aflatoxigenic *Aspergillus* in Kitui was *A. flavus S* while the least was *A. flavus* L (Figure 3.4). In Meru the most frequently isolated aflatoxigenic *Aspergillus* was *A. flavus* L and the least was *A. parasiticus* (Table 3.3). In Machakos *A. flavus* L was the most dominant while *A. flavus* S strain was the least. *Aspergillus flavus* L strain which is less atoxigenic did not vary significantly in Kitui though LM4 and LM5 which are the lowest AEZs in Kitui had the highest. Upper Midland 3 (UM3) had the highest incidence of *A. flavus* L strain in Meru while the other AEZ did not vary significantly (Table 3.2). In Machakos UM4 and LM4 had the highest of *A. flavus* L strain which significantly varied from UM3 (Table 3.2).

Aspergillus flavus S strain did not vary significantly in all AEZ of all the regions at 95% confindence interval. In Kitui, however, LM5 had the highest of *A. flavus* S strain while in Meru UM3 had the highest (Table 3.3). Lower Midland 4 (LM4) of Machakos had the highest *A. flavus* S strain. Except in Meru, *A flavus* S was more frequently isolated in lowest AEZs of Kitui and Machakos (Table 3.3). *Aspergillus parasiticus* did not vary significantly in Kitui and Meru while in Machakos UM4 had the highest population that was significantly different from the rest of AEZs (Table 3.3).

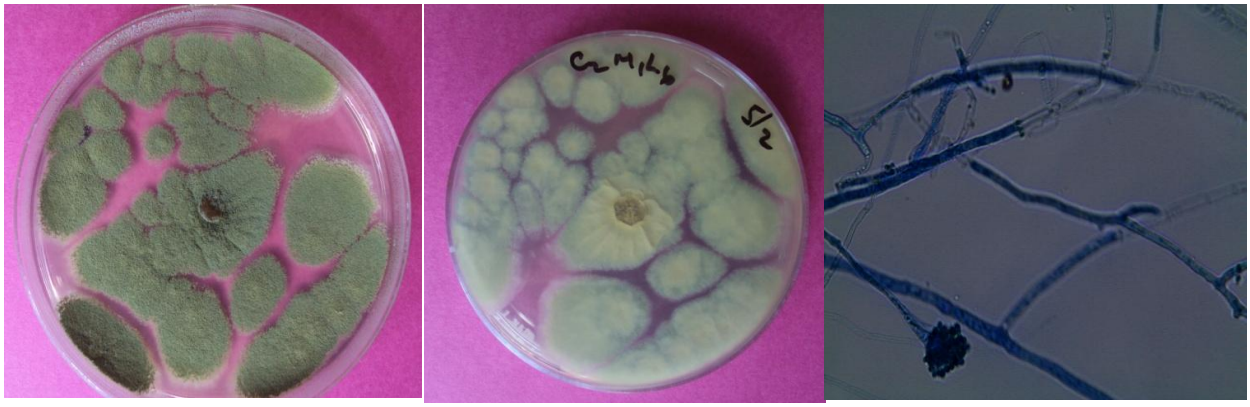
Vesicles of *A. flavus* L isolated were uniseriate while those of *A. flavus* S were biseriate and all had hyaline conidiophores (Figure 3.5). *Aspergillus parasiticus* had conidial heads mostly biseriate and conidia globose (Figure 3.5). On PDA *A. parasiticus* and *A. flavus* S appeared blighter and more raised with large white colony margins while *A. flavus* L was darker and with very smaller white colony margin (Figure 3.5).

Upon incubating the three at room temperature for more than 7 days *A. flavus* S produced clear liquid droplets within its mycelia this characteristic was also observed with *A. parasiticus* when incubated at 30^{o}c on both PDA and Rose Bengal media. On Rose Bengal media *A. flavus* S produced black scelerotia within its mycelia that were arranged in roughly circular manner and this was used as one of the major differential for identification of this strain.

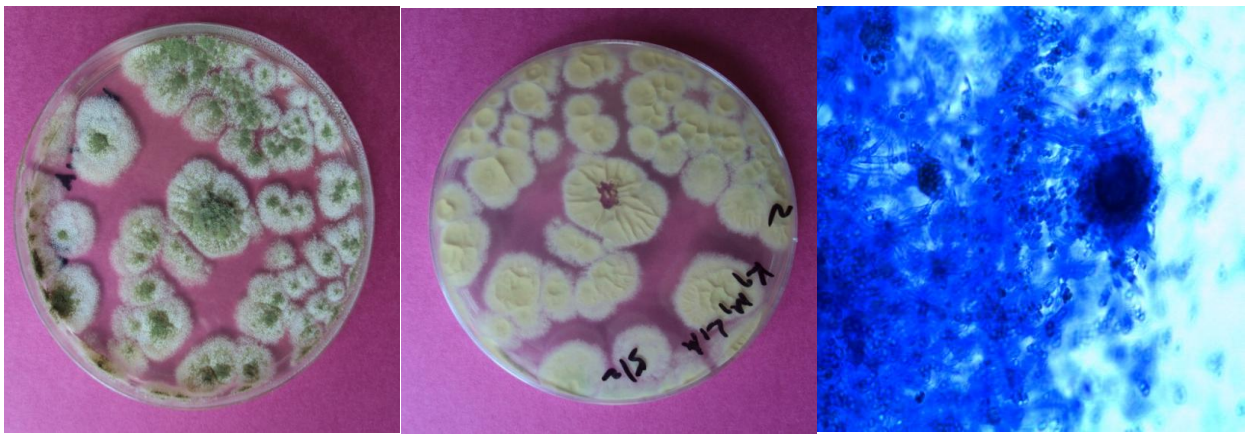
Table 3.3: The population (CFU/g) of aflatoxigenic *Aspergillus* spp. isolated from maize grain collected from different agroecological zones in three Counties of Eastern Kenya during the long rains 2013.

County	**AEZ**	***A. flavus L***	***A. flavus S***	***A. parasiticus***
Kitui	UM4	38a	823a	75a
	LM4	227a	1200a	195a
	LM5	241a	1517a	267a
	Mean	169	1180	179
	LSD	226	1649	258
	CV	41	6	29
Meru	LM3	56a	222a	0a
	UM1	188a	21a	0a
	UM2	203a	204a	204a
	UM3	833b	354a	125a
	Mean	320	200	82
	LSD	481	311	215
	C.V	39	12	45
Machakos	LM3	218a	372a	218a
	UM4	524ab	175a	999b
	LM4	1276b	464a	130a
	Mean	672	337	449
	LSD	872	404	741
	C.V	44	18	13

AEZ: Agro-Ecological Zones, LM: Lower Midland, UM: Upper Midland, LSD: Least significant difference, C.V: Coefficient of Variation.



Aspergillus flavus L strain



Aspergillus flavus S strain

Aspergillus parasiticus

Figure 3.5: *Aspergillus* species isolated on potato dextrose agar (PDA media) from maize kernels and corresponding microscopic slides prepared from modified Riddell mounts at 400 times magnification.

Other *Aspergillus* fungi isolated from all the regions were *A. niger and A. tamarii* while *A. ochraceous* which produces ochratoxins and *A. candundus* were isolated in Meru and Kitui samples only. *Aspergillus niger* did not vary in Kitui and Meru AEZs but in Machakos UM4 had the highest isolation which was significantly different from LM3 and LM4 (Table 3.4). *Aspergillus niger* and was most frequently isolated in Machakos while Kitui had the highest isolation of *A. tamarii, A. candundus* and *A. ochraceous. A. tamarii* was produced in all the regions (Table 3.4). Kitui and Machakos had the highest isolation of *A. tamarii.*

Table 3.4: The population of (CFUs/g) other *Aspergillus* spp. isolated from maize samples collected in different agroecological zones in three Counties of Eastern Kenya during the long rains harvest of 2013.

County	**AEZ**	***A. niger***	***A. ochraceous***	***A. candudus***	***A. tamarii***	**Others**
Kitui	UM4	19a	38a	19a	1a	13a
	LM4	53a	40a	0a	40ab	92a
	LM5	104a	218a	264b	161b	129a
	Mean	93	99	95	67	78
	LSD	59	218	219	137	164
	CV	68	86	74	24	80
Meru	LM3	28a	56a	56a	0a	0a
	UM1	42a	125a	0a	0a	125a
	UM2	0a	0a	0a	0a	18a
	UM3	146a	104a	208b	21a	62a
	Mean	54	71	66	5	52
	LSD	205	136	119	28	129
	C.V	105	13	24	178	72.
Machakos	LM3	128a	-	-	77a	-
	UM4	397b	-	-	32a	-
	LM4	73a	-	-	87a	-
	Mean	199	-	-	65.	-
	LSD	265	-	-	91	-
	C.V	18	-	-	13	-

AEZ: Agro-Ecological Zones, LM: Lower Midland, UM: Upper Midland, LSD: Least significant difference, C.V: Coefficient of Variation.

3.3.3 *Fusarium* species isolated from maize grain samples

Fusarium. verticillioides, F. proliferatum and *F. oxysporum* were isolated in all the regions (Figure 3.4). *Fusarium verticillioides* was the most frequently isolated species while *F. oxysporum* was the least isolated in all the regions (Figure 3.6). There was significant variation of *F. verticillioides* isolation across the three regions with Kitui having the highest and Machakos with the least (Figure 3.6). *Fusarium proliferatum* varied across the regions and was most frequently isolated in Meru and least in Machakos (Figure 3.6). *Fusarium oxysporum* was most frequently isolated in Kitui and least in Machakos (Figure 3.6). *Fusarium verticillioides* did not significantly vary in AEZs of Meru but varied significantly in Kitui and Machakos (Table 3.5). In Kitui *F. verticillioides* was high in UM4 and least in LM5 while in Machakos it was highest in UM4 and least in LM3 (Table 3.5). *F. proliferatum* varied significantly in AEZs of Kitui and Meru but did not significantly vary in Machakos. In Meru *F. proliferatum* was highest in UM2 and least in LM3 while in Kitui it was highest in UM4 and least in LM4 (Table 3.5). In all the AEZs within the three regions most *F. proliferatum* was isolated from the highest zones which were UM1 in Meru, UM4 in Kitui and UM3 in Machakos (Table 3.5). *Fusarium oxysporum* in Meru did not significantly vary within the AEZs, while in Kitui the variation occurred in LM4 which had the highest isolation and in Machakos UM4 had the highest isolation of *F. oxysporum* (Table 3.5).

There were some variations in the *F. verticillioides* isolated from Meru County, although all had similar microscopic features that are used to in distinguishing *F. verticillioides* others in the same category (Figure 3.7). *Fusarium verticillioides* had abundant micro conidia occurring in chains with very rare macro conidia (Figure 3.7). *Fusarium proliferatum* had abundant macro and micro conidia while *Fusarium oxysporum* had both conidia types and chramydospores on mycelia. Those characteristic features were the major differentials used to differentiate the species.

Table 3.5: The population (CFUs/g) of *Fusarium* species contaminants from maize samples collected in different agroecological zones in three Counties of Eastern Kenya during the long rains 2013.

County	AEZ	*F. verticillioides*	*F. proliferatum*	*F. oxysporum*
Meru	UM1	6875a	313ab	792a
	UM2	9056a	1203b	407a
	LM3	10511a	0a	0a
	UM3	14396a	278ab	1750a
	Mean	10209	447	737
	LSD	8156	986	2294
	C.V	8.	57	116
Kitui	LM5	21609b	207ab	23a
	UM4	24757b	481b	80a
	LM4	5987a	133a	2400b
	Mean	17451	274	834
	LSD	9637	324	2017
	C.V	185	396	809
Machakos	LM3	1564a	103a	167a
	LM4	2623ab	232a	73a
	UM4	4746b	64a	1126b
	Mean	2988	133	455
	LSD	2242	180	645
	CV	15	41	12

AEZ: Agro-Ecological Zones, LM: Lower Midland, UM: Upper Midland, LSD: Least significant difference, C.V: Coefficient of Variation.

