# EFFECTIVENESS OF DENSITY SORTING IN REDUCING AFLATOXIN B1 AND FUMONISINS IN MAIZE GRAIN

**BY**

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN PLANT PATHOLOGY**

**DEPARTMENT OF PLANT SCIENCE AND CROP PROTECTION**

**FACULTY OF AGRICULTURE**

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**2020**

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This thesis is my original work and has not been submitted for the award of a degree in any other university.

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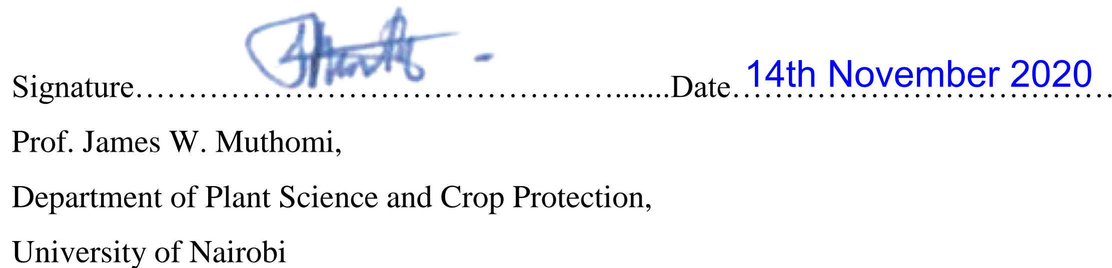

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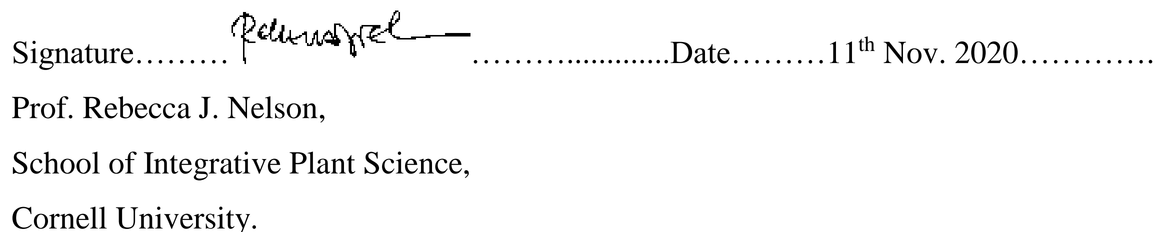

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# DEDICATION

This thesis is dedicated to:

My parents who have greatly supported me in this Msc. Course, my beloved husband for his great support and guidance in this work as well as my dear siblings for the encouragement through it all. God bless you all.

# ACKNOWLEDGEMENTS

I wish to acknowledge the following institutions for making this research a reality; Cornell University under the guidance of Prof. Rebecca Nelson for funding part of the work, International Livestock Research Institute (ILRI) Biosciences eastern and central Africa (BecA) for giving me the chance to use their facilities for part of this research and Kenya Plant Health Inspectorate Service (KEPHIS) Plant Quarantine and Biosecurity Station (PQBS) Muguga for allowing me to carry out part of the laboratory analysis in their facilities. My sincere thanks to my wonderful supervisors Prof. James Muthomi and Prof. Rebecca Nelson for their guidance, support and critique during this work. I wish to acknowledge Dr. Francis Ngure for his contribution and guidance through the work as well as Miss Grace Rozinah Achieng' for being of great help in laboratory work and data analysis.

My appreciation goes to my parents Mr. and Mrs. Ngure for their great support financially and morally in this course and educating me with a deep motivation to pursue higher levels of education. I greatly appreciate my dear husband Dr. Matunda Cedric for his moral and financial support through this work. I am thankful to my dear brother and sister for their support through this course. My sincere gratitude to the mycology staff and the entire staff of KEPHIS Muguga for making it easy for me to achieve my goals as part of this research. Last but not least, to the KEPHIS Managing director Dr. Esther Kimani, Mrs. Florence Munguti and Mr. George Ngundo of KEPHIS Muguga PQBS for allowing me to do part of the work in their facilities.

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# LIST OF ACRONYMS AND ABBREVIATIONS

BecA : Biosciences East and Central Africa

CFU : Colony forming units

Cm : Centimeters

$^0C$ : Degrees

DNA : Deoxyribonucleic Acid

ELISA : Enzyme Linked Immunosorbent Assay

ESI : Electron Spray Ionization

GC/MS : Gas Chromatography Mass Spectrometry

g : Grams

HPLC : High Pressure Liquid Chromatography

ILRI : International Livestock Research Institute

ISO : International Organization for Standardization

KCL : Potassium chloride

Kg : Kilograms

$KH_2PO_4$ : Potassium dihydrogen phosphate

$KNO_3$ : Potassium nitrate

LC : Liquid Chromatography

LCMS : Liquid Chromatography Mass Spectrometry

$MgSO_4.7H_2O$: Magnesium sulfate heptahydrate

ml : Milliliters

MS : Mass Spectrometry

NIR : Near Infra-red

Nm : Nanometer

OD : Optical density

PDA : Potato Dextrose Agar

ppb : Parts per billion

ppm : Parts per million

Psi : Pounds per square inch

QC : Quality Control

SNA : Synthetic Nutrient Agar

Spp. : Species

TFC : Total Fungal Count

USA : United States of America

µl : Microliters

µm : Micrometer

# GENERAL ABSTRACT

Mycotoxins such as aflatoxins and fumonisins are prevalent contaminants of maize, which is a major staple food in Kenya. *Aspergillus flavus, Aspergillus parasiticus* and *Fusarium verticilloides* are the major producers of carcinogenic aflatoxins and fumonisins respectively. Currently there are no effective methods of decontaminating grains and whole consignments have to be destroyed. This study sought to determine the effectiveness of density sorting in reducing aflatoxin B1, fumonisins, *Aspergillus* spp. and *Fusarium* spp. populations in maize grains.

Samples (n=206) were collected during the 2017 harvest crop from markets in eight counties in Western and Nyanza regions of Kenya. Sample numbers differed across counties ranging from 10-30 per county. All samples were analyzed for mycotoxins using an ELISA assay. Ten samples with more than 50 ppb of aflatoxin B1 and 4 ppm of fumonisins were weighed into 300 g with two replicates and sorted using a density sorter into heavy and light fractions constituting 65-75% and 25-35% of the original weight respectively. Bulk density was determined by filling a container of given weight and volume with kernels and the weights were determined for the heavy and light fractions. Kernel weight for each of the heavy and light fractions was determined by weighing 100 kernels. The effectiveness of density sorting in reducing mycotoxin-producing fungi was determined by isolation from 20 samples of the unsorted and 80 samples of the sorted heavy and light fractions. Finely ground maize flour was serially diluted and plated on PDA and Rose Bengal Modified Dichloran media. Single isolates of *Fusarium* spp. and *Aspergillus* spp. colonies were counted after five days and the number of colony forming units determined. Each fraction was analyzed for aflatoxin B1 and fumonisins by ELISA then reduction of the toxins in the heavy fractions determined in comparison to the unsorted samples.

The unsorted maize samples had up to 765±0 ppb aflatoxin B1 and 16±0 ppm fumonisins. The Majority (68%) of the samples showed a co-existence of the two toxins with aflatoxin B1 being more prevalent. Bulk density and kernel weights of the fractions were higher in the heavy fractions and lower in the light fractions. Mycotoxin-producing fungi isolated from unsorted and sorted samples were *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. Prevalence of *Aspergillus flavus* was higher in 93% of the samples followed by *Penicillium* spp. at 85% and *Fusarium verticilloides* at 67%. Population of *Aspergillus flavus* and *Fusarium verticilloides* significantly ($p<0.05$) varied among the unsorted, heavy and light fractions, with the light fractions exhibiting

highest populations and the unsorted grains exhibiting the lowest. There was no significant ($p>0.05$) reduction in the populations of *Aspergillus flavus* and *Fusarium verticilloides* in the heavy fractions. Density sorting did not effectively lower the fungal populations in the heavy fractions though the light fractions had evidently higher populations than the unsorted and the heavy fractions. Density sorting reduced fumonisins in 100% of the samples with an average of 71% reduction and aflatoxin B1 in 50% of the samples while the levels increased in the rest of the samples averaging the percentage change at -12.8%. Bulk density and aflatoxin B1 levels exhibited a strong correlation. Bulk density and fumonisins levels in light fractions had a strong correlation while in the heavy fractions the correlation was weak.

Density sorting can be used to reduce fumonisins and aflatoxin B1 effectively in maize grain but had no effect on mycotoxin-producing fungi. The density sorter machine should be improved for large scale use at a commercial level.

**Key words:** *Aspergillus*, Aflatoxins, Density sorting, Fumonisins, *Fusarium*, Mycotoxin

# CHAPTER ONE: INTRODUCTION

## 1.1 Background information

Maize is a staple food in Kenya. About 75% of the maize production is provided by small scale farmers out of the 90% produced by households in the rural areas (Kang'ethe, 2011) while 25% is produced by large scale farmers. Maize contributes to most households as a main food source and as an income earner especially in Western and Rift valley regions of Kenya. Statistics from Kenya Maize Development Program show that maize consumption levels are at 103 kg per person per year contributing to 35% of the dietary consumption per day (Kang'ethe, 2011). Maize production has been fluctuating in Kenya over the last 10 years, with the production levels being 24 million bags to 33 million bags per annum. The consumption levels are estimated at over 36 million bags. Maize farming is faced by major challenges at the pre-harvest stages which include pests and diseases such as Maize Lethal Necrosis Disease and Fall Army Worm whereas post-harvest challenges are majorly inclined to fungi producing mycotoxins (Hell and Mutegi, 2011). Mycotoxins pose threats to food safety, requiring great interventions as food safety enhances people's health and productivity.

Mycotoxins are secondary metabolites produced by many species of fungi during the pre-and post-harvest periods. High levels of contamination cause the maize to be very unsafe for animal and human consumption (Wagacha and Muthomi, 2008). Currently the issue of mycotoxins is of primary concern in the Kenyan maize value chain. The major fungi of interest are *Aspergillus* and *Fusarium* species causing aflatoxins and fumonisins respectively (Kang'ethe, 2011). Aflatoxins are classified as B1, B2, G1 and G2 with Aflatoxin B1 being the most prevalent and the most potent (Kang'ethe, 2011).

## 1.2 Problem statement

Many fungi attack maize in the field which include *Aspergillus, Fusarium, Alternaria, Cladosporioum* and *Cochlioboulus.* Some fungi start attacking maize in the field and in storage causing major threats to food safety as well as serious post-harvest losses (Mutiga *et al*., 2015). *Aspergillus* spp. and *Fusarium* spp. are major threats to maize causing *Aspergillus* and *Fusarium* ear rots respectively, which reduce grain quality. In storage they tend to cause discoloration and shriveling. In addition these fungi produce mycotoxins as their secondary metabolites (Wagacha

and Muthomi, 2008). *Aspergillus flavus* produces aflatoxin which is highly toxic and carcinogenic. Chronic exposure to aflatoxins is associated with liver cancer and currently Kenya ranks 76$^{th}$ globally for this type of cancer (Chai and Jamal, 2012). Acute exposure to aflatoxins in humans leads to hepatic failure and can kill very fast. In 2010, 2.3 million bags of maize were declared unsafe for human consumption by the Kenyan government due to high levels of aflatoxins (Mutegi, Cotty and Bandyopadhyay, 2018). Over 60% of maize produced in Eastern Kenya has unsafe levels of aflatoxins in some years (Mutegi, Cotty and Bandyopadhyay, 2018). Major aflatoxicosis outbreak was reported in Kenya in 2004 with minor outbreaks being reported in 2005, 2006 and 2009 (Muthomi *et al*., 2009).

*Fusarium verticilloides* is associated with production of fumonisins which are also carcinogenic. Chronic toxicity arising from exposure of small amounts in humans over a long period of time leads to esophageal cancer (Mutiga *et al*., 2015). They have also been associated with disruption of sphingolipid metabolism. Currently, Kenya ranks 8$^{th}$ globally in esophageal cancer cases (Chai and Jamal, 2012). Fumonisins have been associated with blind staggers a condition known as leukoencephalemalocia in animals (Cardwell and Henry 2004). Co-exposure of aflatoxins and fumonisins have been shown to increase human morbidity and stunted growth in children (Smith *et al*., 2012).*Aspergillus* and *Fusarium* require high moisture content to thrive especially in storage (Kang'ethe, 2011). Grain damage in the field facilitates fungal colonization which can be prevented by interventions at the pre-harvest and post-harvest stages. Interventions have been attempted in Kenya by improving storage conditions which are not always enhanced by farmers, so the two fungi tend to thrive (Kang'ethe, 2011). Visual sorting has been attempted by most farmers as they believe that visual characteristics can enable them distinguish between the clean and infected samples, which is not always possible. It has been documented that maize kernels that look very clean can be highly toxic with either aflatoxins or fumonisins or both (Mutiga *et al*., 2015). Maize samples that are traded in the local markets can be very toxic and are then sold to the millers who sell the flour for household consumption. The rate of maize flour consumption in Kenya is very high, which can lead to continuous exposure to the toxins present. Farmers as well as millers do not have a specific way of distinguishing between the clean and infected maize grains. In some cases, some of the grains tend to have a natural co-occurrence of both *Aspergillus* and *Fusarium*, being highly contaminated with fumonisins and aflatoxins increasing the negative health effects of consumption (Kimanya *et al*., 2015). Mycotoxins remain be a major threat to

human and animal health and their great impact has informed several approaches to lowering their levels to below the acceptable limit for consumption. The present study adds onto the knowledge gap in the area of sorting maize grains for fungal and mycotoxin contamination.

## 1.3 Justification

Food safety is vital in any society and ensuring that food consumed enhances nutritional status rather than causing harm. Food that is contaminated only leads to predisposing factors that can lead to chronic illnesses or even death. Continuous intake of aflatoxins in small quantities is suspected to have implications for human nutrition and can lead to chronic problems such as stunted growth and liver cancer. Fumonisins have been associated with esophageal cancer. Fumonisins also affect sphingolipid metabolism. The threats posed to human health by intake of aflatoxins and fumonisins in humans either in small quantities over time or in high quantities at once are very high. It is important to reduce this intake in economically viable ways so that everyone can have access to safe maize. Management of the fungi at the farming and storage levels has not guaranteed safe and clean maize.