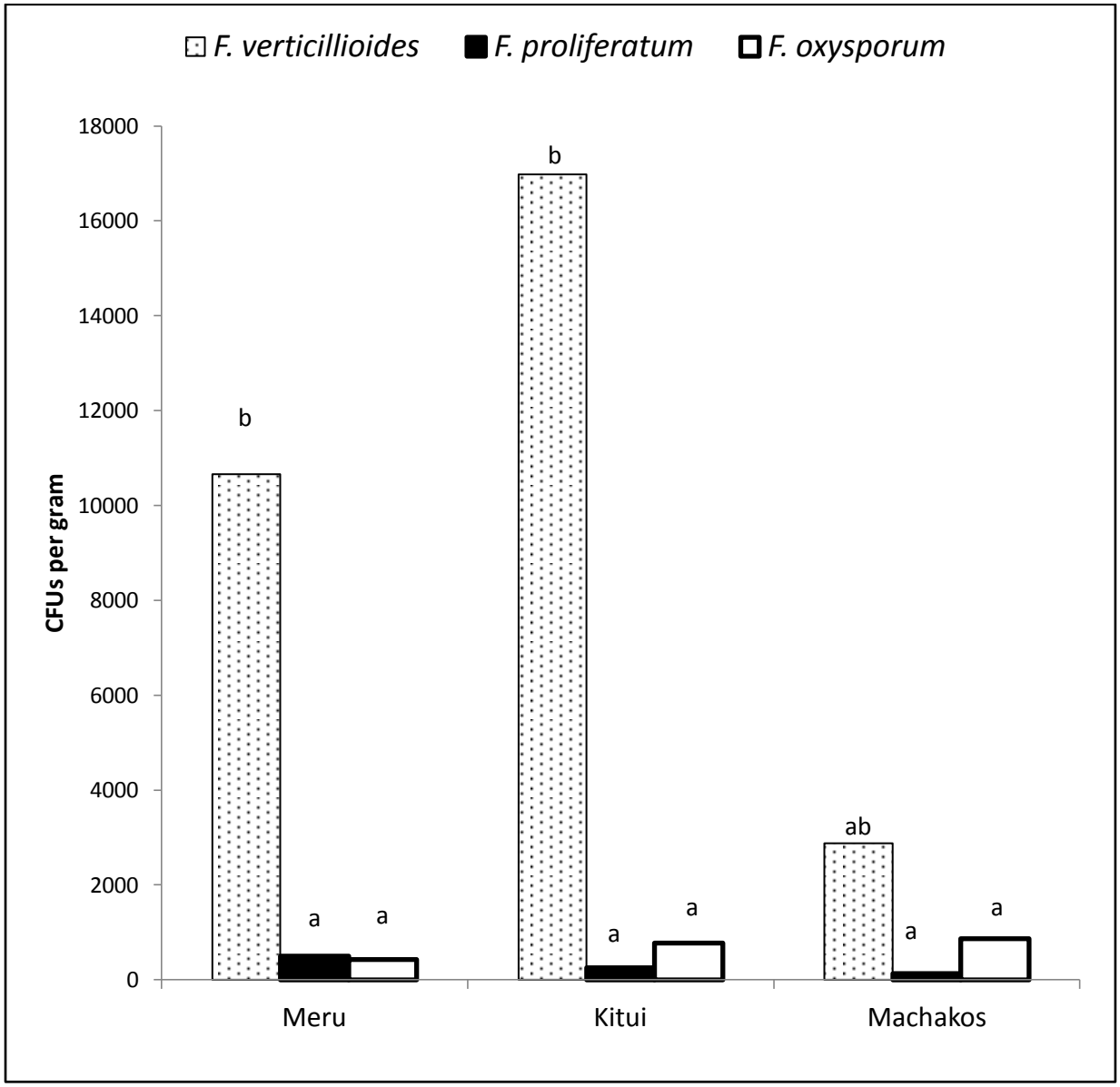
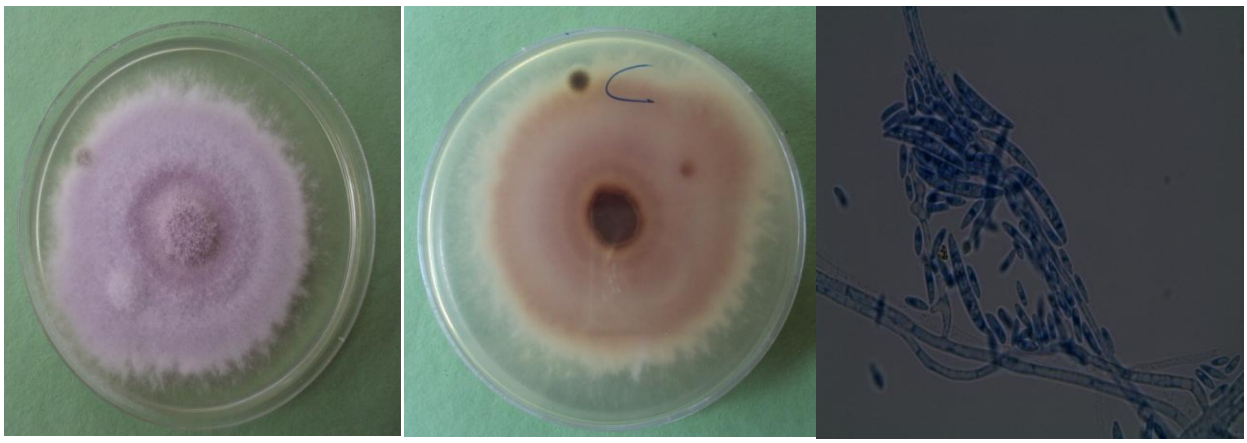
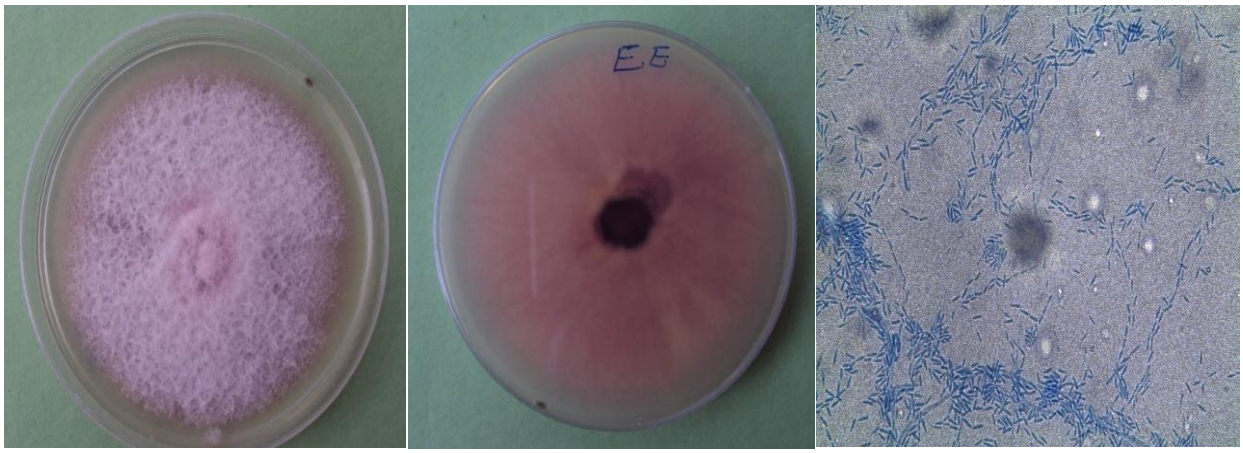


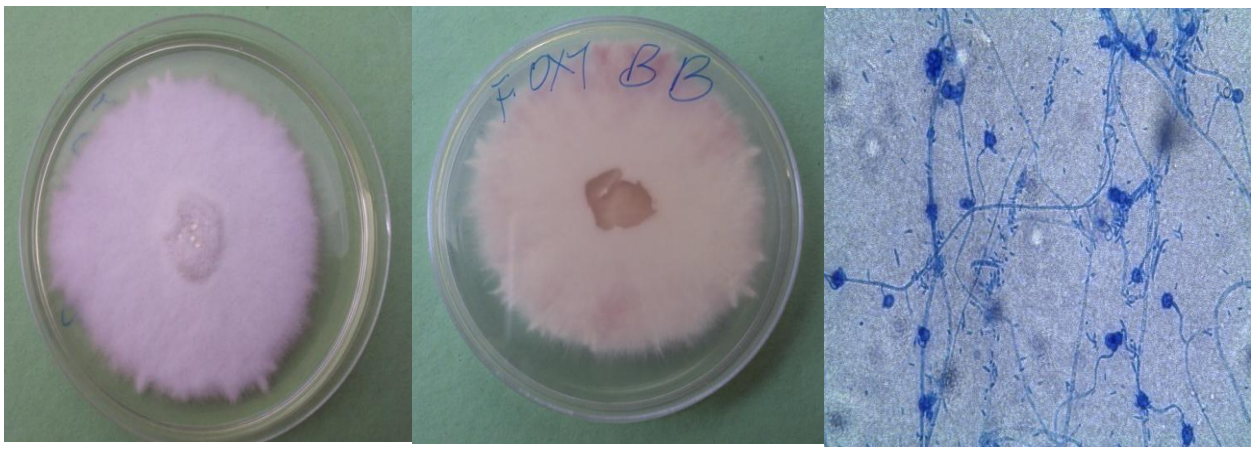
Figure 3.6. The population (CFUs/g) *Fusarium* from maize samples collected in three Counties of Eastern Kenya during the long rains 2013



Fusarium proliferatum



Fusarium verticillioides



Fusarium oxysporum

Figure 3.7: *Fusarium* species isolated on potato dextrose agar (PDA media) from maize kernels and corresponding microscopic slides prepared from synthetic nutrient agar (SNA) at 400 times magnification.

3.3.4 Aflatoxin and fumonisins levels in maize grain samples

On average aflatoxin was detected beyond acceptable levels in Meru, Kitui and Machakos (Figure 3.8). Kitui had the highest levels of aflatoxin detected while Machakos had the least (Figure 3.8). Fumonisins was detected in levels above the acceptable limits in Meru and though detected in Kitui and Machakos the levels were within the acceptable limits (Figure 3.9). The fumonisins levels did not match the *F. verticillioides* isolated since Kitui had the highest isolation. In Meru the higher the AEZ the fewer the samples with aflatoxin levels above recommended limits. This trend was roughly seen in all the regions with minor disparities such as in Machakos where LM3 has the least number of samples with aflatoxin levels above the recommended (Table 3.7). Machakos had the least number of samples with fumonisins above the recommended levels (Table 3.7) which agrees with the isolation results in section 4.1.3 above.

Aflatoxin levels from all AEZs in Kitui had similar means that were higher than the recommended levels. LM5 was the lowest AEZ sampled in Kitui and had the highest toxin recorded. In Meru UM1 and LM3 had the least aflatoxin levels which were actually lower than the recommended level (Table 3.6). UM2 had the highest toxin followed by UM3 (Table 3.6). UM2 in Meru had the highest aflatoxin recorded compared to the rest of AEZs in all the sampled regions. This could be attributed to its proximity to the major Nairobi - Meru road since this road cuts across UM2 zone and most of the sampled markets in this zone were along this road. This observation can be confirmed by looking at the number of samples that had aflatoxin levels above the recommended from Table 3.6. Only 6% of the samples had aflatoxin levels above that recommended and among them, majority had aflatoxin levels higher than 100ng/kg (Table 3.6). In Machakos, it was only UM4 that had toxin levels below the recommended levels. LM3 and LM4 had toxins above the recommended levels and levels

that were significantly different from UM4. UM4 zone in Machakos was the highest sampled zone in the region

Fumonisins levels in Kitui and Machakos were not of major phytosanitary concern since all the AEZs in these two regions had fumonisins levels below that recommended (Figure 3.9). The range of fumonisins in Kitui and Machakos was between 20ng/kg to 550ng/kg. In Meru however, fumonisins was a major phytosanitary threat. All the AEZs in Meru had fumonisins levels ranging from 1139ng/kg to 2008ng/kg which was above the acceptable limits. The levels of fumonisins in Meru increased from the highest AEZs to the lowest. UM3 the transition coffee zone and LM3 which is mainly the sorghum / cotton zone had the highest fumonisins levels (Table 3.6). Comparing the three regions as it is in Figure 3.7 Meru had by far the highest fumonisins levels.

Table 3.6: Aflatoxin and fumonisins (ng/kg) in maize samples collected in different agroecological zones three Counties of lower eastern Kenya during the long rains 2013.

	aflatoxin			**Fumonisins**		
AEZ	**Kitui**	**Machakos**	**Meru**	**Kitui**	**Machakos**	**Meru**
LM5	87.0a	-	-	550.9a	-	-
LM4	49.1a	46.6b	-	522.0a	20.0a	-
UM4	52.2a	2.1a	-	269.3a	173.6b	-
LM3	-	26.6ab	5.1a	-	40.7a	2008.00b
UM1	-	-	0.3a	-	-	1139.0a
UM2	-	-	115.7b	-	-	1203.0a
UM3	-	-	17.2ab	-	-	1735.0a
Mean	63	25.1	35	447	78.1	1697
LSD $_{(p\leq0.05)}$	84.4	35.44	98.9	478.7	97.09	863.2
C.V(%)	449.7	447.8	753.1	358	393.7	134

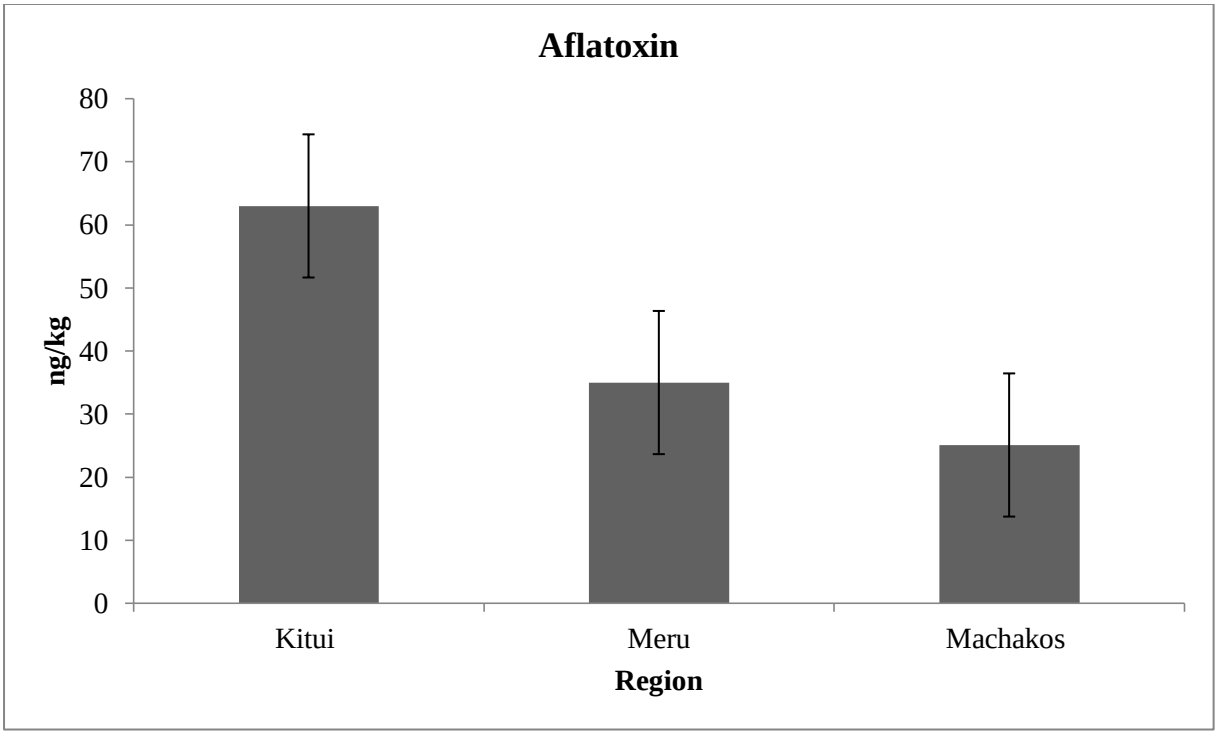


Figure 3.8. Amount of aflatoxin (ng/kg) from maize samples collected in the three Counties of lower eastern Kenya during the long rains 2013.

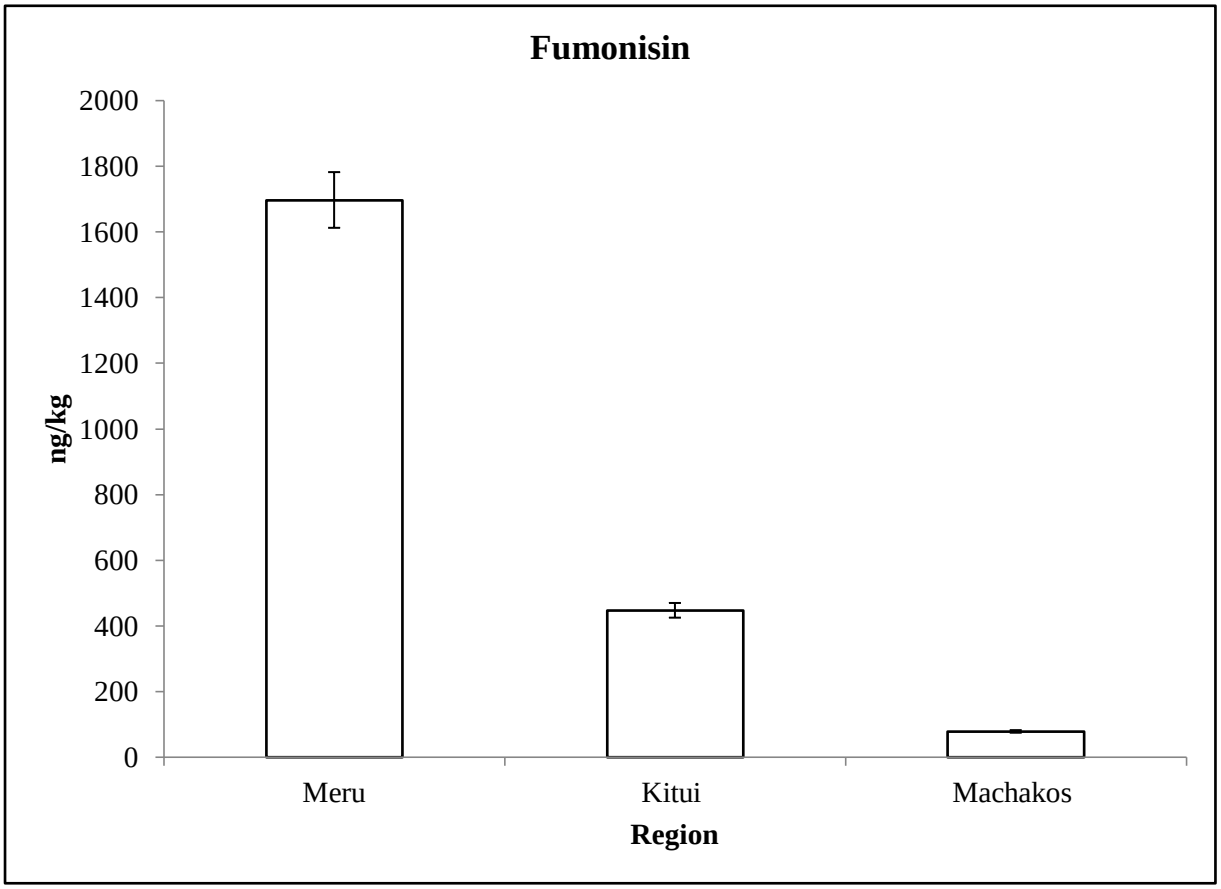


Figure 3.9. Amount of fumonisins levels (ng/kg) from maize samples collected in the three Counties of lower eastern Kenya during the long rains 2013.

Table 3.7: Percentage of Aflatoxin and fumonisin contaminated maize samples from different agroecological zones in three counties of eastern Kenya

			Aflatoxin			fumonisin		
	Number		Above	Below	Not	Above	Below	Not
Region	of samples	AEZ	10ng/kg	10ng/kg	detected	100ng/kg	1000ng/kg	detected
		UM1	0	31	69	31	13	56
		UM2	6	50	44	33	17	50
		UM3	13	38	50	44	19	38
Meru	67	**LM3**	25	25	50	50	25	25
		LM3	4	27	69	0	31	69
		LM4	22	30	48	0	17	83
Machakos	70	**UM4**	10	43	48	10	0	90
		LM4	20	12	68	8	12	80
		LM5	17	17	66	14	10	76
Kitui	72	**UM4**	17	11	72	6	17	78

3.4 Discussion

3.4.1 Fungi contamination in maize

Three fungi genera isolated from Meru, Kitui and Machakos were *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. The successful detection of fungal could be one of the indicators mycotoxin contaminations. This agrees with the findings of Alakonya (2009); Mureithi *et al,* (2011); Muthomi *et al,* (2012); Probst *et al,* (2009); Wangari (2013); Probst *et al,* (2011); Pitt *et al,* (2000) and Bii *et al,* (2012) who working on maize from different parts of Kenya isolated similar spectrum of fungal contamination. Out of the three fungi genera isolated from the maize samples in all the regions, the genus with the highest populations overall was *Fusarium*. Alakonya (2009) while working on maize ear, reported higher prevalence of

Fusarium species in all varieties he was working with which were readily isolated from both rotten and symptomless maize kernels. Similar observations were made by Bii, *et al,* (2012) and Muthomi *et al,* (2009) who reported wide spread infestation and contamination of maize by *Fusarium* species in aflatoxin 'hot spots' in Kenya and Kadera, *et al,* (1999) who while working on fumonisins contamination in western Kenya reported higher population of *Fusarium* spp. comparing to other fungal types in the sampled maize. *Fusarium* spp. is one of the major causes of maize ear rots in the maize plantations in Kenya (Bii, *et al, 2012*; Alakonya 2009; Nooh , *et al,* 2014).

Higher *Fusarium* spp contamination was reported from higher agro-ecological zones that experience higher rainfall and relatively warmer such as UM4 in Kitui, LM3 and UM3 in Meru. This agrees with the findings of Velluti, *et al,* (2000); who working *in vitro* on fungal competition on maize reported that the growth rate of *Fusarium* was higher at a temperature of 25^{o}c whereas at 15^{o}c, growth was much lower (Kimanya *et al,* 2008; Fandohan *et al,* 2004) . This however is not the case with fumonisins production since cooler agro-ecological zones produced more fumonisins (Kimanya *et al,* 2008; Hinojo *et al,* 2006; Nelson *et al,* 1992; Nelson *et al,* 1993), making Meru the highest producer of fumonisins in comparison with other study areas. Hinojo *et al, (*2006) established that fumonisin production was the highest at 20^{o}c and lowest at 37^{o}c. These findings indicate a wide spread infestation of maize by *Fusarium* spp species across the study areas.

Fusarium spp. can survive well on maize crop residues such as stalks, roots, seed, soil, silk of plants after harvesting (Bii, *et al, 2012*; Nelson 1992; Fandohan, *et al,* 2004). This could be the most possible source of inoculums. *Fusarium* fungal structures like mycelium, chlamydospores, macrospores, microspores and thickened hyphae can survive for longer periods in unfavourable environmental conditions (Munkvold, 2003; Fandohan 2004; Dragich and Nelson 2014. Infection of maize by *Fusarium* species and contamination with

fumonisins are generally influenced by many factors including environmental conditions such as climate, temperature, humidity, insect infestation and pre- and postharvest handling (Shephard *et al,* 1996, Marasas *et al,* 2001, and Fandohan *et al,* 2003). These factors do not influence infection independently but most often a complex of interactions (Dragich and Nelson 2014; Fandohan *et al, 2003*; Manninger, 1979; Emerson and Hunter 1980, Riley and Norred, 1999; Odvody *et al,* 1990).

Although *Aspergillus* populations were not as high those of *Fusarium* in the study area, there was variation in population from high AEZs to the lowest. *Aspergillus* in Meru was least in UM1 and this can be attributed to cool climatic conditions it that zone. The high *Aspergillus* population can be attributed to post harvest handling of maize that predisposes it to fungal contamination. Typically maize comes off the field with moisture levels of between 16 - 20% moisture (Aflastop, 2013; Alakonya and Monda, 2013). *Aspergillus* flourishes in warm humid conditions (Muthomi, 2009; Wagacha and Muthomi, 2008; Bandyopadhyay, 2014). The grain is then often stored into rooms or stores not suitable to either the grain or the weather conditions (Aflastop, 2013; Nyaga, 2010) since they lack proper airflow which would remove heat and humidity from the maize while in store. These storage conditions allow *Aspergillus* to grow easily. Bandyopadhyay (2014) reported that the optimum temperature for growth is 37^{o}c but the fungus readily grows between the temperatures of 25-42^{o}c, and will grow at temperatures from 12-48^{o}c.

Other fungal species commonly isolated in the three regions was *Penicillium* spp. which was more cosmopolitan and its population did not vary across agro-ecological zones of Meru and Machakos but did vary significantly in Kitui. The findings of this study agree with those of Muthomi, *et al, (*2009) who while working in eastern Kenya predominantly isolated *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. Contamination of maize grains by three fungi makes them unfit for human and animal consumption due to discolouration, reduced

nutritional value and of important poisoning by the mycotoxins produced by some species of *Aspergillus* spp. and *Fusarium* spp. (Wagacha and Muthomi 2008).

3.4.2 *Aspergillus* species isolated from maize grain samples

Aspergillus flavus S and L strains and *A. parasiticus* were the mostly isolated aflatoxigenic fungi from the maize samples in Meru, Kitui and Machakos. *Aspergillus* section *Flavi* contains three known aflatoxigenic species, *A. flavus, A. parasiticus* and *A. nomius* (Samson, *et al*, 1995) and the non-aflatoxigenic species *A. tamarii, A. caelatus*, and *A. oryzae* (Ito *et al*, 1999, Goto *et al*, 1997 and 1996). *Aspergillus flavus* has two morphotypes (or strains), the S strain and the L strain, that differ in aflatoxin-producing ability (Probst *et al*, 2011 and Klich 2007), and other characteristics. Fungal communities on maize dominated by the S strain of *A. flavus* have repeatedly been associated with acute aflatoxin poisonings in Kenya (Probst *et al*, 2011). In this study, the two strains differed significantly in their ability to produce sclerotia and aflatoxins. *Aspergillus flavus* S produced numerous small sclerotia when cultured on Modified Dichrolan Rose Bengal media at 33^{o}c, while L strain produced none and *A. parasiticus* produced fewer and larger sclerotia. This agrees with the findings of Probst *et al,* (2011), Klich (2007) and Cotty (1989) who found out that S strain isolates produce numerous, small sclerotia (average <400 µm) and, on average, higher levels of aflatoxins than L strain isolates. In other study by Probst *et al, (*2011), L strain is said to produce very few and large sclerotia, a feature that was not noticed in this study.

The presence of aflatoxigenic *Aspergillus* spp. in all the agroecological zones sampled did not necessarily indicate presence of aflatoxin. The key ecological determinants pre- and post-harvest production of aflatoxin is water availability and temperature (Milani 2013; Bandyopadhyay 2014). In warm and humid conditions maize ears are ideal conditions for colonisation and dominance of A. flavus/parasiticus species, resulting in the formation of

aflatoxins (Milani 2013; Muthomi *et al*, 2012; Mureithi *et al, 2011*; Wangari 2013; Bandyopadhyay 2014). In good storage conditions therefore, the fungi will not produce aflatoxin. The distribution of aflatoxigenic *Aspergillus* and aflatoxin was largely found to be temperature dependent and this explains the variations between warm agro-ecological zones and the cooler agro-ecological zones. This agrees with the findings of Muthomi *et al*, (2012) who attributed the distribution of *A. flavus* and its respective mycotoxins in semi arid eastern to the warmer temperature conditions of 25 – 35^{o}C prevalent in this region compared to the colder North Rift region of 18 – 25^{o}C where they were carrying out similar experiments. Schmidt-Heydt *et al,* (2010) and Mutungi (2008) also showed that high temperature of above 27^{o}C was a key factor for aflatoxin production.

Other *Aspergillus* Species isolated were *A. niger, A. ochraceous, A. candudus* and *A. tamarii*. Among them *A. niger* and *A. tamarii* were the most abundant and they were isolate din all the regions. Muthomi, *et al, (*2009) reported a similar spectrum of *Aspergillus* species while working in semi-arid Eastern Kenya during the long and short rains of 2008. *A. tamarii* falls under section *Flavi* and has been implicated with production of aflatoxin B1 and B2 by Goto *et al, (*1996) but later was exonerated by the same authors in 1999. *A. ochraceous* section *Circumdati* (Bayman, *et al,* 2002) produces achratoxins which are also mycotoxins that contaminate grains, coffee and dried fruits (Frisvad, 1995, Pitt, 2000, Romani *et al,* 2000).

3.4.3 *Fusarium* species isolated from maize grain samples

Fusarium verticillioides, F. proliferatum and *F. oxysporum* were the only fumonisins producing fungi isolated during this study. The populations of *F. verticillioides* was the highest in all the agro-ecological zones studied making a very cosmopolitan species. Among the fumonisins producing species *F. verticillioides* is the most common and the highest producer of fumonisins (Bii, *et al,* 2012 and Fandohan, *et al,* 2003). *F. verticillioides* is

thought to exist systemically and asymptomatically in most maize fields (in roots, stalk tissues, and kernels) and to be passed from parent to progeny by seed-borne infection (Wilke, *et al,* 2007). In addition attack by *F. verticillioides* is often associated with damaged host tissues from insects (Maiorano, *et al,* 2009; Munkvold and Hellmich, 2000) or mechanical harvesting (Munkvold, 2003).

Fusarium verticillioides isolated varied in colour shades and density of mycelia on PDA media indicating a possibility of different strains of the same species. Venturini, *et al,* (2013) and Coverelli, (2012) noted variation of *F. verticillioides* strains based on pathogenicity, ability to produce fumonisins and the type of fumonisins produced and identified 181 different strains. Similar studies were carried out by Alakonya, *et al,* (2008) who found out *F. verticillioides* isolates from different parts of western Kenya varied significantly based on the amount of fumonisins they produced.