Several approaches have been applied in trying to reduce the toxicity levels in maize to ensure that the maize is safe for consumption. Mitigation processes that have been tried at pre- and post-harvest periods include;

Harvesting practices are highly critical and most farmers have tried harvesting when the maize is fully mature and dry (Kang'ethe, 2011). The approach seeks to ensure that the maize has the right moisture content to reduce the prevalence of any fungal development. However, most farmers don't have the right information as to when they are supposed to harvest. Some of the approaches such as harvesting at the onset of rain causes most of the maize to accumulate moisture creating a favorable environment for the fungi producing mycotoxins to infect the maize during storage (Alakonya *et al*. 2009). Climatic conditions that tend to change over time really affect planting and harvesting seasons (Santiago *et al*. 2015).

Maize drying is one of the most critical steps in reducing moisture content in maize, which reduces probabilities of fungal growth and consequently, fumonisins and aflatoxin production (Hell and Mutegi, 2011). However, some farmers tend to harvest maize when natural sun drying is not highly effective and when moist maize is placed into storage, fungal development is very high. Other

regions have very high relative humidity combined with high temperatures. In such places maize is not fully dried and in storage, mycotoxin accumulation is very high. This approach has not been very effective in curbing the problem of mycotoxins.

Maize sorting has also been practiced by farmers whereby most farmers sort the maize during the harvesting process as they get rid of the rotten maize cobs (Kang'ethe, 2011). The cobs that are classified as highly rotten are shelled separately and their grains used to make animal feeds. The maize cobs that pass through the grading system as clean are shelled separately and considered fit for consumption or sale. The sorting approach has a limitation in that not all maize kernels that are highly contaminated are visibly moldy. Some of the highly contaminated samples appear clean when observed visually, so contaminated maize is still consumed in the Kenyan households. The maize grain lots tend to be heterogeneous for the toxins. Most of the toxins tend to be in a minority of the kernels thus mycotoxin contamination is highly skewed greatly reducing sorting effectiveness (Stasiewicz et al. 2017).

Mycotoxins, as a major threat to food safety and human health, have attracted significant interest. In this context, this project seeks to come up with a cost effective intervention method at a farmer's and miller's level to reduce toxicity in the maize set for consumption. The project seeks to test the effectiveness of density sorting in reducing toxicity caused by aflatoxin B1 and fumonisins in maize grain. The approach is because maize grain with high levels of toxins has a lower density compared to the maize grains with lower levels of toxins (Shi *et al*. 2014; Morales *et al*. 2019). The difference is attributed to the fact that the fungi in the maize grains feeds on the sugars and carbohydrates in the maize, which lead to the production of secondary metabolites which are the mycotoxins (Nelson, 2016; Lewis *et al*., 2005). This reduces the bulk density of the kernel thus sorting out a portion of the lighter maize grain from a larger sample may reduce toxicity of the maize.

### 1.4 Objectives

The broad objective of the study was to evaluate a low-cost sorter as a possible technology in reducing mycotoxin contamination in maize that is used for consumption at a household and consumer level through sorting thus enhancing food safety in Kenya.

The specific objectives were:

i. To determine the effectiveness of density sorting in reducing *Aspergillus* and *Fusarium* populations in maize grain.
ii. To determine the effectiveness of density sorting in reducing aflatoxin B1 and fumonisins levels in maize grain.

**1.5 Research hypotheses**

i. The heavy fractions of sorted maize kernels have lower populations of *Aspergillus* and *Fusarium* species.
ii. Kernels that have high levels of aflatoxin B1 or high levels of fumonisins are lighter in weight compared to the ones that are less toxic.

# CHAPTER TWO: LITERATURE REVIEW

## 2.1 Key maize production areas in Kenya and their risk of mycotoxin contamination

Maize in Kenya is produced in small and large farms with most of it being produced in small scale in the Rift valley and Western regions (Okoth *et al*., 2017). Maize production has been fluctuating over the years and in the year 2019, 3800 thousand tonnes were produced in Kenya with 95% of the maize being for subsistence needs (Okoth *et al*., 2017). Other maize production areas are Nyanza, Eastern and Central regions with varying altitudes and climatic conditions. Nyanza is characterized by a tropical humid climate, being hot and with high rainfall whereas Western region has a hot and humid climate (Climate and Agriculture, 2018). Rift valley region has a cool and humid climate, Eastern region is hot and dry while Central region is cool and wet.

Production of mycotoxins in maize grain is largely influenced by temperature and relative humidity among other agronomic factors and practices (Santiago *et al*., 2015). Aflatoxins prevalence is linked to drought conditions characterized by hot and dry climate whereas fumonisins production is prevalent in hot and humid conditions (Mutiga *et al*., 2015). The different maize production areas have varying risks of mycotoxin contamination. Eastern region has been reported to have a high prevalence of aflatoxins with several outbreaks in 2004, 2005 and 2006 (Mutegi, Cotty and Bandyopadhyay, 2018; Muthomi *et al*., 2010). Western and Nyanza regions have been reported to have a high prevalence of fumonisins and in some cases maize is contaminated with aflatoxins owing to fluctuating weather patterns (Mutiga *et al*., 2015; Njeru *et al*., 2019).

## 2.2 Prevalence of mycotoxins in Kenya

Mycotoxicosis has been reported in various places worldwide with major outbreaks having been reported in humans, poultry and dogs. Cases of serious mycotoxicosis have been reported in Kenya in the last decade whereby in 2004, 125 people died in Eastern and Central regions of Kenya. About 317 people were affected by the outbreak which was as a result of aflatoxicosis from contaminated maize (Muthomi *et al*., 2009). The 2004 outbreak was major followed by other smaller outbreaks in 2005 and 2006 leading to 53 deaths (Muthomi *et al*., 2009). In 2010, 2.3 million bags of maize were declared unsafe for human consumption by the Kenyan government (Mutegi, Cotty and Bandyopadhyay, 2018). Over 60% of maize produced in Eastern Kenya has unsafe levels of aflatoxins. Maize is a staple food in Kenya and it is easily distributed among

farmers within a small network. It is easily purchased from the shops and markets thus controlled trade is very difficult. In some instances, high rainfall may occur around the harvesting time increasing the moisture content of the kernels during storage (Staciewicz *et al*. 2017). This acts as a risk factor for mycotoxin accumulation. Poor storage conditions dominate most farmers' households' thus increasing mycotoxin accumulation in storage. The serious cases of outbreaks have led to regulations that now govern the acceptable legal limits of mycotoxins in maize for human consumption and animal feeds.

### 2.3 Mycotoxins occurring in maize

Mycotoxins are secondary metabolites that are produced by fungi and are poisonous causing major health effects on animals and humans. Mycotoxins are chemically diverse and several of them can be found in animal feeds and human food such as grains and seeds for planting. Mycotoxins are associated with moldy grains and feeds (Bennett and Klich, 2003), but the signs may not be visible despite high levels of mycotoxins being present. The mycotoxins that are frequently occurring in food and animal feed include deoxynivaleons, fumonisins (B1, B2 and B3), aflatoxins (B1, B2, G1 and G2), zearalenones and ochratoxins (A, B and C) (Bennett and Klich, 2003). Other mycotoxins include citrinins, patulins and ergot alkaloids. Aflatoxin B1 and B2 are produced by *Aspergillus flavus* and is found occurring in maize, peanuts, cotton seeds, animal feeds and many other commodities. *Aspergillus parasiticus* produces aflatoxin B1, B2, G1 and G2 in maize and peanuts. Fumonisins B1, B2 and B3 are produced by *Fusarium verticilloides* and are mostly found on maize (Bennett and Klich, 2003). Ochratoxins (specifically ochratoxin A; OTA) are produced by *Aspergillus ochraceus* and *Penicillium verrucosum* (Bennett and Klich, 2003). Ochratoxins can be found in wheat, barley and products such as chicken feeds and other animal feeds.

Zearalenones are produced mostly by *Fusarium graminearum, F.culmorum* and *F.crookwellense.* They are mostly found in maize and wheat as well other commodities. Deoxynivalenols are found to be produced by *F. graminearum, F. culmorum* and *F. crookwellense* and mostly found in wheat, maize and barley (Bennett and Klich, 2003). Trichothecenes are produced by *F.graminearum*, *F. sporotrichiodes*, *F. poae* and *F. equiseti* on many grains such as wheat, oats or maize (Bennett and Klich, 2003). Most of the economically important mycotoxins are produced by the *Aspergillus* and *Fusarium* fungi. The mycotoxins of *Aspergillus* and *Fusarium* have a high impact on human and animal health. Citrinin has been found to be produced by *Penicillium citrinum* mostly associated with rice (Bennett and Klich, 2003). However, citrinin has also been found to be produced by a

range of *Aspergillus* species. Ergot alkaloids are produced by *Claviceps purpurea* mostly affecting grains and cereals. Patulins are produced by *Penicillium expansum* and some species of *Aspergillus* mostly being found in moldy fruits such as apples and fruits as well as vegetables. Despite the wide range of mycotoxins occurring in human food and animal feeds, aflatoxins and fumonisins are more prevalent causing major impacts on human and animal health.

**2.4 Fungi producing aflatoxin B1 and fumonisins**

The fungi causing aflatoxin B1 are found in the *Aspergillus spp.* They are widely spread in nature causing high levels of aflatoxin in human and animal food. *A. flavus* and *A. parasiticus* are associated with production of aflatoxin in the field, during harvest, in storage and even in the processing. *Aspergillus flavus* is an ascomycete fungus that is found mainly in cotton seed, in maize and tree nuts whereas *Aspergillus parasiticus* is highly dominant in peanuts. *Aspergillus flavus* has conidia, mycelia or sclerotia and can grow at temperatures between 12 $^{0}$C and 48$^{0}$C (Kumar *et al*., 2017). Growth of the fungus almost ceases at 5-8$^{0}$C and it produces a high number of airborne propagules that are infective. The pathogen in grains infects the embryo reducing germinability as well as increasing the number of infected plants in the field. The prevalence of the fungus in maize can be associated with several factors such as drought related stress, stalk rot, severe leaf damage, insect injuries, high temperatures and excessive moisture in storage. The colonies of *Aspergillus flavus* are powdery and consisting of yellowish green spores mostly on the upper side of the culture whereas the lower side is characterized by reddish gold spores. Branching hyphae grow in a thread like manner and they are septate and hyaline. The fungus breaks down nutrients enabled by the degradative enzymes produced by the mycelia. Conidia producing thick mycelia produce conidiospores during reproduction. The conidiophores are rough and colorless whereas the philiades are both uniseriate and biseriate.

The fungus has L strains and S strains with the L strains being more aggressive and toxigenic. The fungus has its propagules in the soil and on decaying matter in form of mycelia or sclerotia (Wagacha and Muthomi, 2008). Sclerotia germinate to produce additional hyphae and conidia. These conidia act as primary inoculum for *Aspergillus flavus*. The propagules in the soil are dispersed by wind and insects such as stink bugs or lygus bugs. The conidia can land on and infect either grains or legumes. The spores enter the maize through the silks and thus infect the kernel. Conidiophores and conidia are produced in sclerotial surfaces. There is a secondary inoculum for *Aspergillus flavus*, which is conidia on leaf parts and leaves. *Aspergillus parasiticus* is also an

ascomycete that is associated with production of aflatoxin B1, B2, G1 and G2 mostly occurs on peanuts. Colonies of *Aspergillus parasiticus* incubated at $25^0$C attain a diameter of 2.5-3.5 cm within 7 days. The fungus produces a dense felt of green conidiophores. The conidial heads radiate and the conidiophores are hyaline and rough walled. The vesicles are sub-globose and the phialides are born directly on the vesicles which are usually hyaline to pale green. The conidia are rough-walled and globose with a yellow-green appearance. The conidial heads are radiate and uniseriate.

*Fusarium verticelloides,* formerly known as *Fusarium moniliforme*, produces fumonisins (Oren *et al*., 2003). The fungus infects the crops in the field, sometimes causes asymptomatic effects and sometimes manifesting symptoms with all plant parts having complete rotting. The fungus causes fusarium ear rot disease, which is of great economic importance before harvest. Routes of infection in maize are diverse with the first one being through airborne conidia that infect the silks and finally infecting the kernels. Systemic infections may also be due to crop residues in the soil. In storage, presence of Fusarium infections can be manifest in high levels of fumonisins production affecting the quality of maize kernels and reducing food safety. The colonies growing are usually pale whitish to cream, or bright colored in yellow, brownish, pink, reddish, violet or lilac shades (Oren *et al*., 2003). The fungus has aerial mycelia that is felty, cotton diffuse and at times absent. The conidiophores are branched, the phialides are often slender, tapering as the conidia form false slimy heads. The fungus has microconidia, which may occur either in chains or singly.

### 2.5 Diseases caused by *Aspergillus flavus* and *Fusarium verticilloides* in maize

*Aspergillus flavus* and *Aspergillus parasiticus* are the fungi that produce aflatoxin B1 and causes *Aspergillus* ear rot disease of maize in the field. Other *Aspergillus* species may be involved in the ear rot complex. These Aspergillus species include; *Aspergillus niger, Aspergillus glaucus, Aspergillus ochraceus, Aspergillus toxicarius, Aspergillus oryzae*. The disease is very important in maize and it is a precursor to development of aflatoxins in maize during storage. The disease is highly prevalent in maize in hot dry areas and especially those that are exposed to drought stress and other stresses such as nutrition deficiency. Feeding damage caused by insects also leads to injuries that lead to disease development. Infection by the *Aspergillus* species is favored by dry conditions and high temperatures of about $30^0$C during pollination and grain fill period (Masibonge *et al*., 2015). The spores overwinter in the soil and on decaying matter.

High temperatures in hot and dry weather favor the proliferation of the spores and are easily transmitted by wind and insects facilitating infection rates in the field. Spores land on the silks, germinate and grow down the silk as they colonize the surface of the developing kernels (Masibonge *et al*., 2015). During the stage of physiological maturity, the moisture content is about 32% and this facilitates the fungus to colonize the internal tissues of the kernels and this continues until the moisture content is about 15% (Masibonge *et al*., 2015). The fungi are a serious storage problem especially when maize is stored in high moisture. This leads to accumulation of aflatoxin leading to impacts on food safety for animal and human consumption. The disease causes symptoms of powdery masses of spores on and between kernels. The spores are typically olive-green and turn dark green to brown as the colonies mature (Masibonge *et al*., 2015). Infection usually occurs close to the ear tip though the kernels may not always show symptoms but they may appear shriveled, dull and discolored near the tip of the ear. The yellow tint of powdery masses is the distinguishing characteristic for the ear rot caused by *Aspergillus* species from the denim-blue ear rot caused by *Penicillium*.

The effects of the disease on maize are increased yield losses that may be at the pre-harvest period or the post-harvest period. Management of the disease is not easy as there are currently no commercial hybrids that have been released as being resistant to the disease. It can however be managed by using hybrids that can tolerate water stress or drought stress. Appropriate nitrogen fertilizer should be applied and the correct fertility of the field maintained. The use of hybrids that have reduced susceptibility to insect damage are preferable as this reduces disease development. Preventive management practices combined with good grain management systems at harvest reduces the impact of *Aspergillus* ear rot on grain quality and yield.