3.4.4 Aflatoxin and fumonisins levels in maize grain samples

The mean aflatoxin levels detected in Meru, Kitui and Machakos were beyond the acceptable limits of 10ng/kg. Kitui had the highest levels of aflatoxin detected while Machakos had the lowest. Cooler temperatures in this agro-ecological zone could be one of the major reasons for this since the same trend was noted in Machakos where UM4 had the least aflatoxin contaminated maize. Lower Midland three in Meru had toxin levels below acceptable limits and this agrees with earlier findings of this study where LM3 had the least population of aflatoxigenic fungi. This anomaly could be as a result of numerous entry of foreign maize into the markets along the Meru Nairobi highway. The market samples collected from this area may not necessarily be from the agro-ecological zone. Cotty, *et al,* (2006) observed that aflatoxin levels in Ugandan maize samples were higher in more humid areas compared to the drier areas and similar results were obtained in maize samples from Nigeria (Atehnkeng, et

al, 2008). Drought and high temperatures after silking generally enhance the potential for aflatoxin contamination in maize (Payne, 1992).

Concerns over food safety and economic losses associated with aflatoxin contamination have led to development of strategies to control aflatoxin in maize (Cotty, *et al*, 2008, Hell, *et al*, 2008). Atoxigenic isolates of *Aspergillus* have been explored as possible sources of biological control designed to compete and outdo the aflatoxin producers (Atehnkeng, *et al*, 2008, Cotty, *et al*, 2006). In pea nuts for example Dorner and Horn, (2007) showed that inoculation of atoxigenic *A. flavus* to soil reduced aflatoxin in kernels to about 93%.

Fumonisins was detected in levels above the acceptable limits in Meru and though detected in Kitui and Machakos the levels were within the acceptable limits. The fumonisins levels did not match the *F. verticillioides* population since we had Kitui with the highest population. This could be attributed to differences in climatic conditions as well as the pathotype. Fumonisins are found more concentrated in the pericarp and germ of the grain than in the endosperm; so that removal of those outer parts by mechanical processes such as dehulling can significantly reduce the toxin in maize (Charmley and Prelusky, 1995; Sydenham, *et al*, 1995).

CHAPTER FOUR: EFFECTIVENESS OF NEAR INFRA RED (NIR) SORTING MACHINE IN SORTING AFLATOXIN AND FUMONISINS CONTAMINATED GRAINS OF MAIZE

4.1 Introduction

The ability to sort and recover clean maize from mycotoxin contaminated lot can save farmers a lot of economic losses such as what happened when 31,000 bags and 1,213 bags of maize were condemned in Mbeere and Bura irrigation Scheme in 2009 (Nyaga 2010). In Kenya efforts to manage aflatoxin emphasise on pre-contamination measures (Martins *et al,* 2014; Bandyopadhyay 2014; Bandyopadhyay and Cotty 2013; Atehnkeng *et al,* 2008) and very little is done post contamination. Measure of pre-contamination commonly used include resistant cultivars, biological control, for eaxample aflasafe (Bandyopadhyay and Cotty 2013; Atehnkeng *et al,* 2008), irrigation, good crop management (Milani 2013), insect control, drying and storage (Kangethe 2011, Aflastop 2013, Wagacha and Muthomi 2008). After contamination the options of reducing exposure are only left to condemnation, sorting or processing measures such as detoxification and dehaulling.

The last line of defence to eliminate grain contaminated with mycotoxins is post-harvest cleaning and sorting (Pearson *et al,* 2009). Sorting maize using visual grain characteristics does not reduce mycotoxin contamination effectively since mycotoxin contaminated grains may not necessarily show visible fungal discolouration. There has been technology for non-destructive measurement of single-kernel mycotoxin attributes such as fumonisins and aflatoxin levels (Pearson *et al,* 2013) protein content (Delwiche and Hruschka 2000; Rittiron, *et al* 2004), insect damage (Dowell, *et al,* 1998) hardness (Maghirang and Dowell, 2003) and colour (Dowell, 1998). Pasikatan and Dowell, (2004) reported segregating large samples by protein content using a commercial high-speed colour sorter with a combination of colour

and NIR filters. Procedures have been developed to analyze whole visible and near infrared spectra to select optimal filters to distinguish toxin levels using sorting machines (Pearson *et al*, 2004; Haff and Pearson, 2006; Pearson, *et al*, 2009).

Using this procedure, it was found that aflatoxin and fumonisin in yellow corn could be reduced by 81% and 85%, respectively, by optically sorting the corn using filters centered at 750 and 1200 nm (Pearson *et al*, 2004; Haff and Pearson, 2006). Only one pass through the sorter was required, and only about 5% of the corn was rejected to achieve these levels of mycotoxin reduction. The goal of this study was to calibrate and test this optical sorting technology using Kenyan maize and establish to what extent the technology could effectively reduce both fumonisins and aflatoxin.

4.2 Materials and methods

4.2.1 Description of the U.V and NIR sorting machine

The USDA-ARS LED Vis/NIR Seed Sorter System, also known as VolkSorter, is a light emitting diode (LED)-based instrument designed to sort individual grain kernels for various grain quality characteristics, such as colour, protein content, damage, kernel hardness (Figure 4.2). The instrument was designed to be low-cost, rapid, and able to measure light using the selected nine visible/NIR region spectral bands. These performance specifications are met by pulsing nine different LEDs at a rate of approximately 12 KHz such that a complete cycle through all nine LEDs could be performed at a rate of approximately 2 KHz. The reflectance from a kernel is measured as it drops off a 20 cm feeder chute inclined at 45° above the horizontal. At this angle and chute length, the kernels travel at a speed of approximately 1.5 m/s. Thus, 12 cycles of nine LED pulses are acquired in the time that a kernel travels a distance of approximately 0.75 mm (Figure 4.1). A microcontroller (ATmega328P Atmel Corp., San Jose, CA, USA) is used to control the timing of the LED pulses, digitize the

analog signal from the photodiode, process the signal, perform the classifications, and activate an air valve which diverts kernels according to the classification. Use of this microcontroller is expected to maintain a throughput of approximately 20 kernels / s.

The machine is programmed to detect images of the kernels that glow with a bright green-yellow fluorescence (BGYF) and Bright orange fluorescence (BOF) under the U.V (black) light. BGYF fluorescence is roughly indicative of aflatoxin levels while BOF is roughly indicative of fumonisin levels. After detecting this fluorescence release of a compressed gas from an external source is triggered and pushes these kernels to a reject container underneath. Good kernels fall off to another container.

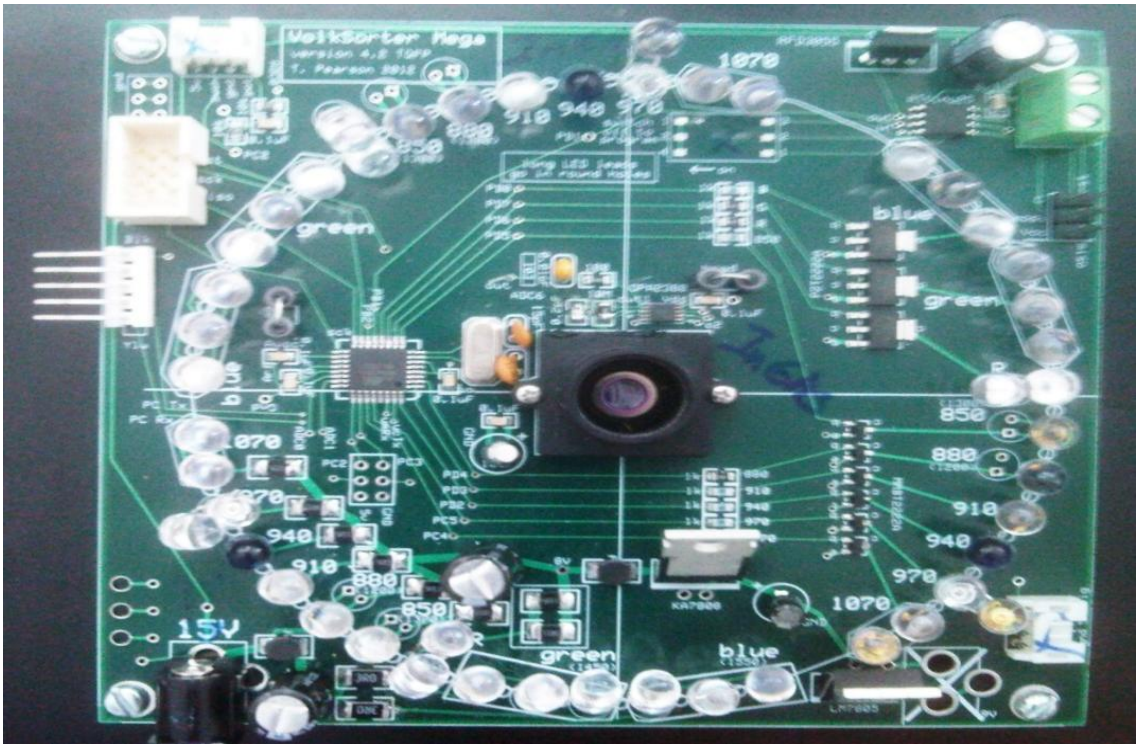


Figure 4.1: Circuit board with LEDs, microcontroller, transimpedance amplifier, and optics. Note that three sets of 12 LEDs provide even illumination about the kernel

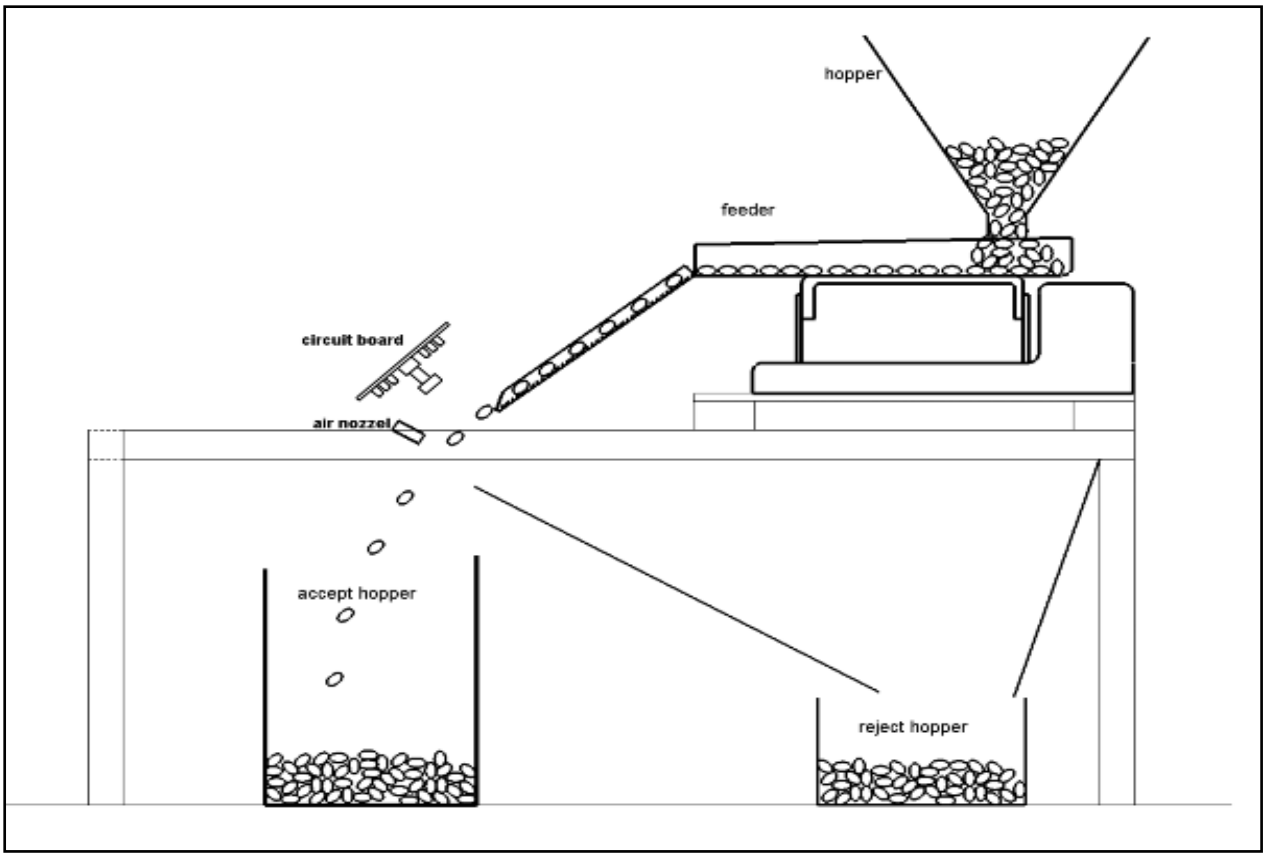


Figure 4.2: Schematic of the main sorter mechanical components (Pearson *et al,* 2013)

4.2.2 Performing Calibration

Calibration of the machine was done to enable sort maize based on certain desired grain characteristics. Since the machine can be used to sort grain for various qualities it was important to calibrate to ensure it sorts maize based on the level of fumonisin and aflatoxin contamination.

For sorting, all of the data was processed on a micro-controller mounted on the LED sensor circuit board (Pearson *et al,* 2004; Pearson *et al,* 2009). Data from 402 kernels comprising of 201 kernels with aflatoxin levels greater than the recommended 10 ppb that needs to be rejected and 201 kernels with levels of aflatoxin lower than 10 ppb that need to be accepted was used (Pearson *et al,* 2004; Pearson *et al,* 2009). In order to do this, the controller was fed with calibration data for the specific product and the characteristic that was desired to be sorted. The calibration file was created on a computer connected to the sorter using data from

201 kernels that you want to categorize as “accept” and 201 kernels that you want to categorize as “reject”. For fumonisins, calibration was also done using 402 kernels comprising of 201 kernels with fumonisins less than 1000ppb and 201 kernels with fumonisins above 1000 ppb. These were collected from field samples inoculated with the toxin prior to sorting and samples that were proved to be free of aflatoxin or with levels lower than the acceptable levels.