*Fusarium* ear rot disease is caused by *Fusarium verticilloides,* formerly known as *Fusarium moniliforme.* It is very common in maize. Other fungi species may form the ear rot complex including *Fusarium proliferatum* and *Fusarium subglutanins* (Oren *et al*., 2003). The fungus can infect the kernels and remain for long periods without causing symptoms. The fungi can survive in maize residues in the field and on other grasses. *Fusarium* ear rot can indicate presence of fumonisins. Infection can occur in the field under a wide range of environmental conditions. The disease is more severe when the weather is warm and dry. The fungus enters the ear through wounds from insect feeding. Airborne spores can also infect the kernels through silk channels. The fungus could also be seed borne or soil borne.

The symptoms of *Fusarium* ear rot include scattered kernels that are severely affected (Pearsons, 2008), mold that is white and can be pink or salmon colored. Infected kernels may also appear tan or brown and a starburst pattern may also be present on infected kernels, whereby they appear with light colored streaks radiating from the top of the kernels where silks were attached (Pearsons, 2008). Diseased crops have reduced grain quality and yield. Severe infections may cause the infected kernels to be completely consumed by the fungi, leaving lightweight husks that are cemented to the kernels by mycelia. Presence of the fungus promotes accumulation of fumonisins that are hazardous to human and animal health. The disease can be managed by use of hybrids that are resistant to the fungi causing *Fusarium* ear rot (Pearsons, 2008). Crop rotation is practiced at least for a year to reduce primary inoculum in the field. Residual crop debris is managed to reduce inoculum in the field. Integrated with cultural practices is the use of hybrids that are not highly susceptible to insect damage. Chemical control is yet to be proved as to whether it is effective enough to manage the disease.

**2.6 Factors that contribute to aflatoxin and fumonisins accumulation**

Maize that is stressed by various factors can be vulnerable to high levels of aflatoxins which have been found to be more prevalent in maize compared to wheat and other cereals. Certain factors predispose maize to accumulation of high levels of aflatoxin and particularly B1 which is highly potent. One of the factors is the use of cultivars that are highly susceptible to *Aspergillus*. However, cultivars have been found to have some resistance and breeding programs using DNA markers are underway to develop more resistant cultivars (Santiago *et al.,* 2015). Another factor is the use of already infected plant material whereby most farmers fail to use certified seeds. Environmental conditions, especially high temperatures, and moisture stress cause pathogen growth and development leading to accumulation of aflatoxins (Kumar *et al*., 2017). Maize ears tend to be highly colonized by *Aspergillus flavus/parasiticus* species in warm and dry conditions with temperatures ranging from 25-35$^{0}$C. The temperatures and moisture content during storage should be well regulated to curb growth of storage pathogens such as *Aspergillus* (Santiago *et al.,* 2015). Cultural practices such as lack of sanitation contribute to the infections and high levels of aflatoxin accumulation in storage. Debris left in the field can act as a source of inoculum for the next season leading to infections in the field and higher levels of aflatoxin accumulation during storage. High moisture content in the grains is also a predisposing factor. Maize harvested for storage should be dried before storage at least to a moisture content of 12-14% (Kumar *et al*., 2017). Most of the

*Aspergillus* species affect maize in the field and the effects are accelerated by damaged kernels, drought related stress and insect wounds caused by field pests or birds.

*Fusarium* infects the maize in the field due to its high preference for high moisture content and high humidity. It is a quiescent fungus and persists for a long time in storage, leading to fumonisins production and accumulation in maize. Prevailing climatic conditions during growth, insect damage and plant characteristics are determinants for *Fusarium verticilloides* and fumonisins accumulation in maize (Santiago *et al.,* 2015). Higher temperatures and drier weather during flowering enhance *Fusarium* ear rot infection. High temperatures during kernel maturation and more rainfall before harvest increase ear rot levels and fumonisins levels at harvest (Santiago *et al.,* 2015). Insect species such as corn borers have been associated with increased fumonisins contamination as they disperse the fungus and create routes of entry into the kernels (Santiago *et al.,* 2015). Kernel and ear characteristics such as kernel humidity, pericarp thickness and husk tightness may create a barrier or make it easier for fungal penetration into the kernel (Mutiga *et al*, 2018). Suitable agronomic as well as proper field sanitization practices lower risk of infection. Late planting and harvest, high plant density, accumulated crop residues in the field and lack of pest control are additional factors that enhance *Fusarium* ear rot and fumonisins contamination and accumulation. High moisture content above 14% in the kernels enhances fumonisins accumulation in storage.

### 2.7 Health effects of aflatoxin B1 and fumonisins on human and animals

Aflatoxins have major effects on humans and animals once ingested through food and animal feeds. Continued exposure of aflatoxins to humans and domestic animals in small amounts leads to chronic aflatoxicosis. In animals, aflatoxins have been found to be secreted in milk, increasing the exposure to humans through milk consumption (Kumar *et al*., 2017). Aflatoxin B1, being highly potent, has been found to have carcinogenic effects being associated with liver cancer in both humans and animals (Mutegi *et al*., 2013). High levels of aflatoxins have led to death due to aflatoxicosis (Muthomi *et al*., 2009). In 2004 people from the Eastern region of Kenya died due to high consumption of aflatoxins from highly contaminated maize. Long periods of exposure to aflatoxin in humans affects food digestion and absorption leading to stunted growth and in some cases, it affects the immune system (Cardwell and Henry, 2004). Exposure to aflatoxins in Kenya starts at an early age with infants being breastfed on contaminated milk (Mutegi *et al*., 2013). A high proportion of mothers ranging from 56.7% in Makueni county and 86.7% in Nandi county

tested positive for aflatoxin $M_1$ (Kang'ethe et al., 2017). Urine samples tested in children under the age of 2.5 years had high levels of aflatoxin in the two counties. Both counties had stunted and severely stunted children above the national averages of 26% and 11% respectively (Kang'ethe *et al*., 017). High doses fed to young ducklings leads to death whereas low doses fed to pigs, cows and sheep over a long period of time leads to development of body weakness, intestinal bleeding, reduced feeding, frequent abortions, reduced growth and nausea (Cardwell and Henry, 2004)..

Fumonisins ($B_1$ and $B_2$) are cancer promoting metabolites with a long chain hydrocarbon unit that plays a role in their toxicity (Zain, 2011). Chronic exposure of fumonisins in humans has been associated with throat cancer, esophageal cancer as well as disruption of sphingolipid metabolism (Smith *et al*., 2012). Fumonisins $B_1$ has been associated with reduced uptake of folate in different cell lines thus being implicated in neural tube defects in human babies (Zain, 2011). Fumonisins have been associated with "blind staggers" in equids a condition known as leukoencephalemalacia as well as porcine edema (Cardwell and Henry 2004). This mostly occurs in donkeys, horses and mules, which can die in extreme cases of fumonisins toxicity. In rats and mice, fumonisins have been linked to liver cancer and neural degeneration respectively (Afolabi *et al*., 2006)

## 2.8 Management of aflatoxins and fumonisins

Management of aflatoxins and fumonisins has been attempted using various approaches such as ensuring grains are dried to the recommended moisture content before storage, use of preservatives before storage, application of soil amendments, reducing grain damage, use of biocontrol agents, use of inbred lines and application of good agricultural practices. These approaches have been effective especially in reducing ear rot infection and accumulation of aflatoxins and fumonisins. However, in the event of ear rot infection in the field and accumulation of the toxins in storage, sorting becomes the last line of defense.

One of the most preferred methods is to dry the grain to at most 12% of moisture content (Bennett and Klich, 2003). Fungi cannot grow in effectively dried foods and for this reason, sufficient drying should be done on maize to reduce prevalence of *Aspergillus* and *Fusarium* in storage. The drying should be done immediately after harvest and as rapidly as feasible. Maintaining the proper moisture content can be difficult especially in the tropics due to quite high levels of humidity (Mutegi *et al*., 2013). Use of polythene bags to store the grain slows down the drying as the

moisture is retained in the bags. The best approach involves the correct drying procedure and proper storage to ensure that moisture levels reduce (Kang'ethe *et al*., 2017).

Farmers in Kenya apply different preservatives such as botanicals, ash, and synthetic pesticides to prevent storage losses (Mutiga *et al*., 2015). Mutiga et al. (2015) established that use of preservatives in storage was associated with a reduction in fumonisins contamination. The preservatives are intended to reduce insect damage during storage. Insects and other pests create microclimates in storage thus increasing humidity which favors production of the toxins (Kang'ethe *et al*., 2017).

The nature and fertility of soil helps in reducing crop infections (Kang'ethe *et al*., 2017). Soil that is able to hold moisture is better in lowering aflatoxin accumulation in maize making loamy soils better as they are able to reduce moisture stress compared to sandy soils (Kang'ethe *et al*., 2017). Application of fertilizers and manure enhances the crop's nutrition and ability to resist fungal infection. According to Hell and Mutegi. (2011) lime application, use of cereal crop residues and farm yard manure lowers the level of *Aspergillus flavus* contamination as well as aflatoxin accumulation by 50-90%. Mutiga et al. (2018) established that soils with high nitrogen amendment lead to high crop vigor, enhancing its defense against fungal pathogens and consequently lowers risk of toxin production and accumulation. Maize grown under low nitrogen soils suffers nitrogen stress and has been reported to have higher aflatoxin levels (Mutiga *et al*., 2018).

Reduced grain damage is also a good way of managing accumulation of aflatoxin and fumonisins (Bennett and Klich, 2003). Damaged grain is more prone to infection by the fungus thus leading to mycotoxin accumulation. It is vital to avoid grain damage before drying, during drying and in storage. This has been achieved by drying the maize before shelling it (Kang'ethe *et al*., 2017). Field insects and pests cause injuries on the grain leading to ease of infection of the grains by the fungi. They should be controlled in the field as well as in storage. High numbers of insects during storage lead to increased activities that lead to moisture increase and temperature facilitating fungal growth (Hussaini *et al*., 2012; Kang'ethe *et al*., 2017). Insects should be controlled in storage to reduce grain damage.

Use of biocontrol agents has also been embraced especially in the USA for competitive exclusion of the toxigenic *Aspergillus flavus*. *Bacillus subtilis*, *Lactobacillus* spp., *Pseudomonas* spp., *Ralstonia* spp., and *Burkholderia cepacia* have been used effectively to manage aflatoxins (Kumar

*et al*., 2017). *Trichoderma spp.* is being used to control aflatoxins in peanuts. This approach has been adopted even in Kenya with the recent commercialization of aflasafe by KALRO to enhance its use.

Use of inbred lines that are associated with resistance to aflatoxin has also been employed (Kumar *et al*., 2017). Resistant varieties will be the best solution in curbing *Fusarium* and aflatoxin infections. However, it takes quite some time before a variety is released. Breeding work has been emphasized much on *Fusarium* and aflatoxins related mycotoxins with lots of work ongoing to achieve varieties that are resistant. Current studies show that kernels with lower bulk density are more susceptible to *Fusarium* ear rot infection and fumonisins accumulation (Morales *et al*. 2019). Use of genomic technology has helped in identification of the genes that are responsible for aflatoxin production and accumulation thus their manipulation can alter the biosynthetic pathway. Use of transgenic maize modified with *Bacillus thurigiensis* has been advocated for to control wounding effects that enhance *Aspergillus* and *Fusarium* infections (Kumar *et al*., 2017). Use of good agricultural practices has been greatly advocated for and has been practiced by most farmers to reduce the occurrence of aflatoxins and fumonisins in maize. These practices include timely planting, which translates to the right time of harvest with the correct prevailing climatic conditions, thus enhancing proper drying of the maize (Alakonya *et al*., 2009). Providing adequate plant nutrition such as nitrogen is also paramount as the plant survives through much stress during the planting growth and development of the plant (Kumar *et al*., 2017; Mutiga *et al*., 2018). Controlling weeds is also important, reducing competition for nutrients with the maize in the field. Crop rotation is also advisable to reduce accumulation of primary inoculum over time. Removal of crop debris after harvesting is vital to reduce chances of the fungi's infective propagules accumulating in the soil.

### 2.9 The use of sorting in the management of aflatoxin B1 and fumonisins

Sorting has also been tried at a farmer's level where the farmers seek to reduce the number of infected maize kernels by sorting based on visual characteristics of the maize kernels (Bennett and Klich, 2003; Kang'ethe *et al*., 2017). This is mostly based on the broken grains, discoloration of the kernels, moldy appearance on the kernels and rottenness (Afolabi *et al*., 2006). Visual sorting can lower the level of aflatoxins by 40%-80% (Kang'ethe *et al*., 2017). However, it has been documented that visual characteristics do not always translate to the level of toxicity of the maize

for either fumonisins or aflatoxins (Nelson, 2016). This has not been reliable although it works in some cases especially in reducing fumonisins but not aflatoxins (Mutiga *et al*., 2014). Aflatoxins and fumonisins tend to affect relatively few seeds compared to the whole sample size that is in question (Nelson, 2016). This has led to the idea of sorting a larger sample of maize seeds aimed at removing those seeds that have high levels of toxins.Multispectral kernel sorting to reduce aflatoxins and fumonisins has been attempted; this achieved 83% mean reduction of each toxin in the maize (Stasiewicz *et al*., 2017). Further developments are underway to make the process easier and manageable by the consumers and millers. High speed sorting of wheat grains based on color or texture using a high speed image based sorting device has been attempted with an accuracy of 96% in separating red wheat from white wheat (Pearson, 2010). The high speed sorting technology also achieved a 92% of accuracy in separating brown flax from yellow flax and 93% accuracy in separating durum from barley (Pearson, 2010). Gravity sorting has also been used in the seed industry where samples are drawn three times from four discharge fractions of heavy, heavy or medium, medium and light in an attempt to sort corn seed for quality (Krueger *et al*., 2007). Quality of the heavy fractions was the highest with the light fractions exhibiting the lowest quality. Krueger *et al*. (2007) established that removal of the light fraction from commodity corn lots resulted in reduced broken corn and foreign material. Density sorting has been opted in other parts of the world, aiming at separating maize kernels based on the bulk density of each kernel. The method operates on the fact that the toxin producing fungi invade the oil rich germ thus reducing the bulk density of the kernels (Shi *et al*., 2014; Leslie and Logrieco, 2014). The method seeks to separate the heavy and light kernels with an assumption that heavy kernels have less aflatoxins and fumonisins while the lighter kernels have higher levels of aflatoxins and fumonisins. Density sorting has been attempted using a pair of filters A750 and A1200 (Leslie and Logrieco, 2014) as well as using a gravity table (Shi *et al*., 2014). The method uses the concept of free fatty acid content of the grain as the index of incipient grain deterioration. Density sorting of kernels with above 100 ppb of aflatoxin has been attempted using this method and 98% accuracy has been achieved (Leslie and Logrieco, 2014). High speed sorting using this method sorted out 5% of the grains that had above 10 ppb and this removed 82% of aflatoxin contamination. High speed sorting using a dual wavelength sorter for fumonisins achieved 88% reduction in fumonisins levels and this applied even for lowly contaminated samples (Leslie and Logrieco, 2014). More developments are underway to modify this sorting technique and increase its effectiveness.