Due to communication speed limitation between the computer and controller, kernels were fed slowly so that data collected from these kernels could be transferred to the computer. After the data was collected, the computer analyzed the data and then created a calibration file that was uploaded to the controller so that high speed sorting could be done. The microcontroller was fed in a way to detect both mycotoxins within one run by running a combined calibration of 804 kernels free and infected with each mycotoxin as well as infected by both mycotoxins.

The air nozzle angle was adjusted by loosening the screw on top of the air nozzle support block. The air nozzle was rotated a few degrees and the support block screw tightened. If all kernels were not diverted at this angle, the air nozzle was rotated some more degrees. By trial and error method, the angle was adjusted so that the highest percentage of seeds was diverted. The support block screws were tightened and the air pressure increased until all seeds were diverted.

Individual mycotoxins test was done on all the 402 kernels after calibration to determine the efficiency of the calibration. A calibration validation was done by use field samples tested in chapter three. Categories of samples that showed highest and lowest levels of aflatoxin and fumonisins and those that are infected by aflatoxin and fumonisins as well as those free from both were used in the validation process.

4.2.3 Sorting of the grains by use of the U.V and NIR sorting machine

Three categories of maize were sorted that is those with high toxins above acceptable limits of 10ng/kg for aflatoxin and 1000ng/kg for fumonisins, those with medium toxins between 1ng/kg and 10ng/kg for aflatoxin and 1ng/kg and 1000ng/kg for fumonisins and those whose toxin levels were not detectable. Each of these had two subsets of aflatoxin and fumonisins except for low toxin category which had undetectable levels for both toxins. More emphasis was put on samples which had toxins beyond acceptable levels before sorting, because of their phytosanitary significance. About 30 grams per minute was fed on the sorter. The speed of feeding the maize kernels to the sorter was regulated by adjusting the vibratory feeder to the feeding rate. Adjusting the gap at the bottom of the feeder was also done to regulate the feeding rate. The pressure was set at 90 psi appropriate for sorting maize kernels. After sorting each sample both rejected and accepted kernels' weight was recorded and both kept in separate paper bags and labelled appropriately. The sorter was dusted off in readiness for the next sample. The reject and accept bundles was weighed and tested for aflatoxin and fumonisins using ELISA technique. The levels of toxin reduction in the accepted kernels were compared to that got before sorting.

4.2.4 Evaluation of the effectiveness of the U.V and NIR sorting machine in reducing fungal contamination

Mycological tests were conducted on the sorted maize to evaluate the effectiveness of the sorter to reduce fungal contamination. Both rejected and accepted kernels of each sorted sample were ground in fine floor and mycological analysis done as in the previous section. The number of fungal colonies of different species was identified and recorded.

4.2.5 Evaluation of the effectiveness of the U.V and NIR sorting machine in reducing aflatoxin and fumonisins in grain

Fumonisin and aflatoxin analysis was done for both accept and reject samples using competitive ELISA method described in chapter 3. The standard curves that were used to interpret the results are shown in figure 3.1 and 3.2 for aflatoxin and fumonisin respectively. The obtained data was compared with the toxin results before sorting and correlated with the rejection rates during sorting.

4.3 Results

4.3.1 Sorting of maize samples using NIR sorting machine.

The machine sorted maize into reject and accept fraction. Not all samples sorted had grains sorted as reject. Samples with high aflatoxin ranged from 22ng/kg to 2824ng/kg before sorting and this was reduced to slightly above the acceptable limits in the accept fraction. Some high aflatoxin samples had fumonisins although only one was beyond the acceptable limits before sorting. All samples which had fumonisins in the high aflatoxin category were cleaned up to zero fumonisins except one in the accept category. Two other samples which had no fumonisins tested positive after sorting in the accept fraction. This could be attributed to errors while sorting the two samples (Table 4.1).

Out of nine samples with high aflatoxin (above 10ng/kg) that were sorted six had aflatoxin levels reduced to acceptable limits in the accept stream. This includes a hundred percent reduction in five samples and over 67% reduction in the rest (Table 4.2) meaning the machine still reduced the toxin though not to the acceptable limits. Aflatoxin enrichment in the reject category occurred in five out of nine samples with high aflatoxin while in the same category fumonisins enrichment occurred in eight out of the nine samples and only one among the

eight had fumonisins levels within acceptable limits. In the reject category five samples had fumonisins and aflatoxin occurring together and beyond the acceptable limits.

High fumonisins samples had fumonisins as high as above 9000ng/kg before sorting and this was reduced to a highest of slightly less than 6000ng/kg on the accept fraction (Table 4.1). Six samples out of nine had fumonisins reduced to below accepted limits on the accept fraction with four of them having a hundred percent reduction. One of the three with fumonisins levels above accepted limits had significant toxin reduction (Table 4.1). Five samples in the reject stream had fumonisins levels enriched to over 6000ng/kg. Six out of the nine samples with high fumonisins had the levels reduced to within acceptable limits in the accept stream while six had fumonisins levels enriched beyond acceptable limits in the reject stream (Table 4.1). In this category there was no aflatoxin detected before sorting and after sorting aflatoxin was only detected in five samples of the reject fraction. There was a 60.8% average fumonisins reduction in the high fumonisins samples and this is despite an outlier sample that significantly reduced the levels (Table 4.2). Rejection rate of the sorted maize was not correlated to toxin levels before sorting but was related to toxin levels in the reject stream after sorting. Samples with high fumonisins or aflatoxin in the reject stream had recorded the highest percentage of maze grain sorted as reject (Table 4.2).

Table 4.1: Mycotoxin levels before and after sorting for samples that had the highest fumonisins and aflatoxin and their percentage grain sorted as reject for each sample.

	Mycotoxin level before sorting		% grain sorted as reject	Mycotoxin level after sorting			
				Accepted portion		Rejected portion	
Sample ID	Aflatoxin	Fumonisins		Aflatoxin	Fumonisins	Aflatoxin	Fumonisins
High Aflatoxin (>10ng/kg)							
M2-M3-L2	136	1,201	18	45	0	274	35,033
K9-M3-L1	2,824	693	4	60	0	308	17,526
K6-M3-L1A	23	319	2	0	0	29	21,093
M10-M1-L1A	370	370	4	0	1,429	34	1,833
K1-M2-L2A	818	272	1	12	0	0	0
K7-M2-L2	928	-	1	0	0	0	1,587
K10-M3-L1A	243	-	3	0	0	2	1
K5-M1-L1A	49	-	1	0	2,656	6	5,490
C4-M1-L1A	43	-	2	1	3,523	113	2,081
Mean	604	317	4	13	845	85	9,405
High fumonisins (> 1000ng/kg)							
M2-M1-L1A	-	9,589	6	-	2,184	1	9,592
M6-M2-L2	-	4,624	2	-	0	0	26,123
K3-M2-L1	-	3,938	2	-	0	0	0
M7-M1-L1	-	3,413	2	-	3,770	0	9,100
M8-M2-L1A	-	3,605	7	-	0	76	10
M4-M2-L1	-	2,723	5	-	427	16	2,111
C9-M2-L1A	-	1,225	6	-	13	23	22,038
K7-M3-L1A	-	1,025	3	-	0	0	0
M3-M3-L2	-	1,013	15	-	5,813	9	7,832
Mean	-	3,462	5	-	1,356	14	8,534

Table 4.2: Percentage mycotoxin reduction on the accepted fraction after sorting maize samples using the NIR machine

		% Reduction in mycotoxin in accepted fractions	
Sample ID	% of grain sorted as accept	Aflatoxin	Fumonisins
High Aflatoxin (>10ng/kg)			
M2-M3-L2	82	67	100
K9-M3-L1	96	98	100
K6-M3-L1A	98	100	100
M10-M1-L1A	96	100	-286
K1-M2-L2A	99	99	100
K7-M2-L2	99	100	0
K10-M3-L1A	97	100	0
K5-M1-L1A	99	100	0
C4-M1-L1A	98	97	0
Mean	96	98	13
High fumonisins (> 1000ng/kg)			
M2-M1-L1A	95	-	77
M6-M2-L2	98	-	100
K3-M2-L1	98	-	100
M7-M1-L1	98	-	11
M8-M2-L1A	93	-	100
M4-M2-L1	95	-	84
C9-M2-L1A	94	-	99
K7-M3-L1A	98	-	100
M3-M3-L2	85	-	-474
Mean	95	-	60.8

4.3.2 Mycotoxin and fungal contamination levels in rejected maize fractions

All the samples in the category of high aflatoxin and fumonisins had some percentage of rejected (Table 4.3). In the high aflatoxin category four samples out of nine had one of the aflatoxin producing *Aspergillus* while six had one of the fumonisins producing *Fusarium* (Table 4.3). Five samples in this category had aflatoxin above the acceptable limits while seven had fumonisins beyond acceptable limits. In high fumonisins category seven samples had fumonisins producing *Fusarium* while only three had aflatoxin producing *Aspergillus*. Only one sample had none of the fungi and any of the toxins in this category. One other

sample had *Fusarium proliferatum* but did not have the corresponding toxin. Six samples high fumonisins category had fumonisins level above the acceptable limits and only two had aflatoxin levels above the acceptable limits (Table 4.3). Out of 18 samples sorted in the two categories 14 had fumonisins and aflatoxin occurring together while 7 out of these 14 had both toxins occurring in levels beyond the acceptable limits (Table 4.3).

In the medium aflatoxin category nine samples were sorted and the percentage of the rejected seeds ranged from 0% to 24.8% (Table 4.4). Six out of nine samples had aflatoxin producing *Aspergillus* while only four had fumonisins producing *Fusarium*. Six samples in this category had aflatoxin levels beyond the acceptable limits meaning there was actually toxin enrichment in the reject fraction while only one had fumonisins levels beyond the acceptable limit (Table 4.4). One sample had aflatoxin and no aflatoxin producing *Aspergillus* was isolated while one other sample had fumonisins and no fumonisins producing *Fusarium* was isolated. The samples with medium fumonisins had percentage rejection range of 1.1 to 13.3 (Table 4.4). Aflatoxin producing *Aspergillus* was isolated from only four samples out of nine while *Fusarium* was isolated from six samples. This means there was fumonisins enrichment in the rejected fraction. Three samples had some fumonisins but no fumonisins producing *Fusarium* was isolated. Nine samples were sorted for the low toxin category and the machine did not reject anything for six samples. This means the machine was roughly rejecting along toxin levels. Only one sample had any of the aflatoxin producing *Aspergillus* while only two had any of the fumonisins producing *Fusarium*. Eight samples had no detectable aflatoxin while only two samples that had fumonisins (Table 4.5). The fact that that these two samples had very high fumonisins levels and were rejected means the machine was actually rejecting based on toxin levels. These two samples had a corresponding fumonisins producing *Fusarium* isolated.

Table 4.3: The popualtion (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in rejected portions of maize samples with high aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
Sample ID	% grain sorted as reject	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
High Aflatoxin (>10ng/kg)								
M2-M3-L2	18	1,000	0	0	11,000	0	274	35,033
K9-M3-L1	4	30,000	667	16,000	0	0	308	17,526
K6-M3-L1A	2	0	0	0	31,333	0	29	21,093
M10-M1-L1A	4	0	0	0	0	2,667	34	1,833
K1-M2-L2A	1	0	0	0	0	0	0	0
K7-M2-L2	1	0	333	0	333	0	0	1,587
K10-M3-L1A	3	0	0	0	0	0	2	1
K5-M1-L1A	1	1,000	0	0	12,333	0	6	5,490
C4-M1-L1A	2	0	0	0	2,000	0	113	2,080
Mean	4	3,556	111	1,778	6,333	296	85	9,405
High fumonisins (> 1000ng/kg)								
M2-M1-L1A	6	0	0	0	54,333	0	1	9,592
M6-M2-L2	2	0	0	0	10,000	0	0	26,123
K3-M2-L1	2	0	0	0	0	667	0	0
M7-M1-L1	2	0	0	0	35,333	0	0	9,100
M8-M2-L1A	7	2,667	0	0	0	0	76	10
M4-M2-L1	5	0	0	0	0	44,667	16	2,111
C9-M2-L1A	6	0	0	667	4,333	0	23	22,038
K7-M3-L1A	3	0	0	0	0	0	0	0
M3-M3-L2	15	1,667	12333	0	1,333	0	9	7,832
Mean	5	482	1370	74	11,704	5,037	14	8,534

Table 4.4: The population (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in rejected portions of maize samples with medium aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
Sample ID	% grain sorted as reject	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
Medium Aflatoxin (1-10 ng/kg)								
C2-M1-L5	2	3,000	0	0	0	0	33	0
C7-M2-L3	2	0	0	0	0	0	0	0
K3-M1-L3	10	0	1,000	1,333	0	0	13	0
C10-M2-L3	2	4,000	0	0	667	0	13	0
M8-M3-L2A	25	1,333	0	667	0	333	15	487
K7-M1-L2	0	0	0	0	0	0	0	0
K9-M3-L1	6	0	0	24,333	0	0	219	323
K7-M1-L1A	6	0	0	0	0	20,667	7	271
K10-M2-L1	5	1,000	0	1,333	11,000	0	14	3,664
Mean	8	467	0	5,267	2,200	4,200	51	949
Medium fumonisins (1- 1000 ng/kg)								
M3-M3-L3	13	2,000	0	0	0	0	47	1,113
M6-M1-L3	4	0	0	0	3,000	0	0	1,452
M9-M3-L3	1	0	0	0	0	0	0	1,007
M7-M1-L1	1	0	1,000	0	3,000	0	0	1,622
M5-M3-L3	1	0	0	0	0	4,000	0	1,231
K2-M1-L3	4	0	0	0	0	0	0	1,057
C10-M1-L1	10	333	3,000	1,000	1,333	0	22	875
C10-M1-L2	8	0	0	0	1,000	1,333	0	963
M1-M2-L1A	9	667	4,000	0	1,667	667	0	700
Mean	6	333	889	111	1,111	667	8	1,113

Table 4.5: The population (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in rejected portions of maize samples with low aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
sample ID	% grain sorted as reject	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
Low aflatoxin and fumonisins (< 1 ng/kg)								
K5-M2-L1A	0	0	0	0	0	0	0	0
K3-M2-L2	0	0	0	0	0	0	0	0
C4-M2-L1	1	0	1,333	0	0	0	0	0
C8-M2-L1	3	0	0	0	48,000	0	0	27,541
M5-M3-L0	5	0	0	0	0	13,667	1	1,482
K8-M2-L1	0	0	0	0	0	0	0	0
K10-M2-L4	0	0	0	0	0	0	0	0
K6-M2-L2	0	0	0	0	0	0	0	0
K8-M2-L2A	0	0	0	0	0	0	0	0
Mean	1	0	148	0	5,333	1,519	0	3,225

4.3.3 Mycotoxin and fungal contamination levels in accepted maize fractions

The percentage of maize sorted as accept in the high aflatoxin stream ranged from 0.6 to 18.2% (Table 4.7). Only three samples had any of the aflatoxigenic *Aspergillus* and only three had aflatoxin levels beyond the accepted limits (Table 4.7). The three samples however had a significant percentage reduction of aflatoxin at 66.9%, 97.9% and 98.5% from their initial toxin levels (Table 4.6). Six samples had at least one of the fumonisins producing *Fusarium* and only three had fumonisins (Table 4.7). The fact that there was consistent reduction of toxins on this category means that the machine was detecting and rejecting contaminated kernels on a dose dependent manner (Figure 4.5). The rejection is not necessarily related to the fungal contamination since there was no consistent reduction in the population before and after sorting (Figure 4.6).