### 2.10 Regulations of aflatoxins and fumonisins

Presence of mycotoxins in grains, other staple foods and animal feeds has a great impact on human and animal health. Most countries have set the maximum limits for presence of certain mycotoxins in human food and animal feed. The regulations have an impact on international trade as most countries, especially the developed ones, cannot allow an import of food or feeds that have mycotoxins beyond the specified levels. The regulations have been set based on the risk assessments that have been carried out on humans and animals for the specific mycotoxins. About 100 countries have set regulations to control levels of mycotoxins in animal and human food (Van Egmond, 2002). The European Union has set the limits at 5 ppb for aflatoxin B1 in human food. USA has set the limit for aflatoxin B1 in human food at 5 ppb except for milk where the limit is at 0.5 ppb (Van Egmond, 2002). Fumonisins have a set legal limit of 1 ppm. The set regulations for the animals are set at higher levels with the values varying from one type of feed to another and from one animal to another.

In East Africa, there are set regulations and standards that have been necessitated to harmonize the requirements that ensure food safety. The standardized requirements also aim at ensuring that trade barriers within the community are curbed thus enhancing regional trade. The set standards are based on the East African Committee standards that have representatives from the national partner states, private sectors and consumer organizations. The set standards cut across the grain quality and characteristics that enhance food safety for trade and consumption (Cardwell and Henry, 2004). Standards for aflatoxins and fumonisins are part of the set regulations for the East African region. Total aflatoxins are regulated at 10 ppb, aflatoxin B1 is regulated at 5 ppb whereas fumonisins are regulated at 1ppm. The set standards are regulated according to ISO 16050 (East African Standard, 2011). The set regulations in the East African region apply to each country that is a member and Kenya being one of them sets the standards at 10 ppb for total aflatoxins though it has been 20 ppb for a long time until recently with more problems and health hazards being associated with the aflatoxins, it has been reduced to 10 ppb (Mutegi, Cotty & Bandyopadhyay, 2018). Aflatoxin B1 being more potent is regulated at 5 ppb and fumonisins at 1ppm. The regulations also factor in other characteristics such as surface contaminants, rottenness levels, insect damage, foreign matter, discolorations and moisture content regulated at around 12% for stored grains (East African Standard, 2011). These regulations have achieved a certain level of grain quality and food safety in the region and in the country.

## 2.11 Detection and quantification of aflatoxin B1 and fumonisins

Different methods have been used for detection and quantification of aflatoxin and fumonisins apart from the visual examination of the maize kernels. Antibody-based assays and chromatography techniques have been used to detect the presence of these mycotoxins. DNA-based assays have been used to detect the presence of the fungi whether or not they are toxigenic. The methods that are currently used widely for aflatoxin detection are described below.

Enzyme Linked Immuno-Sorbent Assay (ELISA) is an antibody-based assay that is used to quantify mycotoxins. Sample preparation requires grinding of the maize and extraction process, which requires 70% methanol for aflatoxin B1 and 90% methanol for fumonisins. The technique is usually a competitive assay in which the mycotoxin of interest from a sample competes with a labeled mycotoxin for a limited number of specific antibody-binding sites (Wacoo *et al*., 2014). Test wells (96 per plate) are used, where each sample and standard is loaded into its own well in duplicate. Incubation steps are involved to allow for the enzymatic based reactions to take place in the presence of the conjugate and substrate (Hosseini *et al*., 2017). Since the assay is competitive, the presence of the toxin is usually measured by the absence of color and it is one of the more affordable methods for detecting mycotoxins. There are many commercially available kits that are used for the detection of different mycotoxins. In line with this, there are specific ELISA kits that have been developed to detect aflatoxin B1 and fumonisins as well as others that are specific for other mycotoxins. Every kit has six standards of known mycotoxin levels that are used in producing the linear regression curve to estimate toxin levels. ELISA is robust and sensitive as well as less laborious as it does not require pure isolations of the pathogen (Wacoo *et al*., 2014).

High Pressure Liquid Chromatography (HPLC) and Gas Chromatography/Mass Spectrometry (GC/MS) are two of the most widely used methods for mycotoxin detection and quantification in food safety laboratories. High pressure liquid chromatography separates a mixture of compounds on a stationary column using a carrier solvent such as methanol or acetonitrile, and the mycotoxins are detected and quantified in the sample as they pass through a specific detector (Wacoo *et al*., 2014). Gas chromatography separates a mixture of compounds on a stationary column using a carrier gas such as helium, and the mycotoxins are detected and quantified using a mass spectrometer. High pressure liquid chromatography and GC/MS require expensive equipment and technical support, but they offer a detection limit of less than 0.05 ppm for many mycotoxins.

Multiple mycotoxins can also be detected using Liquid Chromatography Mass Spectrometry (LCMS). Liquid chromatography coupled with mass spectrometry has been greatly used to detect multiple mycotoxins. Several conditions are used to achieve the right LCMS results and there seems not to be universally accepted conditions. The choice of ion source and mobile phase is dependent on the compounds used in the method. Electron Spray Ionization (ESI) is the most commonly used ion source (Wacoo *et al*., 2014). Liquid chromatography has many advantages in that it has high selectivity, it is highly sensitive and it can detect a wide range of mycotoxins. However, the challenge remains being able to develop conditions that are suitable for all mycotoxins (Rahmani *et al*. 2009). It is not easy to reduce the matrix effects thus the method development and validation must be well established. The method combines the physical separation of liquid chromatography with the mass analysis of mass spectrometry. Liquid chromatography in this case separates mixtures with multiple components as the mass spectrometry identifies the individual components based on the molecular specificity and detection sensitivity. The method makes use of an interface that transfers the separated components from the LC column to the MS ion source (Rahmani *et al*. 2009). The liquid chromatography system uses pressurized liquid as a mobile phase and the MS system uses a vacuum. The difference in operation system makes it difficult to transfer the eluate from the liquid chromatography column to the mass spectrometry source. The interface plays the role of transfer removing a portion of the mobile phase and preserving the chemical identity of the products.

# CHAPTER THREE

# EFFECTIVENESS OF DENSITY SORTING IN REDUCING *ASPERGILLUS* AND *FUSARIUM* INFECTION IN MAIZE GRAIN

## 3.1 Abstract

*Aspergillus flavus, Aspergillus parasiticus* and *Fusarium verticilloides* are the major mycotoxin producing fungi in maize grain. This study sought to determine the effectiveness of density sorting in reducing *Aspergillus* spp. and *Fusarium* spp. contamination in maize grain. Samples (n=206) from markets in eight counties in Western and Nyanza regions of Kenya were analyzed for mycotoxins using an ELISA assay. Samples with more than 50 ppb of aflatoxin B1 and 4 ppm of fumonisins were weighed into 300g and subjected to density sorting using a density sorter into heavy and light fractions constituting 65-75% and 25-35% of the original weight respectively. Sorted samples comprising of 80 heavy and light fractions and 20 unsorted samples were selected for isolation of fungi. Finely ground maize flour from the selected samples was serially diluted and plated on PDA and Rose Bengal Modified Dichloran media. Single isolates of *Fusarium* spp. and *Aspergillus* spp. colonies were counted after five days and the number of colony forming units determined. Density sorting concentrated *Aspergillus* spp. and *Fusarium* spp. in the light fractions with reduction in 35% of the samples for the two fungi when comparing the unsorted and heavy fractions. *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. were predominantly isolated with *Aspergillus flavus* being more prevalent than other fungi occurring in at least 93% of the samples followed by *Penicillium* spp. at 85% and *Fusarium verticilloides* at 67%. *Aspergillus* spp. populations in the light fractions increased by 64% and 37% when compared to the unsorted and heavy fractions respectively. *Fusarium* spp. populations in the light fractions increased by 134% and 73% when compared to the unsorted and heavy fractions respectively. At individual sample level, reduction of the fungal populations in the heavy fraction was not effective for both *Aspergillus flavus* and *Fusarium verticilloides*. However, in the case of *Aspergillus parasiticus* and *Fusarium graminearum,* heavy fractions had the least populations while the light fractions had the highest. Density sorting did not reduce the fungal populations effectively. Further research work is recommended in the area of lowering toxigenic fungi in maize grain.

**Key words:** *Aspergillus flavus*, density sorting, *Fusarium verticilloides,* grain quality

## 3.2 Introduction

Mycotoxin producing fungi, specifically *Aspergillus flavus, Aspergillus parasiticus* and *Fusarium verticilloides* have been reported in various maize samples and different agro-ecological zones in Kenya (Kang'ethe *et al*., 2017, Mutiga *et al*., 2015, Njeru *et al*., 2019, Okoth *et al*., 2012, Stasiewicz *et al*., 2017). Generally higher levels of *Aspergillus* have been reported compared to other fungi (Maxwell *et al*., 2017) though cases of higher *Fusarium* have been reported especially in Western region of Kenya (Mutiga *et al*, 2015, Alokanya *et al*, 2009, Njeru *et al*., 2019). Many factors have been found to have a great effect on possible contamination of maize by these toxigenic fungi especially at pre-harvest stage such as agronomic practices, prevailing climatic conditions and varietal differences (Mutiga *et al*., 2018, Santiago *et al.,* 2015). In most cases, infection of maize kernels occurs when the maize is still in the field and the levels could become worse at post-harvest when the maize is stored in conducive temperatures and high moisture content in the kernels.

Post-harvest handling has been emphasized by observing proper drying before storage to the recommended moisture content of 12% and also observing proper storage conditions (Kang'ethe *et al*., 2017). However many factors have still enhanced prevalence of these mycotoxigenic fungi especially *Aspergillus* whose levels tend to be higher in drought prone areas (Mutiga *et al*., 2015) or in cases where drought conditions (hot and dry) prevail during kernel filling and drying in the field. The fungus is found in crop and soil debris and infects the crops in the field producing aflatoxins in storage. *Fusarium* tends to thrive in warm temperatures and could infect the crop via systemic infections from the seed (Santiago *et al.,* 2015). It could also be found in crop debris and its effects at pre-harvest stages contribute to Fusarium ear rot disease which is of great economic importance. At post-harvest, its presence is mostly asymptomatic however it leads to accumulation of fumonisins (Morales *et al*., 2019).

At post-harvest, control of continued growth of these fungi requires strict observation of all set guidelines at harvest, shelling, drying and storage. Kang'ethe *et al*. (2017) reported use of polypropylene bags by farmers in Makueni and Nandi. These bags have been reported to increase moisture content enhancing fungal growth and subsequent production of mycotoxins (Mutegi *et al*., 2013). Kang'ethe *et al*. (2017) reported that at least 39% of the farmers in Nandi and Makueni areas used recommended storage methods to try and alleviate fungal growth. Further approaches to try and reduce further fungal growth in storage and at post-harvest have been attempted, but

density sorting has not been applied to lower fungal populations in maize. Pearson *et al.* (2004); Shi *et al*. (2014); Stasiewicz *et al*. (2017) in their sorting work aimed at reducing mycotoxins reported no effect of sorting on fungal contamination. This study therefore, sought to determine the effectiveness of density sorting in reducing the levels of *Aspergillus* and *Fusarium* in maize grain.

### 3.3 Materials and Methods

#### 3.3.1 Description of the average rainfall and temperatures where samples were collected

The area of study involved three regions: Rift valley, Western region and Nyanza region where aspects of the prevailing weather conditions and farmer practices were considered. The samples whose origin could be traced were collected in July 2017 having been stored for a period ranging from 1 to 6 months and the sampling targeted markets within the different counties. Maize samples were collected from eight counties, with the following sample size for each county, Homabay -27, Siaya – 39, Vihiga – 20, Busia- 31, Bomet- 18, Kericho- 11, Kisumu – 34 and Kakamega- 26 samples. The sample size was 206 collected from the markets each weighing 2 kg. Samples were collected from the traders based on the number of markets available in the county. The samples from the same market were collected based on the type of grain and origin. The more the markets and the different grain varieties, the larger the sample size. The sampling design was convenience sampling accommodating the locally available type of grain in each county. Below is a summary of the annual climatic conditions for different counties where the samples were collected.

**Table 3.1 Annual average temperature and rainfall of the counties where samples were collected**

| County | Average temperature | Annual rainfall |
|---|---|---|
| Homabay | 22.5$^0$C | 1226 mm |
| Siaya | 21.7$^0$C | 1572 mm |
| Kisumu | 22.9$^0$C | 1321 mm |
| Kakamega | 20.4$^0$C | 1971mm |
| Vihiga | 20.0$^0$C | 1921mm |
| Busia | 22.0$^0$C | 1691mm |
| Kericho | 18.1$^0$C | 1735mm |
| Bomet | 17.5$^0$C | 1247mm |

### 3.3.2 Density sorting of the selected maize samples

In this procedure, 300g of the samples selected for sorting were weighed twice from the bulk 2kg sample and subjected to sorting as collected from the field (without drying). The sorting was done by taking the sample and pouring onto the feeder of the drop sort machine which was connected to a blower (Figure 3.1) which blew out air from the machine thus reduced pressure on the side to which it was connected. This setting helped the lighter kernels to move to the side of reduced pressure. The machine has two collection points below it that are divided by a lever and this distinguishes between the heavy and the light fraction (Figure 3.1). The lighter fractions were collected on the side with reduced pressure while the heavier fractions that are presumed to fall straight were collected into the collection point directly below the feeder. The lighter fractions were weighed after every sort at different sorting regimes until 25% -35% of the 300g had been sorted into the light fraction. The same was repeated for a replicate sample and percentage grain lost from the heavy fraction calculated by taking the original weight subtracting the final weight of the heavy fraction , divided by the original weight multiplied by 100, [300-HF]/300X100.

After sorting, bulk density of the samples for the fractions was determined by filling a measuring cylinder of 100ml with grains from each fraction and the weight determined. The procedure was done twice for each fraction using a different subsample from the same fraction and the average calculated. Kernel weight was determined by taking 100 grains twice from the each fraction and weighing them then the average calculated. The sorted heavy and light fractions of the maize grain were weighed and ground into fine powder using a Romer miller machine. This was done by pouring the maize into the grain holder and after each sample had been ground, the machine was cleaned to ensure that there is no cross contamination between the samples.

**Figure 3.1: Density sorter machine**

### 3.3.3 Preparation of media

Culturing of *Aspergillus* spp. was done on Rose Bengal Media comprising 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 liter of distilled water modified with 8µl/L of commercially prepared Dichloran fungicide and streptomycin at the rate of 50ppm (Mutegi, 2009; Mutegi, 2012; Probst *et al*., 2011). The media

was autoclaved for 15 minutes at $121^0$C and 15 PSI pressure, allowed to cool to $40^0$C and then amended with Streptomycin and Dichloran fungicide which were filter sterilized through a 0.25µm filter into the cooled media.

Culturing of *Fusarium* spp. was done on Potato Dextrose Agar (PDA) media which was prepared by Suspending 39g in 1000 ml distilled water in a media bottle. The mixture was heated to boiling to dissolve the media completely and then sterilized by autoclaving at $121^0$C for 15 minutes. The media was allowed to cool to $40^0$C and then amended with streptomycin and tetracycline at the rate of 50ppm and 40ppm respectively to limit the growth of bacteria. The antibiotics were prepared by dissolving 0.05g of streptomycin and 0.04g of tetracycline separately in 5ml of distilled water then added to the cooled media using filter sterilization (a 0.25µm filter was used with a 5ml syringe). *Fusarium* type colonies were sub-cultured on PDA and synthetic nutrient agar (SNA) at the same time. SNA was prepared by weighing; $KNO_3$ 1.0 gm, $KH_2PO_4$ 1.0 gm, $MgSO_4.7H_2O$ 0.5 gm, KCL 0.5 gm, glucose 0.2 gm, sucrose 0.2 gm, agar 20g gm and $dH_2O$ 1000 ml (Singh *et al*., 1991). The flask was then sealed and all the media autoclaved for 15 minutes at 121°C and 15 PSI pressure. The media was then cooled to $40^0$C and then amended with streptomycin and tetracycline at the rate of 50ppm and 40ppm respectively to limit the growth of bacteria.

### 3.3.4 Determination of populations of *Aspergillus* and *Fusarium* in the maize samples

Isolation was done using dilution plate method where 10g of the maize flour previously grounded from the unsorted samples and sorted samples inclusive of the heavy and light fractions for aflatoxin B1 and fumonisins was suspended in 90ml to make a stock solution and serially diluted up to dilution $10^2$, after which 1 ml of the suspension was drawn from this dilution and placed in sterile petridishes in three replicates. Sterile molten media (25ml) was then added into the petridishes and swirled to mix the sample and the media then left to solidify. The plates were then incubated in an inverted position for 3-5 days with frequent observations at a frequency of 24hours from the second day at $30^0$C for samples plated on Rose Bengal media (Embaby et al., 2015). For samples plated on PDA, incubation was done for 3-5days with frequent observations at a frequency of 24hours from the second day at $26\pm2^0$C (Embaby et al., 2015). Fusarium type colonies were identified and sub-cultured on the cooled solidified media PDA and Synthetic Nutrient Agar (SNA) then incubated under near UV light for two weeks.

### 3.3.5 Identification of the isolated *Aspergillus* and *Fusarium* species

After incubation, the plates were examined and identification done based on morphological and microscopic characteristics. This helped in identifying *Aspergillus flavus, Aspergillus parasiticus, Fusarium verticilloides* and *Fusarium graminearum*. Mycelial growth was observed under a stereomicroscope for morphological characteristics and growth habits. Further identification was done under a compound microscope at X400 magnification. The fungal spores were collected from the plate using adhesive tape and placed on a microscopic slide and a drop of distilled water added. The slide was then placed under the compound microscope lenses for identification. Morphological characteristics such as colony color, colony reverse, colony growth and colony texture were observed. Microscopic characteristics such as vesicles, phialides, conidiophores, and conidia were observed. *Aspergillus flavus* was characterized by presence of dense-felt yellow-green conidiophores with conidial heads that are radiate, and becoming dark yellow-green whereas *Aspergillus parasiticus* was characterized by presence of radiate conidial heads with dense felt of dark green conidiophores. The conidiophores for *Aspergillus parasiticus* are rough-walled, spherical in shape and with short conidiophores whereas *Aspergillus flavus* has colorless and rough textured conidiophores. The numbers of single isolates were recorded as well as samples with more than one species of *Aspergillus*. Any other pathogen was also counted and recorded.

To identify *Fusarium verticilloides*, colonies growing fast and initially white changing to violet or lilac shades with age were the point of interest. Aerial mycelium that was felty, cottony and diffuse was observed. Pigmentation varying from grayish orange to violet grey, dark violet or magenta was considered. Further identification was done using the compound microscope where abundant septate oval to club shaped microconidia were observed. The microconidia were observed appearing in chains and others in small aggregates whereas macroconidia were difficult to find. Monophialides were occasionally present giving a rabbit appearance. The identification of *Fusarium* spp. was made using the criteria described in the *Fusarium* laboratory manual (Nelson, Toussoun and Marasas, 1983; Leslie., 2006). Each single isolate for both was counted and recorded on a datasheet then used to calculate the total Colony Forming Units (CFUs) as follows;

$$CFU/g = A*10^n/V$$

Where A = Number of colonies

$10^n$ = Level of dilution at which counting was carried out

V = Volume of inoculation

### 3.4 Data analysis

The data collected on fungal populations for the unsorted, heavy and light fractions was transformed using ($log_{10}X+1$) then subjected to analysis of variance to determine the differences in populations for the fractions. Means were separated using Tukey's least significance difference at a 5% level of significance.

### 3.5 Results

#### 3.5.1. Weights of the sorted fractions for determination of *Aspergillus* and *Fusarium* populations

Bulk density of the heavy fractions, kernel weights of the heavy and light fractions were significantly different ($p<0.05$). (Table 3.2) The heavy fractions exhibited higher bulk densities compared to the light fractions. The Kernel weights were higher in the heavy fractions compared to the light fractions. Weights of the heavy and light fractions, percentage grain lost as well as the bulk density of the light fractions were not significantly different ($p>0.05$).

Bulk densities and kernel weights of the heavy and light fractions were significantly different ($p<0.05$). (Table 3.3) Kernel weights and bulk densities in the heavy fractions were higher compared to the light fractions. Weights in the heavy fractions did not vary significantly. Weights in the light fractions as well as percentage grain lost for each sample did not vary significantly ($p>0.05$).

**Table 3. 2: Weights of the fractions after sorting for determination of *Aspergillus* populations**

| Sample ID | Weight (g) | | | Kernel weight (g/100ks) | | Bulk density (g/100ml) | |
|---|---|---|---|---|---|---|---|
| | Heavy | Light | % grain lost | Heavy | Light | Heavy | Light |
| BSA-057 | 206.5$^{a}$ | 91.5$^{a}$ | 31.2$^{a}$ | 31.5$^{bc}$ | 26.5$^{bc}$ | 79.0$^{ab}$ | 73.0$^{a}$ |
| SYA-070 | 219.5$^{a}$ | 77.5$^{a}$ | 26.8$^{a}$ | 34.0$^{ab}$ | 30.0$^{ab}$ | 78.0$^{ab}$ | 72.5$^{a}$ |
| SYA-072 | 201.0$^{a}$ | 91.0$^{a}$ | 33.0$^{a}$ | 27.5$^{c}$ | 24.0$^{c}$ | 76.5$^{ab}$ | 72.0$^{a}$ |
| SYA-073 | 212.0$^{a}$ | 85.5$^{a}$ | 29.3$^{a}$ | 28.0$^{c}$ | 24.5$^{c}$ | 76.5$^{ab}$ | 71.0$^{a}$ |
| KKA-128 | 218.5$^{a}$ | 78.5$^{a}$ | 27.2$^{a}$ | 38.0$^{a}$ | 32.0$^{a}$ | 76.0$^{ab}$ | 69.5$^{a}$ |
| KKA-130 | 214.0$^{a}$ | 85.5$^{a}$ | 28.7$^{a}$ | 38.0$^{a}$ | 30.8$^{ab}$ | 75.0$^{b}$ | 68.5$^{a}$ |
| KSM-142 | 205.5$^{a}$ | 91.0$^{a}$ | 31.5$^{a}$ | 34.0$^{ab}$ | 26.0$^{bc}$ | 75.5$^{ab}$ | 68.5$^{a}$ |
| KSM-158 | 201.5$^{a}$ | 96.5$^{a}$ | 32.8$^{a}$ | 32.5$^{abc}$ | 27.5$^{abc}$ | 75.0$^{b}$ | 70.5$^{a}$ |
| BMT-187 | 216.0$^{a}$ | 83.0$^{a}$ | 28.0$^{a}$ | 31.0$^{bc}$ | 26.5$^{bc}$ | 79.5$^{a}$ | 73.0$^{a}$ |
| BMT-190 | 203.5$^{a}$ | 89.5$^{a}$ | 32.2$^{a}$ | 34.5$^{ab}$ | 28.0$^{abc}$ | 78.5$^{ab}$ | 70.0$^{a}$ |
| Mean | 209.8 | 87.0 | 30.1 | 32.9 | 27.6 | 77.0 | 70.9 |
| LSD ($p \leq 0.05$) | 19.5 | 21.1 | 6.5 | 3.2 | 2.9 | 2.5 | 3.2 |
| CV % | 4.2 | 10.9 | 9.7 | 4.4 | 4.7 | 1.5 | 2 |
| P value | 0.343 | 0.613 | 0.343 | <.001 | 0.001 | 0.014 | 0.051 |

Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different ks-Kernels, BSA-Busia, SYA-Siaya, KKA-Kakamega, KSM-Kisumu, BMT-Bomet

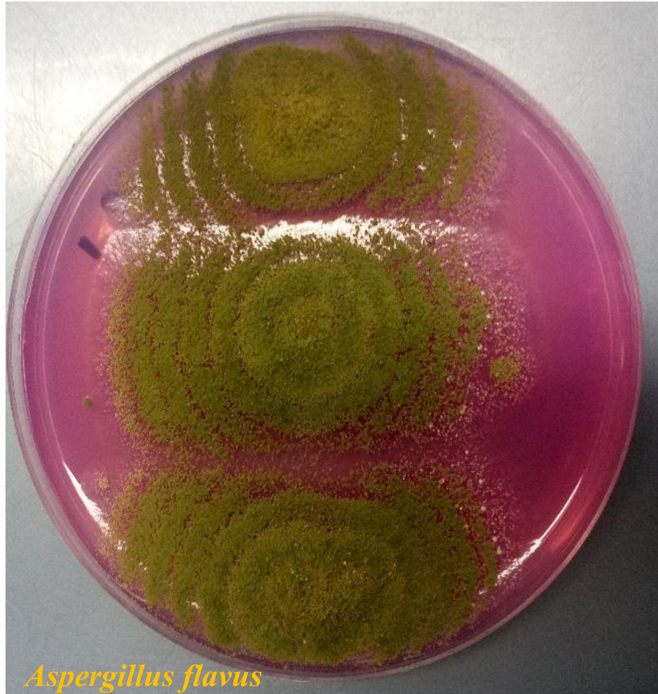
**Table 3. 3: Weights of the fractions after sorting for determination of *Fusarium* populations**

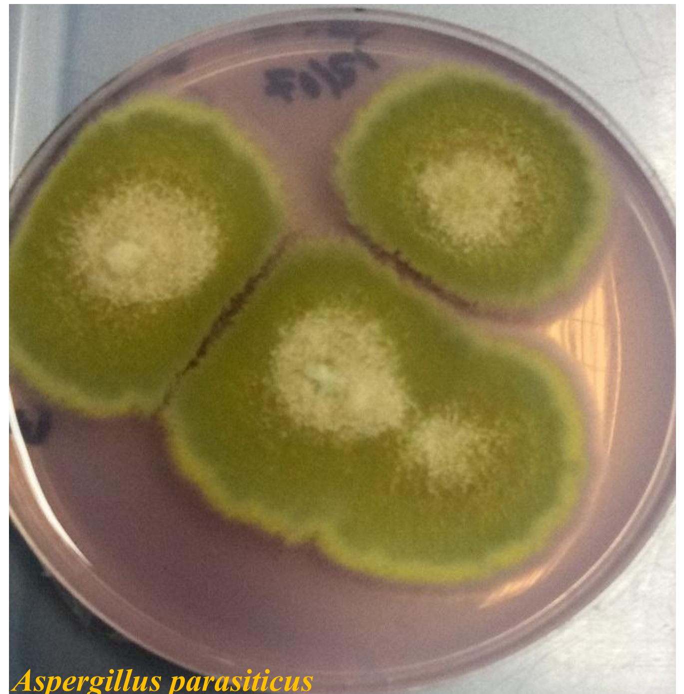
| Sample ID | Weight (g) | | | Kernel weight (g/100ks) | | Bulk density (g/100ml) | |
|---|---|---|---|---|---|---|---|
| | Heavy | Light | %grain lost | Heavy | Light | Heavy | Light |
| HBY-005 | 85.5$^{a}$ | 212.0$^{a}$ | 29.3$^{a}$ | 29.0$^{d}$ | 21.5$^{c}$ | 75.0$^{abc}$ | 66.5$^{ab}$ |
| HBY-011 | 94.5$^{a}$ | 203.5$^{a}$ | 32.2$^{a}$ | 36.0$^{b}$ | 27.5$^{b}$ | 74.5$^{bc}$ | 68.5$^{ab}$ |
| HBY-025 | 85.5$^{a}$ | 212.0$^{a}$ | 29.3$^{a}$ | 35.0$^{bc}$ | 27.5$^{b}$ | 76.0$^{abc}$ | 72.0$^{a}$ |
| BSA-033 | 85.0$^{a}$ | 211.5$^{a}$ | 29.5$^{a}$ | 42.5$^{a}$ | 36.0$^{a}$ | 73.5$^{cd}$ | 68.5$^{ab}$ |
| BSA-052 | 84.5$^{a}$ | 213.5$^{a}$ | 28.8$^{a}$ | 31.5$^{bcd}$ | 25.0$^{bc}$ | 75.5$^{abc}$ | 69.5$^{ab}$ |
| BSA-055 | 84.5$^{a}$ | 212.5$^{a}$ | 29.2$^{a}$ | 30.0$^{cd}$ | 25.0$^{bc}$ | 76.0$^{abc}$ | 69.0$^{ab}$ |
| SYA-075 | 80.0$^{a}$ | 218.0$^{a}$ | 27.3$^{a}$ | 43.0$^{a}$ | 33.5$^{a}$ | 76.5$^{ab}$ | 70.0$^{ab}$ |
| KKA-126 | 93.0$^{a}$ | 205.0$^{a}$ | 31.7$^{a}$ | 31.0$^{bcd}$ | 25.0$^{bc}$ | 74.0$^{bcd}$ | 64.5$^{b}$ |
| KSM-143 | 88.0$^{a}$ | 210.0$^{a}$ | 30.0$^{a}$ | 31.0$^{bcd}$ | 26.0$^{b}$ | 71.5$^{d}$ | 66.5$^{ab}$ |
| KSM-163 | 90.0$^{a}$ | 207.5$^{a}$ | 30.8$^{a}$ | 34.0$^{bcd}$ | 27.5$^{b}$ | 77.5$^{a}$ | 73.0$^{a}$ |
| Mean | 87.0 | 210.6 | 29.8 | 34.3 | 27.5 | 75.0 | 68.8 |
| LSD ($p \leq 0.05$) | 18.2 | 18.7 | 6.2 | 3.2 | 2.3 | 1.6 | 3.7 |
| CV% | 9.4 | 4 | 9.4 | 4.2 | 3.7 | 0.9 | 2.4 |
| P value | 0.791 | 0.835 | 0.835 | <.001 | <.001 | <.001 | 0.011 |

Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different ks-kernels,HBY-Homabay, BSA- Busia, SYA-Siaya, KKA-Kakamega, KSM-Kisumu

### 3.5.2 Population of *Aspergillus* and *Fusarium* species isolated from sorted and unsorted samples

*Aspergillus flavus*, and total *Aspergillus* sp. (Figure 3.2) populations in the samples of unsorted, sorted heavy and light fractions were significantly different ($p<0.05$). (Table 3.4). *Aspergillus parasiticus* populations in the samples of unsorted and sorted light fractions were significantly different ($p<0.05$). *Aspergillus flavus* populations were significantly different ($p<0.05$) when compared among the fractions with the light fractions exhibiting the highest count and the unsorted samples exhibiting the lowest count. *Aspergillus parasiticus* populations exhibited significant differences ($p<0.05$) among the heavy, light and unsorted samples. The highest numbers were in the light fractions and the lowest in the heavy fractions. *Aspergillus* sp. populations had significant variation ($p<0.05$) in the three sets of samples with the highest populations being in the light fractions and the least in the unsorted samples. Mycotoxigenic fungi varied across samples from different regions with Siaya samples exhibiting higher populations of *Aspergillus flavus* while those from Homabay had lower counts. *Aspergillus parasiticus* populations in the samples of the heavy fractions were not significantly different ($p>0.05$).*Fusarium verticilloides, Fusarium graminearum* (Figure 3.3) and their total populations were significantly different ($p<0.05$) in the samples of the unsorted, sorted heavy and light fractions. (Table 3.5).Comparing across the fractions, significant differences ($p<0.05$) were observed in *Fusarium* sp. populations. *Fusarium verticilloides* exhibited highest count in the light fractions while the unsorted samples had the least. *Fusarium graminearum* populations were highest in the light fractions and least in the heavy fractions. In totality *Fusarium* sp. populations were significantly different ($p<0.05$) with the light fractions had the highest numbers while the unsorted samples had the least. Unsorted samples and the heavy fractions did not show significant variation ($p>0.05$). *Penicillium* sp. were also isolated in high numbers with the populations in all the samples of unsorted, heavy and light fractions being significantly different ($p<0.05$). (Table 3.6). Samples with high *Aspergillus flavus* populations exhibited high *Penicillium* populations. *Penicillium* sp. populations were not significantly different ($p>0.05$) when compared across the fractions but the light fractions had the highest while the unsorted samples had the least.





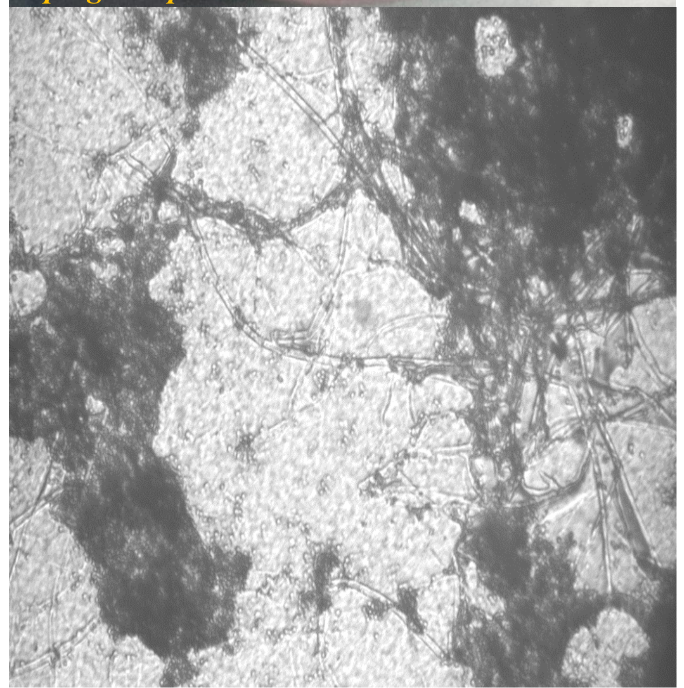
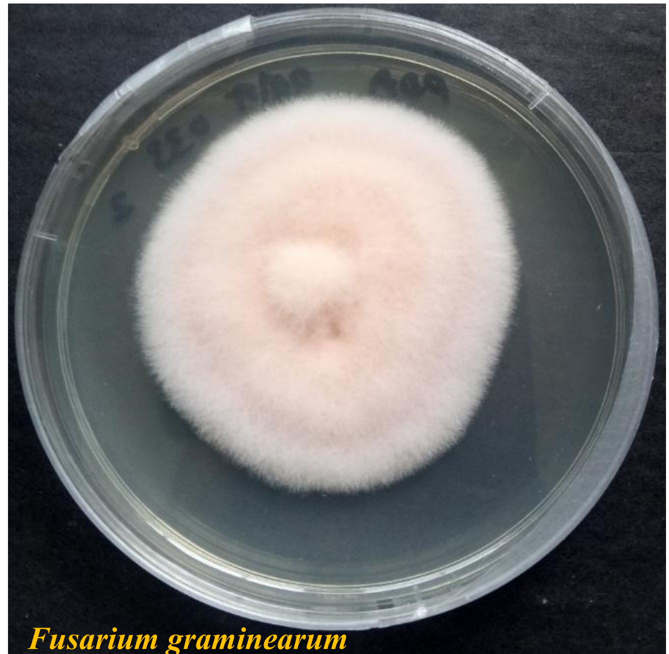
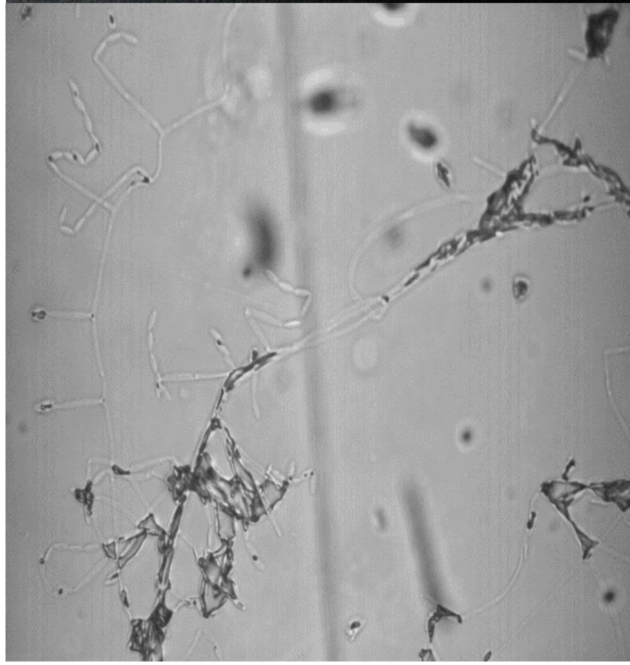


Figure 3. 2: *Aspergillus* species isolated from ground maize on Rose Bengal media modified with Dichloran fungicide



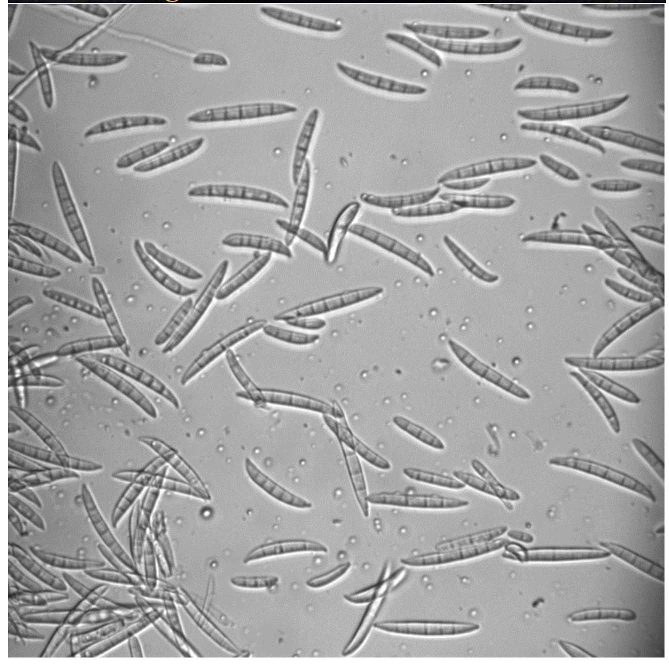


Figure 3. 3: *Fusarium* species isolated on potato dextrose agar (PDA) from ground maize

**Table 3. 4: Population (CFUs g$^{-1}$ x10$^{2}$) of *Aspergillus* spp. isolated from unsorted and sorted ground maize**

| | *Aspergillus flavus* | | | *Aspergillus parasiticus* | | | *Total Aspergillus* spp. | | |
|---|---|---|---|---|---|---|---|---|---|
| **Sample ID** | **Unsorted** | **Heavy** | **Light** | **Unsorted** | **Heavy** | **Light** | **Unsorted** | **Heavy** | **Light** |
| HBY-005 | 25.0$^{abc}$ | 116.7$^{abc}$ | 120.8$^{abc}$ | 8.3$^{a}$ | 4.2$^{a}$ | 12.5$^{ab}$ | 33.3$^{abcd}$ | 120.8$^{abcde}$ | 133.3$^{ab}$ |
| HBY-011 | 16.7$^{bc}$ | 25.0$^{bc}$ | 37.5$^{abc}$ | 8.3$^{a}$ | 0.0$^{a}$ | 4.2$^{ab}$ | 25.0$^{abcd}$ | 25.0$^{cde}$ | 41.7$^{b}$ |
| HBY-025 | 8.3$^{bc}$ | 29.2$^{bc}$ | 37.5$^{abc}$ | 0.0$^{a}$ | 8.3$^{a}$ | 33.3$^{ab}$ | 8.3$^{cd}$ | 37.5$^{bcde}$ | 70.8$^{ab}$ |
| BSA-033 | 0.0$^{c}$ | 33.3$^{abc}$ | 8.3$^{bc}$ | 0.0$^{a}$ | 8.3$^{a}$ | 379.2$^{ab}$ | 0.0$^{d}$ | 41.7$^{abcde}$ | 387.5$^{ab}$ |
| BSA-052 | 16.7$^{bc}$ | 12.5$^{c}$ | 33.3$^{abc}$ | 0.0$^{a}$ | 0.0$^{a}$ | 4.2$^{ab}$ | 16.7$^{bcd}$ | 12.5$^{de}$ | 37.5$^{ab}$ |
| BSA-055 | 0.0$^{c}$ | 16.7$^{c}$ | 0.0$^{c}$ | 0.0$^{a}$ | 0.0$^{a}$ | 0.0$^{b}$ | 0.0$^{d}$ | 16.7$^{e}$ | 0.0$^{c}$ |
| BSA-057 | 716.7$^{ab}$ | 600.0$^{a}$ | 691.7$^{a}$ | 0.0$^{a}$ | 0.0$^{a}$ | 0.0$^{b}$ | 716.7$^{a}$ | 600.0$^{ab}$ | 691.7$^{ab}$ |
| SYA-070 | 500.0$^{ab}$ | 637.5$^{ab}$ | 383.3$^{a}$ | 0.0$^{a}$ | 4.2$^{a}$ | 12.5$^{ab}$ | 500.0$^{ab}$ | 641.7$^{abc}$ | 395.8$^{ab}$ |
| SYA-072 | 300.0$^{ab}$ | 33.3$^{bc}$ | 120.8$^{ab}$ | 0.0$^{a}$ | 0.0$^{a}$ | 8.3$^{ab}$ | 300.0$^{abc}$ | 33.3$^{cde}$ | 129.2$^{ab}$ |
| SYA-073 | 1183.3$^{a}$ | 1320.8$^{a}$ | 1233.3$^{a}$ | 0.0$^{a}$ | 0.0$^{a}$ | 0.0$^{b}$ | 1183.3$^{a}$ | 1320.8$^{a}$ | 1233.3$^{a}$ |
| SYA-075 | 166.7$^{ab}$ | 29.2$^{abc}$ | 133.3$^{abc}$ | 0.0$^{a}$ | 8.3$^{a}$ | 8.3$^{ab}$ | 166.7$^{abc}$ | 37.5$^{abcde}$ | 141.7$^{ab}$ |
| KKA-126 | 150.0$^{ab}$ | 95.3$^{abc}$ | 1083.3$^{a}$ | 33.3$^{a}$ | 4.2$^{a}$ | 25.0$^{ab}$ | 183.3$^{abc}$ | 100.0$^{abcde}$ | 1108.3$^{a}$ |
| KKA-128 | 91.7$^{abc}$ | 166.7$^{abc}$ | 295.8$^{ab}$ | 8.3$^{a}$ | 0.0$^{a}$ | 20.8$^{ab}$ | 100.0$^{abc}$ | 166.7$^{abcde}$ | 316.7$^{ab}$ |
| KKA-130 | 525.0$^{ab}$ | 1254.2$^{a}$ | 1583.3$^{a}$ | 0.0$^{a}$ | 4.2$^{a}$ | 0.0$^{b}$ | 525.0$^{a}$ | 1258.3$^{a}$ | 1583.3$^{a}$ |
| KSM-142 | 833.3$^{ab}$ | 904.2$^{a}$ | 958.3$^{a}$ | 0.0$^{a}$ | 4.2$^{a}$ | 0.0$^{b}$ | 833.3$^{a}$ | 908.3$^{a}$ | 958.3$^{ab}$ |
| KSM-143 | 100.0$^{abc}$ | 516.7$^{ab}$ | 375.0$^{a}$ | 16.7$^{a}$ | 0.0$^{a}$ | 25.0$^{ab}$ | 116.7$^{abc}$ | 516.7$^{ab}$ | 400.0$^{ab}$ |
| KSM-158 | 341.7$^{abc}$ | 525.0$^{ab}$ | 1095.8$^{a}$ | 0.0$^{a}$ | 0.0$^{a}$ | 4.2$^{ab}$ | 341.7$^{abcd}$ | 525.0$^{ab}$ | 1100.0$^{a}$ |
| KSM-163 | 0.0$^{c}$ | 20.8$^{c}$ | 8.3$^{abc}$ | 0.0$^{a}$ | 0.0$^{a}$ | 112.5$^{ab}$ | 0.0$^{d}$ | 20.8$^{e}$ | 120.8$^{ab}$ |
| BMT-187 | 491.7$^{ab}$ | 412.5$^{ab}$ | 362.5$^{a}$ | 0.0$^{a}$ | 4.2$^{a}$ | 4.2$^{ab}$ | 491.7$^{ab}$ | 416.7$^{abc}$ | 366.7$^{ab}$ |
| BMT-190 | 250.0$^{ab}$ | 150.0$^{abc}$ | 287.5$^{ab}$ | 0.0$^{a}$ | 8.3$^{a}$ | 8.3$^{ab}$ | 250.0$^{abc}$ | 158.3$^{abcd}$ | 295.8$^{ab}$ |
| Mean | 285.8$^{b}$ | 345.0$^{ab}$ | 442.5$^{a}$ | 3.8$^{b}$ | 2.9$^{b}$ | 33.1$^{a}$ | 289.6$^{b}$ | 347.9$^{ab}$ | 475.6$^{a}$ |
| LSD($p \leq 0.05$) | 2.05 | 1.57 | 1.54 | 1.65 | 1.23 | 1.77 | 1.85 | 1.55 | 1.34 |
| CV% | 40.2 | 40.5 | 38.9 | 245.8 | 289.7 | 158.7 | 34.9 | 39.5 | 30.3 |
| P value | <.001 | <.001 | <.001 | 0.049 | 0.545 | 0.01 | <.001 | <.001 | <.001 |
| LSDF.($p \leq 0.05$) | 144 | | | 23.6 | | | 142.7 | | |

Data analysis was carried out on transformed data (log(x+1).Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different.