The percentage maize sorted as accept in the high fumonisins category indicated that the contaminated fraction can be very little compared to the total maize sorted (Table 4.7). This means that the machine will save farmers and traders a lot of losses by getting rid of the small infected percentages. None of these samples had aflatoxin or the toxin producing *Aspergillus*. Four samples had some colonies of fumonisins producing *Fusarium* and five had at least some fumonisins and only three out of the five had fumonisins beyond acceptable limits. Although the mean fumonisins was higher than the acceptable limit, there was a significant reduction on the toxin levels before and after sorting on the accept fraction (Table 4.7).

As the other categories the number of samples sorted with medium aflatoxin and fumonisins were nine. The percentage of grain sorted as accept ranged from 75.2% to 100% (Table 4.8). Only four samples had any of the aflatoxigenic *Aspergillus* and only one of the nine samples had aflatoxin beyond acceptable limits while there were two more with some aflatoxin less than the acceptable limits (Table 4.8). There was one sample out of the nine that had some

fumonisins producing *Fusarium* and only one had fumonisins above the acceptable limits and two others had at least some fumonisins.

In the medium fumonisins category the range of percentage grain sorted as accept was 86.7% to 98.9% (Table 4.8). This category had two samples out of nine with some aflatoxin producing *Aspergillus* and none with any fumonisins producing *Fusarium*. All the samples had aflatoxin level below 3ng/kg which was below the acceptable limits (Table 4.8). Only two samples had fumonisins level above the acceptable limits and all samples had some fumonisins.

The low toxin category had only nine samples sorted all of which had zero levels of both toxins. The percentage grains sorted as accept ranged from 94.8% to 100% and six samples having 100% grain accepted (Table 4.9). Out of the nine samples only one had the aflatoxigenic *Aspergillus* and three had *Fusarium* (Table 4.9). There was only one sample out of the nine that had aflatoxin while none had fumonisins.

Table 4.6: The population (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in accepted portions of maize samples with high aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
sample ID	% grain sorted as accept	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisin
High Aflatoxin (>10 ng/kg)								
K9-M3-L3	96	2,000	0	0	0	23,000	45	0
M2-M3-L2	82	0	0	0	0	0	60	0
K6-M3-L1A	98	0	0	0	667	0	0	0
M10-M1-L1A	96	0	0	0	333	0	0	1,429
K1-M2-L2A	99	0	0	0	0	333	12	0
K7-M2-L2	99	0	333	333	333	0	0	0
K10-M3-L1A	97	0	1,000	0	0	0	0	0
K5-M1-L1A	99	0	0	0	0	0	0	2,656
C4-M1-L1A	98	0	0	0	2,667	333	1	3,523
Mean	96	222	148	37	444	2,630	13	845
High fumonisins (> 1000 ng/kg)								
M2-M1-L1A	95	0	0	0	333	0	0	2,184
M6-M2-L2	98	0	0	0	0	0	0	0
K3-M2-L1	98	0	0	0	0	0	0	0
M7-M1-L1	98	0	0	0	0	0	0	3,770
M8-M2-L1A	93	0	0	0	0	0	0	0
M4-M2-L1	95	0	0	0	0	2,000	0	427
C9-M2-L1A	94	0	0	0	0	0	0	13
K7-M3-L1A	98	0	0	0	0	333	0	0
M3-M3-L2	84	0	0	0	3,000	0	0	5,813
Mean	95	0	0	0	370	259	0	1,424

Table 4.7: The population (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in accepted portions of maize samples with medium aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
Sample ID	% grain sorted as accept	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
Medium Aflatoxin (1-10 ng/kg)								
C2-M1-L5	99	0	0	0	0	0	0	0
C7-M2-L3	98	0	0	0	0	0	0	0
K3-M1-L3	90	0	0	0	0	0	0	0
C10-M2-L3	98	333	0	0	0	0	3	0
M8-M3-L2A	75	0	0	0	0	0	0	4
K7-M1-L2	100	667	0	0	667	0	53	1,566
K9-M3-L1	94	0	667	0	0	0	0	0
K7-M1-L1A	94	0	0	0	0	0	1	11
K10-M2-L1	95	0	0	1,667	0	0	0	0
Mean	94	111	74	185	74	0	6	176
Medium fumonisins (1- 1000 ng/kg)								
M3-M3-L3	87	0	1,333	0	0	0	2	636
M6-M1-L3	96	0	333	0	0	0	1	975
M9-M3-L3	99	0	0	0	0	0	0	530
M7-M1-L1	99	0	0	0	0	0	0	1,145
M5-M3-L3	99	0	0	0	0	0	0	754
K2-M1-L3	96	0	0	0	0	0	0	580
C10-M1-L1	90	0	0	0	0	0	0	1,863
C10-M1-L2	92	0	0	0	0	0	2	175
M1-M2-L1A	91	0	0	0	0	0	1	20
Mean	94	0	185	0	0	0	1	742

Table 4.8: The population (cfu/g) of aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* species and their associated mycotoxins in accepted portions of maize samples with low aflatoxin and fumonisins levels before NIR sorting.

		Aspergillus			*Fusarium*		Mycotoxin	
sample ID	% grain sorted as accept	*A. flavus S*	*A. flavus L*	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
Low aflatoxin and fumonisins (< 1 ng/kg)								
K5-M2-L1A	100	0	0	0	0	0	0	0
K3-M2-L2	100	0	0	0	0	0	0	0
K8-M2-L1	100	0	0	0	0	0	0	0
K10-M2-L4	100	0	0	0	0	0	0	0
C4-M2-L1	99	0	0	0	333	0	21	0
K6-M2-L2	100	0	0	0	48,333	0	0	0
C8-M2-L1	97	0	0	0	0	0	0	0
M5-M3-L2	95	333	0	0	0	0	0	0
K8-M2-L2A	100	0	0	0	33,33	0	0	0
Mean	99	48	0	0	7,429	0	3	0

4.3.4 Correlation among rejection percentage, mycotoxin and fungal contamination levels

There was a significant correlation at 0.05 confidence level between aflatoxin and percentage grain sorted as reject in both in both accept and reject stream. The correlation between fumonisins and percentage grain sorted as reject in both streams was highly significant at 0.01 level (Table 4.9). This confirms the findings in the previous sections that the dose dependent sorting of maize was actually based on the two toxins. There was not significant correlation between percentages grains sorted as reject with any of the fungi analysed in both streams (Table 4.9). Aflatoxin was significantly correlated with *A. flavus* S strain and *A. parasiticus* in both accept and reject stream meaning that these were the most atoxigenic strains of *Aspergillus* (Table 4.9)

There was a positive relationship between the percentage of the rejected maize and the toxin levels in the entire samples sorted (Figure 4.3). A similar plot of total aflatoxin producing *Aspergillus* and fumonisins producing *Fusarium* indicated no relationship between *Fusarium* CFUs/g and percentage reject while there was a slight relationship between percentage reject and aflatoxin producing *Aspergillus* (Figure 4.4).

Table 4.9: Pearson correlation coefficient among percentage rejection, fungal contamination and mycotoxin levels in the accepted and rejected fraction after NIR sorting.

Reject stream								
	% rejection	*A. flavus* L	*A. flavus* S	*A. parasiticus*	*F. verticillioides*	*F. proliferatum*	Aflatoxin	Fumonisins
% rejection	1							
A. flavus L	-.097	1						
A. flavus S	.032	.879**	1					
A. parasiticus	.007	.884**	.999**	1				
F. verticillioides	.118	-.311	-.213	-.224	1			
F. proliferatum	.017	-.177	-.134	-.125	-.224	1		
Aflatoxin	.681*	.522	.701*	.684*	-.060	-.158	1	
Fumonisins	.802**	.120	.270	.246	.556	-.230	.723*	1
accept stream								
% rejection	1							
A. flavus L	-.097	1						
A. flavus S	.032	.879**	1					
A. parasiticus	.007	.884**	.999**	1				
F. verticillioides	.118	-.311	-.213	-.224	1			
F. proliferatum	.017	-.177	-.134	-.125	-.224	1		
Aflatoxin	.681*	.522	.701*	.684*	-.060	-.158	1	
Fumonisins	.802**	.120	.270	.246	.556	-.230	.723*	1

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

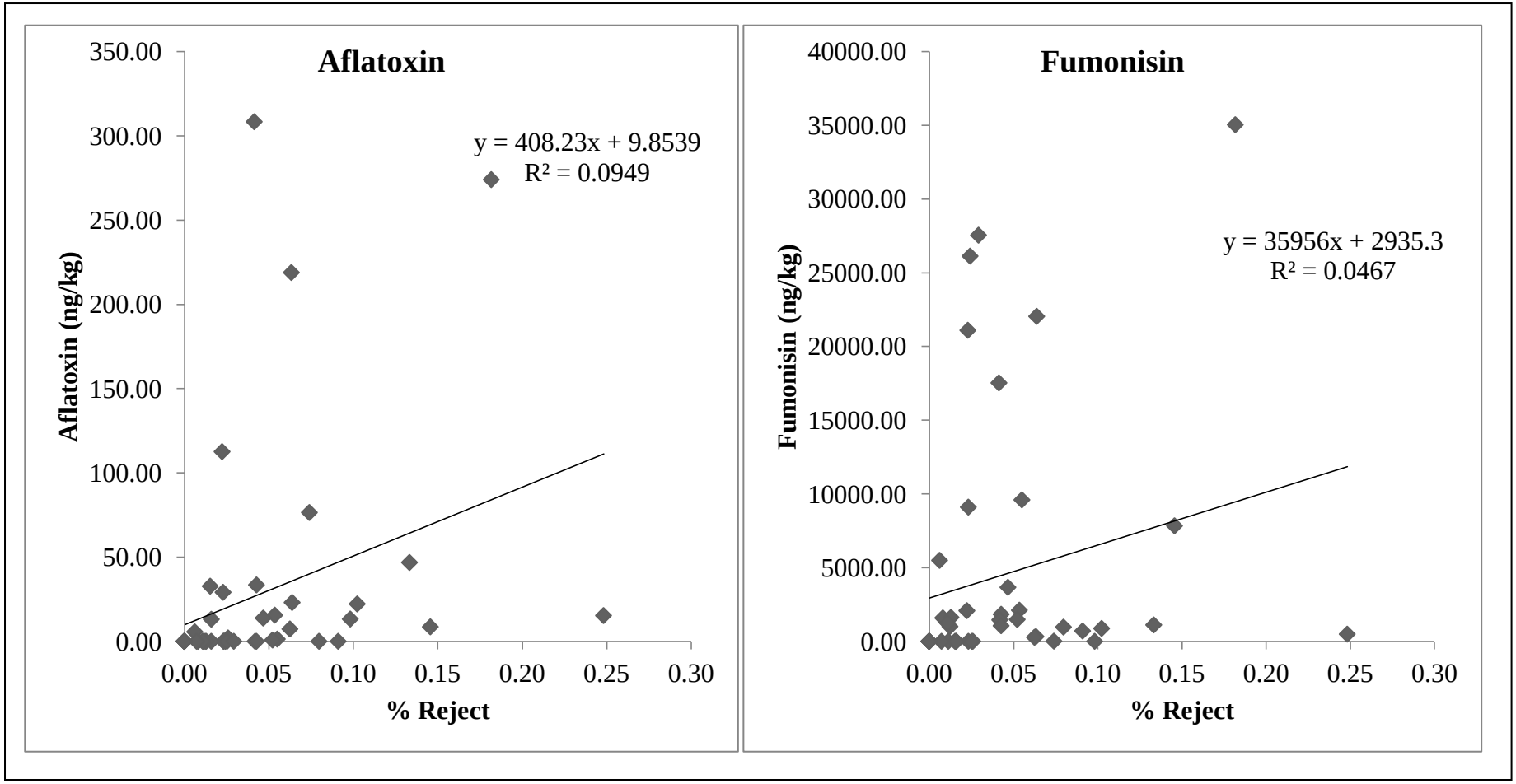


Figure 4.3: Relationship between mycotoxin levels and percentage of grain sorted as reject in the rejected maize grain fractions.

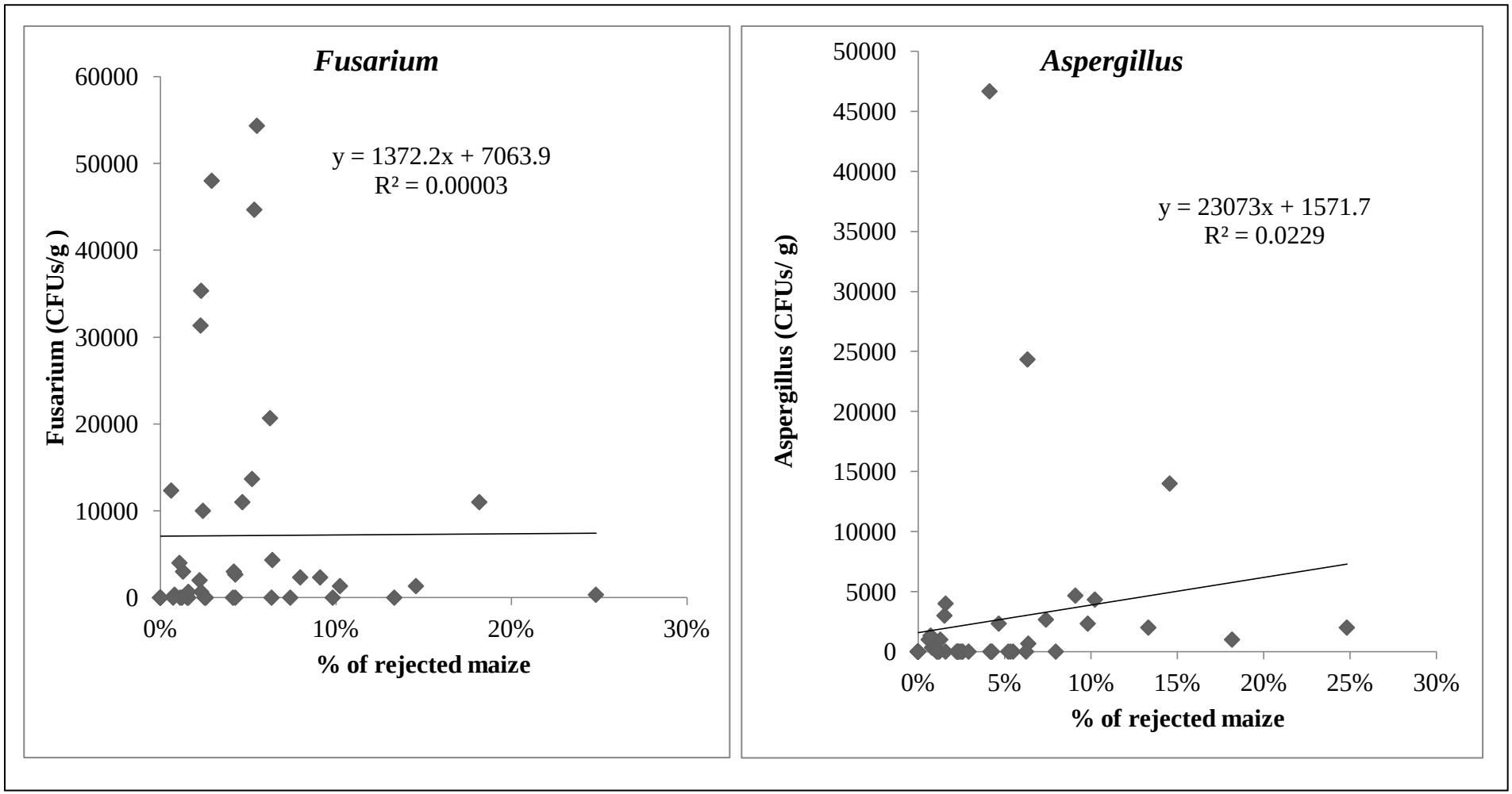


Figure 4.4: Relationship between total *Aspergiluus* and *Fusarium* levels and percentage of grain sorted as reject in the Rejected maize grain fractions.

There was a positive relationship between percentage grain sorted as accept and the fumonisins and aflatoxin levels (figure 4.5). While total *Fusarium* species did not seem to have any relationship with the percentage grain sorted as accept (Figure 4.6). *Aspergillus* however, was positively related with the percentage grain accepted.

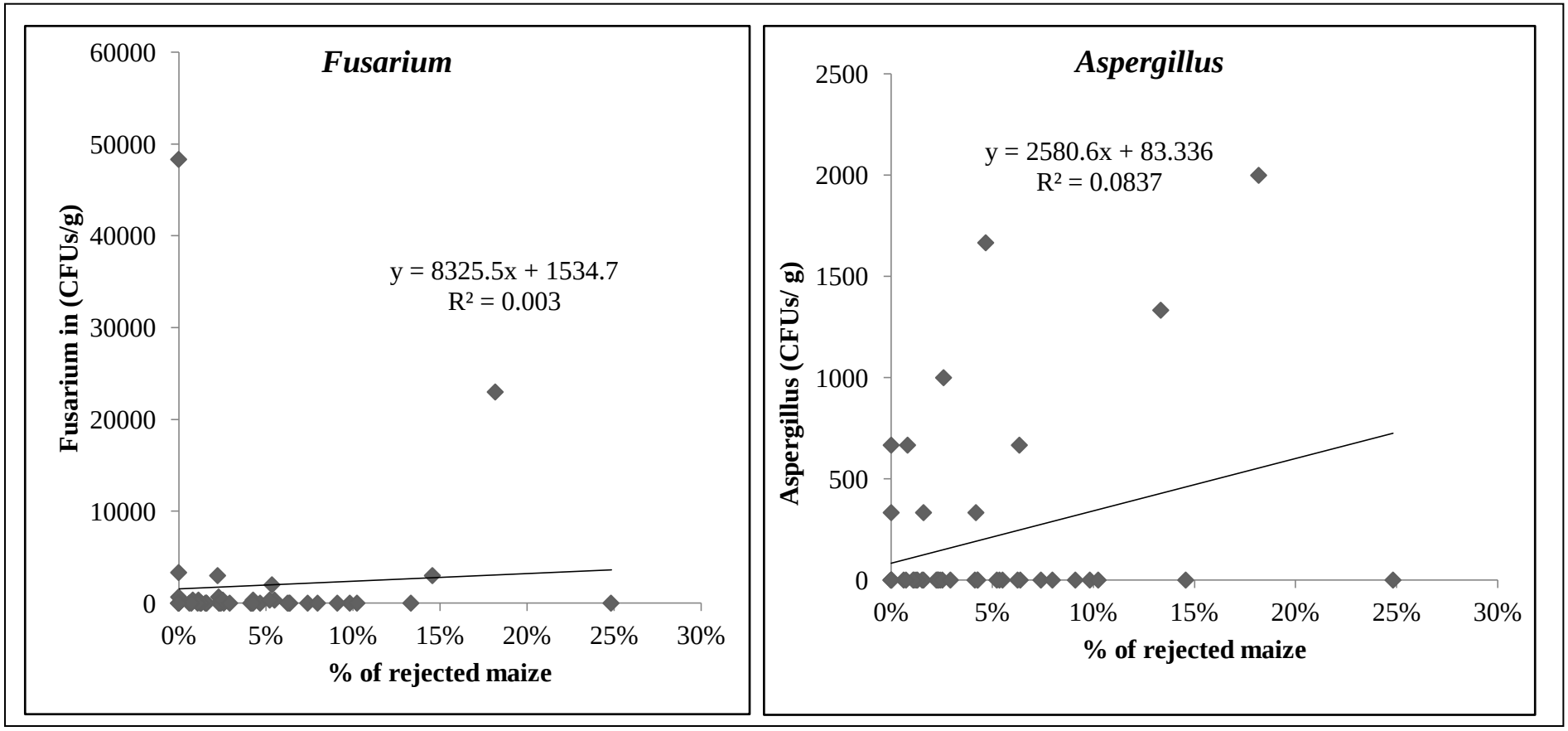


Figure 4.4: Relationship between total *Aspergillus* and *Fusarium* levels and percentage of grain sorted as reject in the accepted maize grain fractions.

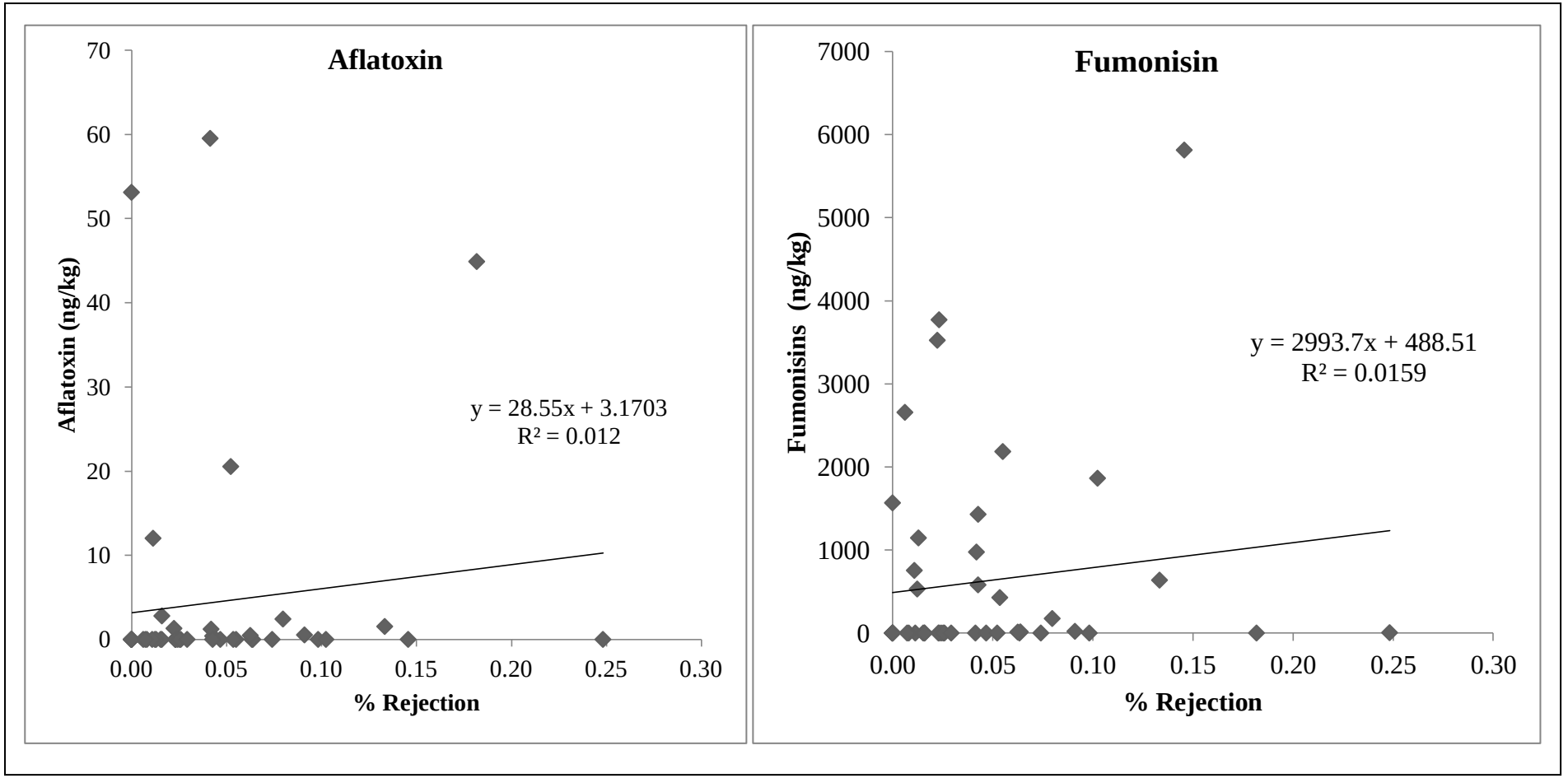


Figure 4.5: Relationship between mycotoxin levels and percentage of grain sorted as reject in the accepted maize grain fractions.

4.4 Discussion

4.4.1 Sorting of maize samples using NIR sorting machine.

The machine separated maize sorted maize samples into two categories of accepted and rejected portions. Generally there was reduction of aflatoxin level in the accepted portion and accumulation of aflatoxin in the reject portion. Toxin enrichment was higher with the fumonisins than the aflatoxin. In an earlier sorting experiment with commercially harvested yellow maize that was contaminated with 75ng/kg aflatoxin, the toxin was reduced to 20ng/kg (Pearson *et al,* 2004). In a similar study Pearson (2009) reported that the optical sorter missed some kernels that were destroyed by insects or mechanically destroyed during sorting. Peiris and Dowel (2011) while working on sorting of single kernel of wheat for *Fusarium* infection reported that the machine could successfully reject contaminated grains. For this to happen however the machine has to be calibrated appropriately.

The instrument was designed to be low-cost, rapid, and able to measure light using the selected nine visible/NIR region spectral bands (Pearson, *et al*, 2010, Dowell, 2009). Use of this machine is expected to maintain an average through put of 20 kernels per second (Pearson and Moore 2013). This means with the right modification and calibration the machine can be a major solution to grain mycotoxin contamination. Grain characteristics such as colour, size, and shape could be having some little effect on sorting (Pearson *et al*, 2010, Wicklow and Pearson (2009).

The NIR machine is promising as an accurate and low-cost instrument for sorting based on the toxin levels of grains tested. Sorting of these grain samples was done within a span of two weeks with no need for further calibration. Pearson (2010) recommends recalibration when the results of sorting one sample in two different times differ to ensure better results. Experience on LED illumination has however indicated that instruments based on this

technology can be highly stable for weeks Pearson 2010). The original calibration can also be saved and reused for recalibration that can be performed within minutes.

While conducting a similar study on deoxynivalenol (DON) and Nivalenol (NIV) of wheat Dowell *et al,* (2013) achieved a reduction up to 0.25 ng/kg NIV and 0.73 ng/kg DON in the healthy fractions, and 25.71 ng/kg NIV and 61.30 ng/kg DON. Pearson (2014) while working on protein content in wheat kernels across seven different samples the machine diverted 4.8% of high protein content portion. The NIR machine separates seeds based on specific quality attributes (Dowell et al, 2006).