Sp.- species,HBY-Homabay, BSA-Busia, SYA-Siaya, KKA-Kakamega, KSM-Kisumu, BMT-Bomet

**Table 3. 5: Population (CFUs $g^{-1}$ x$10^2$) of *Fusarium* spp. isolated from unsorted and sorted ground maize**

| Sample ID | *Fusarium graminearum* | | | *Fusarium verticilloides* | | | Total *Fusarium* spp. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Unsorted | Heavy | Light | Unsorted | Heavy | Light | Unsorted | Heavy | Light |
| HBY-005 | $50.0^{ab}$ | $16.7^{abc}$ | $41.7^{abc}$ | $650.0^{a}$ | $337.5^{abc}$ | $54.2^{abc}$ | $700.0^{ab}$ | $354.2^{abcd}$ | $95.8^{ab}$ |
| HBY-011 | $100.0^{ab}$ | $25.0^{abc}$ | $370.8^{ab}$ | $41.7^{ab}$ | $458.3^{ab}$ | $200.0^{a}$ | $141.7^{ab}$ | $483.3^{ab}$ | $570.8^{ab}$ |
| HBY-025 | $75.0^{ab}$ | $370.8^{abc}$ | $1000.0^{ab}$ | $525.0^{a}$ | $91.7^{abc}$ | $554.2^{a}$ | $600.0^{ab}$ | $462.5^{abc}$ | $1554.2^{a}$ |
| BSA-033 | $0.0^{c}$ | $4.2^{b}$ | $145.8^{ab}$ | $0.0^{c}$ | $491.7^{a}$ | $516.7^{a}$ | $0.0^{c}$ | $495.8^{ab}$ | $662.5^{ab}$ |
| BSA-052 | $50.0^{abc}$ | $208.3^{abc}$ | $33.3^{bcd}$ | $108.3^{ab}$ | $445.8^{abc}$ | $104.2^{ab}$ | $158.3^{ab}$ | $654.2^{abcd}$ | $137.5^{ab}$ |
| BSA-055 | $100.0^{ab}$ | $20.8^{abc}$ | $1112.5^{a}$ | $191.7^{ab}$ | $4.2^{bc}$ | $1237.5^{a}$ | $291.7^{ab}$ | $25.0^{abcd}$ | $2350.0^{a}$ |
| BSA-057 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{c}$ | $0.0^{c}$ | $162.5^{abc}$ | $0.0^{c}$ | $0.0^{d}$ | $162.5^{abcd}$ |
| SYA-070 | $91.7^{bc}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{c}$ | $4.2^{bc}$ | $0.0^{d}$ | $91.7^{abc}$ | $4.2^{cd}$ | $0.0^{e}$ |
| SYA-072 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $58.3^{abc}$ | $687.5^{a}$ | $387.5^{a}$ | $58.3^{abc}$ | $687.5^{ab}$ | $387.5^{ab}$ |
| SYA-073 | $0.0^{c}$ | $8.3^{b}$ | $0.0^{d}$ | $0.0^{c}$ | $4.2^{bc}$ | $0.0^{b}$ | $0.0^{c}$ | $12.5^{bcd}$ | $0.0^{e}$ |
| SYA-075 | $175.0^{abc}$ | $266.7^{ab}$ | $487.5^{ab}$ | $733.3^{c}$ | $775.0^{abc}$ | $1137.5^{a}$ | $908.3^{ab}$ | $1041.7^{a}$ | $1625.0^{a}$ |
| KKA-126 | $0.0^{c}$ | $645.8^{a}$ | $4.2^{cd}$ | $8.3^{bc}$ | $312.5^{ab}$ | $0.0^{d}$ | $8.3^{bc}$ | $958.3^{a}$ | $4.2^{de}$ |
| KKA-128 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $8.3^{bc}$ | $95.8^{abc}$ | $4.2^{cd}$ | $8.3^{bc}$ | $95.8^{abcd}$ | $4.2^{de}$ |
| KKA-130 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $8.3^{bc}$ | $0.0^{c}$ | $20.8^{bcd}$ | $8.3^{bc}$ | $0.0^{d}$ | $20.8^{cde}$ |
| KSM-142 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{e}$ |
| KSM-143 | $8.3^{bc}$ | $37.5^{bc}$ | $4.2^{cd}$ | $0.0^{c}$ | $29.2^{bc}$ | $137.5^{ab}$ | $8.3^{bc}$ | $66.7^{abcd}$ | $141.7^{abc}$ |
| KSM-158 | $0.0^{b}$ | $12.5^{b}$ | $408.3^{bc}$ | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $0.0^{c}$ | $12.5^{cd}$ | $408.3^{bcde}$ |
| KSM-163 | $1116.7^{a}$ | $58.3^{abc}$ | $562.5^{ab}$ | $0.0^{c}$ | $125.0^{abc}$ | $608.3^{a}$ | $1116.7^{a}$ | $183.3^{abcd}$ | $1170.8^{a}$ |
| BMT-187 | $0.0^{c}$ | $4.2^{b}$ | $312.5^{bcd}$ | $0.0^{c}$ | $0.0^{c}$ | $4.2^{cd}$ | $0.0^{c}$ | $4.2^{cd}$ | $316.7^{abcd}$ |
| BMT-190 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{d}$ | $16.7^{bc}$ | $0.0^{c}$ | $0.0^{d}$ | $16.7^{abc}$ | $0.0^{d}$ | $0.0^{e}$ |
| Mean | $88.3^{b}$ | $84.0^{b}$ | $224.2^{a}$ | $117.5^{b}$ | $193.1^{ab}$ | $256.5^{a}$ | $205.8^{b}$ | $277.1^{b}$ | $480.6^{a}$ |
| LSD($p \leq 0.05$) | 1.74 | 1.67 | 1.32 | 1.72 | 1.82 | 1.26 | 2.09 | 1.78 | 1.39 |
| CV % | 74 | 120.1 | 60.8 | 62.4 | 90.9 | 48 | 60.1 | 71.1 | 43.2 |
| P value | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 |
| LSD F.($p \leq 0.05$) | 109.1 | | | 135.1 | | | 200.6 | | |

Data analysis was carried out on transformed data (log(x+1).Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different Sp.- species,HBY-Homabay, BSA-Busia, SYA-Siaya, KKA-Kakamega, KSM-Kisumu, BMT-Bomet,PDA-PotatoDextroseAgar

**Table 3. 6: Population (CFUs $g^{-1}$ $x10^{2}$) of *Penicillium* spp. isolated from unsorted and sorted ground maize**

| | *Penicillium* spp. | | |
|---|---|---|---|
| **Sample ID** | **Unsorted** | **Heavy** | **Light** |
| HBY-005 | $125.0^{a}$ | $54.2^{abc}$ | $108.3^{ab}$ |
| HBY-011 | $25.0^{ab}$ | $37.5^{abc}$ | $100.0^{ab}$ |
| HBY-025 | $16.7^{ab}$ | $58.3^{ab}$ | $37.5^{abc}$ |
| BSA-033 | $100.0^{a}$ | $283.3^{a}$ | $470.8^{a}$ |
| BSA-052 | $91.7^{ab}$ | $345.8^{ab}$ | $62.5^{ab}$ |
| BSA-055 | $41.7^{ab}$ | $20.8^{abc}$ | $45.8^{abc}$ |
| BSA-057 | $0.0^{b}$ | $0.0^{c}$ | $0.0^{c}$ |
| SYA-070 | $33.3^{ab}$ | $29.2^{abc}$ | $12.5^{abc}$ |
| SYA-072 | $0.0^{b}$ | $0.0^{c}$ | $8.3^{bc}$ |
| SYA-073 | $441.7^{a}$ | $300.0^{a}$ | $208.3^{a}$ |
| SYA-075 | $83.3^{ab}$ | $25.0^{abc}$ | $208.3^{abc}$ |
| KKA-126 | $0.0^{b}$ | $208.3^{a}$ | $62.5^{abc}$ |
| KKA-128 | $158.3^{a}$ | $4.2^{bc}$ | $70.8^{abc}$ |
| KKA-130 | $191.7^{a}$ | $125.0^{abc}$ | $120.8^{ab}$ |
| KSM-142 | $0.0^{b}$ | $12.5^{abc}$ | $8.3^{bc}$ |
| KSM-143 | $75.0^{a}$ | $33.3^{abc}$ | $29.2^{abc}$ |
| KSM-158 | $0.0^{b}$ | $104.2^{a}$ | $16.7^{abc}$ |
| KSM-163 | $50.0^{ab}$ | $50.0^{abc}$ | $8.3^{bc}$ |
| BMT-187 | $108.3^{a}$ | $25.0^{abc}$ | $300.0^{a}$ |
| BMT-190 | $0.0^{b}$ | $20.8^{abc}$ | $116.7^{ab}$ |
| Mean | $77.1^{a}$ | $86.9^{a}$ | $99.8^{a}$ |
| LSD(p≤0.05) | 2.03 | 1.77 | 1.69 |
| CV% | 53.1 | 63.6 | 54.4 |
| P value | <.001 | <.001 | <.001 |
| LSDF.(p≤0.05) | 47.8 | | |

Data analysis was carried out on transformed data (log(x+1). Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different Sp.- species,HBY-Homabay, BSA-Busia, SYA-Siaya, KKA-Kakamega, KSM-Kisumu, BMT-Bomet,PDA-PotatoDextroseAgar

## 3.6 Discussion

### 3.6.1 Weights of the sorted fractions for determination of *Aspergillus* and *Fusarium* populations

The density sorter machine sorted maize kernels based on their densities from bulk into heavy and light fractions. Bulk densities and kernel weights were higher in the heavy fractions compared to the light fractions in all the samples. This agrees with the findings of Shi *et al*. (2014) who showed that the healthy kernels which in our case were the heavy fractions had higher densities whereas the unhealthy kernels which in our case were the light fractions had lower densities. The differences in density could be attributed to fungal break down of the cellular structure and nutrient consumption which lowers kernel densities (Shi *et al*., 2014). Morales *et al*. (2019) highlighted that kernels with greater *Fusarium* ear rot symptoms exhibited greatly reduced bulk kernel density. The joint level of soluble carbohydrates in grains attacked by fungal pathogens may be modified both by regulatory mechanisms and pathogen interference (Morkunas and Ratajczak, 2014).The level of sugars at the infection site may be reduced by their uptake by the pathogen while the infected tissues have high substrate requirements for the initiation of defense responses (Morkunas and Ratajczak, 2014). The attributes of bulk density differences informed density sorting as a way of reducing the toxins.

### 3.6.2 Population of *Aspergillus* and *Fusarium* species from sorted and unsorted samples

Density sorting did not have an effect in reducing *Aspergillus* spp. and *Fusarium* spp populations in the heavy fractions. However, light fractions had higher fungal populations than bulk samples and heavy fractions. In the case of *Fusarium graminearum* and *Aspergillus parasiticus*, populations' there was a reduction after sorting with the heavy fractions having the least. *Aspergillus, Fusarium* and *Penicillium* genera were isolated in the sorted and unsorted maize samples with *Aspergillus flavus* being the most frequently isolated in all fractions. The findings on fungal populations reduction after sorting are in line with those of Stasiewicz *et al*. (2017) and Pearson *et al*. (2010) where kernel sorting using Near Infra-Red (NIR) machine and optical sorting did not effectively enrich *Fusarium* and *Aspergillus* contamination in the reject kernels and did not reduce the fungi count in the accept kernels considered to be the healthy kernels. The density sorter was designed to sort kernels based on bulk density and whose difference is linked to the

fungi feeding on the kernel components (Nelson, 2016). High populations of fungi across all fractions would be due to their colonization levels in the kernels. The fungi colonize kernels internally affecting the cellular components thus reducing their bulk density which formed the basis for density sorting thus kernels were not sorted based on their fungi populations count. However, where kernels have visible signs such as moldiness and discoloration, the levels of fungi present could be reduced in the grains sorted visually (Kang'ethe *et al*., 2017; Mutiga *et al*., 2014). However, sorting for the fungi using visible signs could have little or no effect on the toxin levels which could be found in visually clean kernels (Mutiga *et al*., 2015).