4.4.2 Mycotoxin and fungal contamination levels in accepted and rejected maize fractions

The machine consistently enriched fumonisins and aflatoxin in the rejected portions of high aflatoxin samples. It did not consistently enrich *Fusarium* and *Aspergillus* contamination. Sorting is one novel component of a larger effort to reduce mycotoxins in food. The machine was instructed to reject grains with fumonisins and aflatoxin beyond the statutory levels. This explains why there was consistency in sorting for mycotoxin and inconsistency in sorting for fungal contamination. Pearson *et al,* (2009) successfully achieved toxin enrichment in the reject stream but reported no effect to fungal contamination. Major ways to reduce the impact of mycotoxins on the human population are to (Daniel *et al,* 2011) reduce mycotoxin contamination of primary commodities, (De Groote and Kimenju, 2012) detoxify foods contaminated with mycotoxins either through detection and removal of contaminated particles or by removal of the toxin itself from the food, and (De Groote *et al,* 2011) protect consumers from the toxic effects of mycotoxins once ingested (Wagacha and Muthomi 2008). To various degrees these three impact reduction strategies can be achieved through physical (sorting, milling), biological (detoxifying fermentation), or chemical (catalysis of

toxin degradation) means (Kabak *et al*, 2006, Kabak and Dobson 2009). Appropriate interventions can be economically efficient, as health economics show that both pre-harvest biological controls in maize and improved groundnut storage are cost-effective in relation to reducing aflatoxin induced hepatocellular carcinoma (Wu and Khlangwiset 2010).

Sorting as an intervention leverages the biological fact that mycotoxin distribution in maize is highly skewed. A classic (1980) paper examining in distribution of aflatoxin among kernels from 3 ears of maize visibly infested with *Aspergillus* fungus showed that only 32% (63/198) total kernels contained any detectable aflatoxin, and among toxin positive kernels the levels ranged from the level of detection of 100 up to 80,000ng/kg (Lee *et al*, 1980).

CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. General Discussion

Mycotoxin contamination of maize from the studied counties was mainly by aflatoxin. Fumonisin contamination was highest in Meru while Kitui and Machakos were below acceptable limits. The environmental conditions of Machakos and Kitui favour aflatoxin production while in Meru the conditions are more favourable for fumonisin production. In Eastern Kenya there are two planting seasons for maize. The short rains stars from October through to early January. Maize planted during this period is harvested in early March when the long rains are just about to start and therefore the harvested crop does not get enough time to dry up. This is one of the major factors contributing to high rate of aflatoxin contamination in Eastern Kenya. Other studies done in Eastern Kenya by Bii *et al*, (2012); Muthomi *et al*, (2009); Odhiambo *et al*, (2013); Okoth and Kola (2012); Wangari (2013) reported high incidences of aflatoxin contamination. Findings of this study are in line with previous studies by Alakonya *et al*, (2008) who reported near uniform contamination of maize by aflatoxin in Western Kenya. Farmers of Machakos, Kitui and Meru are well aware that maize gets infected with mould (Wangari 2013) that they commonly refer to as aflatoxin. It is known in these areas that after good harvest maize will suffer this mould attack, an expectation that is in line with findings of Hassan (1998). Aflatoxin producers are favoured by warm conditions; thus, global warming, particularly in the tropics, poses a potential problem (Okoth et al, 2012).

Fumonisins were found to contaminate maize from Meru beyond the acceptable level wile in Kitui and Machakos the contamination remained within the acceptable range. Of the three counties, Meru receives higher average rainfall and has a higher humidity levels. Fumonisin

production by *Fusarium* species is favoured by lower temperatures than aflatoxin (Fandohan *et al*, 2004; Milani 2013). Such environmental conditions explain the high fumonisins levels in Meru. When changes in the weather occur, mycotoxins will be affected. Mycotoxins are climate-dependent, plant and storage-associated problems, and are also affected by non-infectious factors, e.g. the bioavailability of micronutrients and insect damage (Milani *et al*, 2013). *Fusarium verticillioides* can colonize and produce fumonisins in the field and, if the maize grain is harvested at high moisture content, conducive to fungal growth and mycotoxin production (Velluti et al, 2001).

The predominant fungi in maize from the three counties were *Fusarium, Aspergillus* and *Penicillium.*The aflatoxigenic *Aspergillus* species commonly found were *A. flavus* L-strain, *A. flavus* S strain and *A. parasiticus*. *Aspergillus* forms sclerotia that allows for saprophytic survival for extended periods in the soil, maize residue and maize-cobs (Wagacha and Muthomi, 2008). The propagules in the soil and crop debris act as the primary source of contamination, infecting maturing *maize crops (Atehnkeng et al,* 2008; Odhiambo 2013). These results are in agreement with the findings of Muthomi *et al*, (2012) where higher *Aspergillus* spp. isolation frequencies were recorded in grain samples from the semi-arid eastern region than those from the humid North Rift regions. These results are in line with the findings of Okoth *et al*, (2012) and Muthomi *et al*, (2012) who reported that A. flavus was the most dominant *Aspergillus* spp. in Makueni and Nandi counties and also in Eastern region and North Rift region, respectively. Total aflatoxin levels were also found to be significantly correlated with the colony counts of *A. flavus* S-strain, with aflatoxin levels increasing with increase in colony counts of *A. flavus* S-strain. A higher per cent of *A. flavus* S-strain was isolated from samples that had higher aflatoxin levels. Therefore, samples with less than 10ng/kg aflatoxin had lower population of *A.flavus* S-strain compared to samples with more than 20 ng/kg aflatoxin content.

Fumonisin producing *Fusarium* specie that was predominant in all regions was *F.verticilloides*. Fumonisins are produced by several closely related species of *Fusarium* that can grow within maize tissues without causing visible symptoms of disease (Leslie *et al*, 2006; Bii *et al*, 2012). *Fusarium verticillioides* population was highly correlated with the levels of fumonisins contamination in each individual maize sample. *Fusarium* species can survive well on maize crop residues, which remain after the harvest (Bii *et al*, 2012, Fandohan *et al*, 2004). Bii *et al*, (2012) reported *F. verticillioides, F. oxysporium* and *F. proliferatum* were the most common Fumonisin producing fungi in Eastern Kenya.

The near infra-red single kernel sorting machine was effective in removing aflatoxin and fumonisin-contaminated kernels from the samples, with an accuracy of up to 97.8% for aflatoxins and 60.8% for fumonisins. While working with a similar machine to separate red wheat from white wheat Pearson *et al*, (2013) indicated that the NIR sorting accuracy was comparable to or better than the colour image-based sorter. For the sorting of red from white wheat, the NIR based instrument removed 98% of the white wheat while also removing 23.7% of the red wheat in two subsequent passes (Pearson 2013). The accepted fractions had statistically lower aflatoxin and fumonisin levels than rejected maize from the same bulk sample while rejecting only 0-15% of the sample. Market maize samples with toxin positive samples had reject rates of 0 to 25% and toxin negative samples having reject rates of 0-1%. These rejection rate data suggest the near infra-red single kernel sorting machine rejects kernels in a dose-dependent manner. There was a positive relationship between percentage accepted grain fractions and fumonisins and aflatoxin levels while total *Fusarium* species did not seem to have any relationship with the percentage accepted grain. Near Infra Red (NIR)

measurements taken with a limited number of spectral bands have been demonstrated as useful in the identification of various grain traits by Pasikatan and Dowell, (2004); Hansen et al, (2006); Delwiche, (2008); Shahin and Symons, (2009); Jaillais et al, (2012).

5.2. Conclusion

The level of aflatoxin varied considerably among the agroecological zones but the majority of agroecological zones had aflatoxin levels beyond the Kenya Bureau of Standards and European Union tolerance levels for total aflatoxins. All the three counties also had average aflatoxin beyond acceptable levels. Fumonisin levels were beyond the acceptable limits in Meru while Kitui and Machakos remained within the limits. The incidence of aflatoxin producing fungi was high including contamination by *A. flavus* S- and L-strain, and *A. parasiticus*. The high levels of aflatoxin producing fungi may exceed tolerance levels if safe pre- and post-harvest practices are not adhered to. Control strategies during maize production should be directed to the Lower Midland 3, Lower Midland 4, Upper Midland 3 and Upper Midland 4 agro-ecological zones. *Fusarium verticillioides, F. oxysporium* and *F. proliferatum* were predominantly found in all agroecological zones. There were variations in morphological features of *Fusarium verticillioides,* from different regions indicating possible variation in ability to produce fumonisins.

The NIR-based sorter performed better than a currently available manual or table sorting. Although the throughput of the NIR instrument was approximately 20 kernels per second, there is great potential in this technology. The NIR-based sorter was able to reject aflatoxin contaminated grain as instructed during calibration and it performs this task at a much higher throughput and potentially at a much lower cost. Additional testing will help determine the optimal configuration of LEDs for use in different applications, and the full utility of the

instrument will be determined by implementing larger-scale and longer-term tests. The throughput of the LED sorter was approximately 20 kernels/s compared with ~200 kernels/s for the image sorter used in other countries. The cost of the LED-based sorter is expected to be the parts for the construction of the LED-based sorter cost approximately 90,000 Kenya Shillings. The LED-based sorter will likely be most effectively employed to separate the mycotoxin contaminated grains of small scale millers.

5.3. Recommendations

1. Development of and improved NIR sorting machine with higher throughput and affordable to small scale millers in Kenya.
2. Further testing of Optical sorting technologies and their application in sorting different types of grains for various grain qualities.
3. Capacity building farmers and millers on effects of feeding on mycotoxin contaminated maize and possible mitigation measures to reduce exposure.
4. Farmers need training on postharvest factors that predispose their grains to aflatoxin and fumonisin contamination

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APPENDICES

Appendix 1: Helica aflatoxin and fumonisin testing protocol

SOP NO:	**SOP description**: HELICA ELISA KIT for *Aflatoxin* B1 detection
Effective date	**Version: 1**
Authors:	**Replaces Version:**
Approval:	**Approved date:**

1.0 Reagents Provided

1.1. 1 x pouch Antibody Coated microwell plate
1.2. Dilution wells
1.3. Aflatoxin Standards
1.4. Aflatoxin HRP-conjugate
1.5. Substrate reagents
1.6. Stop solutions

2.0 Extraction Procedure

2.1. Grind sufficient samples to particle sizes of fine instant coffee
2.2. Collection Container: Minimum 125ml capacity
2.3. Weighting Balance (Scout Pro) : 20g measuring capability (1 dp)
2.4. Graduated cylinder: 100 mL
2.5 Methanol: 70 mL Analytical grade per sample
2.6.Deionized water: 30 mL per sample
2.7. Filter Paper: Whatman #1 filter paper
2.8. Filter Funnel

3.0 Apparatus

3.1 Pipettor with tips: 100µl and 200µl
3.2 Timer
3.3 Wash bottle
3.4 Absorbent paper towels
3.5 Microplate reader with 450nm filter
3.6 Gloves
3.7 N95 face mask

4.0 Precautions and Safety Measures

4.1. Bring all reagents to room temperature (19º - 27ºC) before use.

4.2. Store reagents at 4^0C, and does not use beyond expiration date(s). Never freeze kit components.

4.3. Do not return unused reagents back into their original bottles. The assay procedure details volumes required.

4.4. Adhere to all time and temperature conditions stated in the procedure.

4.5. Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may affect the test results.

4.6. Never pipette reagents or samples by mouth.

4.7. Standards are flammable. Caution should be taken in the use and storage of these reagents.

4.8. The Stop Solution contains acid. Do not allow contact with skin or eyes. If exposed, flush with water.

4.9. Consider all materials, containers and devices that are exposed to samples or standards to be contaminated with aflatoxin. Wear protective gloves when using this kit.

4.10. Dispose of all materials, containers and devices in the biohazard bags after use.

5.0 Extraction Procedure

Note: The sample must be collected according to established sampling techniques

5.1. Prepare the Extraction Solution (70% Methanol) by adding 30 mL of deionized water to 70 mL of methanol (Analytical grade) for each sample to be tested.

5.2. Weigh out a 5g ground portion of the sample and add 25ml of the Extraction Solvent (70% methanol).

Note: The ratio of sample to extraction solvent is 1:5 (w/v).

5.3 Mix by shaking in an orbital shaker for 2 minutes.

5.4. Allow the particulate matter to settle and collect the filtrate into a 50 mL falcon tube to be tested. The sample is now ready for testing.

6.0 Assay Procedure

Note: A multi-channel pipettor should be utilized to perform the assay. If a single channel pipettor is used, it is recommended that no more than a total of 16 samples and standards (2 test strips) are run.

6.1. Bring all the reagents to room temperature before use.

6.2. Place one Dilution Well in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder.

* All samples and Standard should be conducted in duplicate across the plate wells.

6.3. Dispense 200 µL of the Conjugate into each Dilution Well.

6.4. Using a new pipette tip for each, add 100 µL of each Standard and Sample to appropriate Dilution Well containing Conjugate. Mix by priming pipettor at least 3 times.

Note: Operator must record the location of each Standard and Sample throughout test.

6.5. Using a new pipette tip for each, transfer 100 µL of contents from each Dilution Well to a corresponding Antibody Coated microtiter well. Incubate at room temperature for 15 minutes.

6.6. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with deionized water, then decanting the water into absorbent towels. Repeat wash for a total of 5 washes.

6.7. Tap the microwells (face down) on a layer of absorbent towels to remove residual water.

6.8. Measure the required volume of substrate reagent (1 ml/strip or 120 µl/well) and place in a separate container. Add 100 µL to each microwell. Incubate at room temperature for 5 minutes.

6.9. Measure the required volume of Stop Solution (1 ml/strip or 120 µl/well) and place in a separate container. Add 100µl in the same sequence and at the same pace as the Substrate was added.

6.10. Read the optical density (OD) of each microwell with a microtiter plate reader (Synergy HT Biotek; located in Lab 5) using a 450nm filter. Record the optical density (OD) of each microwell

7.0 Interpretation of Results

Send the plate layout and the O.D reading to supervisor (Post-Doc) and save the raw results in the shared CAAREA folder and copy of the same in lab book, including details of samples done. The O.D readings are then analyzed using the logit software.

Information contained on the label of a standard vial refers to the contents of that vial. However, the sample has been diluted at a 5:1 ratio with 70% methanol, and so the level of aflatoxin shown by the standard must be multiplied by 5 in order to indicate the ng of aflatoxin per gram of commodity (ppb) as follows:

Standard ng/mL	commodity (ppb)
0.0	0.0
0.2	1.0
0.5	2.5
1.0	5.0
2.0	10.0
4.0	20.0

The sample dilution results in a standard curve from 1ppb to 20 ppb. If a sample contains aflatoxin at greater than the highest standard, it should be diluted appropriately in 70% methanol and retested. The extra dilution step should be taken into consideration when expressing the final result.