The study's findings on fungal populations in maize kernels contradict those of Njeru *et al*. (2019); Mutiga *et al*. (2015); Alokanya *et al*. (2009) whose work found higher prevalence of *Fusarium* spp. from Western region as compared to *Aspergillus* spp. However, the high numbers of *Aspergillus* spp. isolated in these kernels could have been due to the drought experienced in Kenya from October 2016 and persisted into 2017. The maize kernels selected for density sorting were harvested in June and July 2017 and could have been exposed to drought stress during the filling of the kernels fostering *Aspergillus* prevalence over *Fusarium*. The 2016/2017 drought penetrated into the key agricultural areas of Kenya including upper regions of Western, Nyanza and Central regions (Uhe et *al.*, 2017). According to Mutiga *et al*. (2015), Stasiewicz *et al*. (2017) and Magan *et al*. (2011) *Aspergillus* genera tend to be more prevalent in drought conditions thus their higher prevalence in the maize kernels. Environmental and climatic conditions have a great role in pathogen prevalence and subsequent mycotoxin production (Santiago *et al.,* 2015 and Magan *et al*., 2011). Density sorting significantly concentrated *Aspergillus* and *Fusarium* populations in the light fractions although they were not reduced completely in the heavy fractions. Density sorting has high potential in lowering toxigenic fungi in maize grain with further improvement.

# CHAPTER FOUR

# EFFECTIVENESS OF DENSITY SORTING IN REDUCING AFLATOXIN B1 AND FUMONISINS IN MAIZE GRAIN

## 4.1 Abstract

Mycotoxins such as aflatoxins and fumonisins are prevalent contaminants of maize a major household food in Kenya. This study sought to determine the effectiveness of density sorting in reducing levels of aflatoxin B1 and fumonisins in maize grain. Samples (n=206) from markets in eight counties in Western and Nyanza regions of Kenya were analyzed for mycotoxins using an ELISA assay. Samples with more than 50 ppb of aflatoxin B1 and 4 ppm of fumonisins were weighed into 300g and subjected to density sorting to obtain heavy and light fractions constituting 65-75% and 25-35% of the original weight respectively. Bulk densities and kernel weights for the fractions were determined. Each fraction was analyzed for aflatoxins and fumonisins by ELISA. The unsorted maize samples had up to 765±0 ppb aflatoxin B1 and 16±0 ppm fumonisins. Density sorting reduced fumonisins in 100% of the samples and aflatoxin B1 in only 50% of the samples. The majority (68%) of samples showed a co-existence of the two toxins with bulk density and kernel weights of the fractions higher in the heavy fractions and lower in the light fraction. Bulk density and aflatoxin B1 levels exhibited a strong correlation in this study while in the case of fumonisins for the light fractions, the correlation was strong whereas in the heavy fractions it was weak. The study showed that density sorting can be used to reduce fumonisins and Aflatoxin B1 effectively in maize grain but is more consistently effective to the former. Further research work is recommended in the area of aflatoxin B1 reduction in maize grain.

**Key Words**: Aflatoxins, Density sorting, Fumonisins, Food safety, Mycotoxins

## 4.2 Introduction

Mycotoxin contamination of maize grains is a major problem in Kenyan maize with several intervention strategies tried at pre-harvest stages including a recent possible commercialization of Aflasafe in Kenya. Approaches including use of resistant cultivars, biological control agents, good agricultural practices (Kang'ethe, 2011; Hell and Mutegi, 2011; Kumar *et al*., 2017; Mutiga *et al*, 2017) have been found to contribute to pre-harvest intervention strategies. Newer methods are still underway with aspects such as push-pull cropping system being seen to lower aflatoxins and fumonisins in Western Kenya (Njeru *et al*., 2019). Post contamination mitigation approaches have not been widely explored leading to high levels of aflatoxins and fumonisins in maize that is consumed locally. Maize contaminated with these two mycotoxins ends up contributing to economic losses as is a case in 2010 where 2.3 million bags of maize were declared unfit for human consumption due to the unsafe levels of aflatoxins (Mutegi, Cotty & Bandyopadhyay, 2018).

Owing to the high levels of aflatoxins and fumonisins that still end up in maize for consumption sorting is a last result in trying to lower the levels to the regulatory limits (Stasiewicz *et al*., 2017). Visual sorting of maize grain has been greatly practiced by farmers (Kang'ethe *et al.*, 2017) and in some cases reported to lower the levels of the toxins in some cases but it is not effective due to a very low correlation between apparent moldiness and toxin production (Nelson, 2016, Mutiga *et al*., 2015). Other aspects such as discoloration, grain shriveling and brokenness have been used by local farmers as a way of sorting for grains that are unfit for consumption. Mycotoxin contamination being highly skewed (Shi *et al*., 2014, Stasiewicz *et al*., 2017) reduces effectiveness of visual grain sorting as highly contaminated kernels may still be retained in the larger bulk stored for consumption. Mutiga *et al*. (2015) established that samples collected from the mills had higher aflatoxin levels compared to those at the storage sheds implying that maize ready for consumption or that which is consumed has unsafe mycotoxin levels despite intervention strategies at pre-harvest and sorting before milling.

Sorting approaches have been further studied to try and sort for highly contaminated kernels that are supposed to be discarded from the larger grain sample that is meant for consumption. (Stasiewicz *et al*., 2017) attempted to calibrate and set the set optical sorting technology using near infrared spectra for Kenyan maize. The findings in this work imply that the machine effectively reduced aflatoxins and fumonisins with an accuracy of 98% and 61% respectively. More work in this area of mycotoxins and mitigation strategies have pointed to the fact that bulk density can be

a proxy in aflatoxin and fumonisins levels (Morales *et al.,* 2019; Shi *et al*., 2014). This study sought to determine how kernels sorting using a density sorter based on their differential kernel densities could lower aflatoxin B1 and fumonisins.

## 4.3 Materials and Methods

### 4.3.1 Mycotoxin analysis by Enzyme-Linked Immunosorbent Assay

The 206 samples collected as earlier explained in section 3.3.1 were subjected to analysis by ELISA to determine aflatoxin B1 levels and fumonisins. The data obtained helped in subsampling from the 206 samples for samples having above 50 ppb from the aflatoxin B1 data and above 4 ppm from fumonisins data which were subjected to density sorting.

#### 4.3.1.1 Extraction of Aflatoxins

400g of the maize grain was weighed and ground into flour using the Rommer miller at the BecA laboratories. 5g of the maize flour was weighed and put into a 50ml falcon tube. 70% Methanol was prepared where 300ml of distilled water was measured into a bottle and topped up to 1L by adding 700ml of methanol. 25ml of the 70% methanol was added to each falcon tube containing 5g of the sample. The samples were vortexed for at least 10 minutes to enhance the extraction process achieving a diluted sample.

#### 4.3.1.2 Extraction of Fumonisins

400g of the maize grain was weighed and ground into flour using the Rommer miller at the BecA laboratory. 5g of the maize flour was weighed and put into a 50ml falcon tube. 90% Methanol was be prepared where 100ml of distilled water will be measured into a bottle and topped up to 1L by adding 900ml of methanol. 10ml of the 90% methanol was added to each falcon tube containing 5g of the sample. The samples were vortexed for at least 10 minutes to enhance the extraction process. The samples were diluted by taking 1900µl of distilled water and placing in a 2ml Eppendorf tube followed by 100µl of the sample extract and this too vortexed for at least 5 minutes.

#### 4.3.1.3 Analysis of aflatoxin B1

The protocol used is based on (Hosseini, Vázquez-Villegas, Rito-Palomares & Martinez-Chapa, 2017) as described in the Helica kits protocol, 2017. All reagents were brought to room temperature. The conjugate reagent (green) was poured into the dilution well and 200µl of the conjugate pipetted into each dilution well in the microtiter plate. 100µl of the six standards and the sample extract in the falcons was pipetted into the test wells containing the conjugate reagents

using a different tip for each well. The contents of the test wells was primed three times and 100µl transferred to an antibody microtiter plate which is a replica of the dilution plate. The antibody plate was incubated at room temperature for 15 minutes and covered to avoid direct light. The antibody plate was washed with 200 µl of distilled water for five times and dried to completely remove the contents of the wells. The plate wells were dried by tapping the plate on absorbent paper towels consistently. 100µl of the substrate reagent (blue) was then added into each well and incubated for 5 minutes while covered to avoid direct light. 100µl of the stop solution was then added into each well. The optical density of the plate was read at a wavelength of 450nm in a microtiter plate. The optical densities obtained from the ELISA reader were entered in a template at ILRI BecA to get the exact values of aflatoxin levels for each sample. A linear regression line was achieved and plots that were based on the standard values whose aflatoxin concentration is known and ranges from 0.0 to 4.0 ppb were obtained. Any samples with above 20ppb of aflatoxin B1 were diluted further in a subsequent manner until the exact value was obtained.

#### 4.3.1.4 Analysis of Fumonisins

The protocol used is based on (Hosseini, Vázquez-Villegas, Rito-Palomares & Martinez-Chapa, 2017) as described in the Helica kits protocol, 2017. All reagents will be brought to room temperature. PBST was reconstituted by taking all the contents of the packet and placing in a 1L bottle and topped up with 1L of distilled water. The conjugate solution A (green) was poured into the well and 100µl of the conjugate solution pipetted into each dilution well in the microtiter plate. The clear conjugate solution B was poured into the well and 100µl of the conjugate pipetted into each dilution well in the microtiter plate.100µl of the six standards and the sample extract in the Eppendorf was pipetted into the test wells containing the conjugate reagents using a different tip for each well. The contents of the test wells was primed three times and 100µl transferred to an antibody microtiter plate which is a replica of the dilution plate. The antibody plate was incubated at room temperature for 10 minutes and covered to avoid direct light. The antibody plate was washed with 200 µl of PBST for five times and dried to completely remove the contents of the wells. The plate wells were dried by tapping the plate on absorbent paper towels consistently. 100µl of the substrate reagent was then added into each well and incubated for 10 minutes while covered to avoid direct light. 100µl of the stop solution was then added into each well. The optical density of the plate was read at a wavelength of 450nm in a microtiter plate reader and the results

entered in a template to get the exact values for each sample. The fumonisins data obtained from the ELISA reader was entered into a template at ILRI BecA to determine the exact values of fumonisins levels for each sample. The linear regression obtained was in line with the standards whose fumonisins levels are known ranging from 0.0 to 150ppm. Samples above 6ppm were diluted further in a subsequent manner until the exact value was obtained.

### 4.3.2 Mycotoxin analysis for density sorted maize samples

Samples previously sorted (as described in chapter 3) were used. Aflatoxin B1 for heavy and light fractions was assayed using ELISA as previously described. This was done ensuring that each sample's heavy and light fractions are run on one plate. The sorting was done in the same way for fumonisins samples and the level of fumonisins in the light and heavy fraction determined by running a Fumonisins ELISA assay with the heavy and light fractions being done on one plate.

## 4.4 Data analysis

Data collected on toxin levels from all samples was transformed using ($\log_{10}X+1$) then subjected to analysis of variance and means separated using Tukey's least significance difference at a probability of $p=\leq 0.05$. The data obtained from the ELISA assays of the light and heavy fractions was used to determine the percentage reduction of toxicity in the heavy fraction compared to the unsorted sample's toxicity. Percentage reduction in aflatoxin B1 and fumonisins [(Bulk toxin level - Heavy fraction toxin level)/ Bulk toxin level] *100 was calculated and subjected to analysis of variance. Means were separated using Tukey's least significance difference at a 5% level of significance.

## 4.5 Results

### 4.5.1 Levels of Aflatoxin B1 and fumonisins in unsorted samples and their reduction after sorting

Analysis of fumonisins and aflatoxin B1 in all the collected samples showed a significant variation ($p<0.05$) in the level of fumonisins and in percentage co-existence of aflatoxin B1 and fumonisins (Table 4.1). Variation in fumonisins levels among samples from different regions was evident with Homabay samples having the highest level of fumonisins of up to 16ppm in one of the samples. Vihiga samples had the least fumonisins levels. The percentage of samples with both fumonisins and aflatoxin B1 averaged at 69% across the samples from different counties with Vihiga samples showing the least percentage co-existence and Kericho showing the highest (Table 4.1). The level of aflatoxin B1 in the samples from different counties had no significant variation ($p>0.05$).

The level of aflatoxin B1 in the selected samples before sorting, their correspondent heavy and light fractions after sorting as well as the percentage change in aflatoxin B1 had significant variation ($p<0.05$). All the selected samples had aflatoxin B1 levels of 50ppb and above. The difference in the level of aflatoxin B1 among the fractions was not significant. The percentage change in aflatoxin B1 when comparing the levels before sorting and the heavy fraction after sorting averaged at an increased level of 13% (Table 4.2). Only five samples had a reduction in aflatoxin B1 with the highest being at 97%. The other five samples had an increase in aflatoxin B1 with the highest being at 165%. The change was higher in the samples that showed an increase as compared to those that showed a reduction in the aflatoxin B1.

The level of fumonisins in the unsorted samples was significantly different ($p<0.05$)**.** The levels of fumonisins in the bulk, light and heavy fractions were significantly different ($p<0.05$) with the light fractions depicting the highest level and the heavy fractions depicting the lowest level (Table 4.3). The level of fumonisins in the light fractions, heavy fractions and the percentage change in fumonisins was not significantly different ($P>0.05$).

**Table 4. 1: Aflatoxin B1 and fumonisins levels in unsorted samples from different counties and their co-existence in Western Kenya**

| County | Af B1(ppb) | Fumonisins (ppm) | % co-existence |
|---|---|---|---|
| Bomet | $11.4^{a}$ | $2.2^{abc}$ | $67.0^{c}$ |
| Busia | $18.2^{a}$ | $4.7^{ab}$ | $68.0^{bc}$ |
| Homabay | $1.3^{a}$ | $5.0^{a}$ | $67.0^{c}$ |
| Kakamega | $32.8^{a}$ | $2.0^{bc}$ | $65.0^{cd}$ |
| Kericho | $6.5^{a}$ | $1.3^{bc}$ | $82.0^{a}$ |
| Kisumu | $16.0^{a}$ | $3.2^{abc}$ | $74.0^{b}$ |
| Siaya | $48.4^{a}$ | $3.2^{abc}$ | $67.0^{c}$ |
| Vihiga | $4.4^{a}$ | $0.8^{c}$ | $60.0^{d}$ |
| Mean | 17.4 | 2.8 | 68.8 |
| LSD ($p \leq 0.05$) | 0.4 | 0.2 | 0.26 |
| CV% | 117.6 | 71.7 | 1.8 |
| P value | 0.146 | <.001 | <.001 |

Data analysis was carried out on transformed data (log(x+1).Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different Af B1- Aflatoxin B1, ppb- parts per billion, ppm- parts per million

**Table 4. 2: Aflatoxin B1 (ppb) levels in the unsorted and sorted maize samples**

| Sample ID | Unsorted | Light | Heavy | %change |
|---|---|---|---|---|
| BSA-057 | $247.1^{e}$ | $400.2^{b}$ | $182.6^{c}$ | $26.2^{abc}$ |
| SYA-070 | $662.8^{b}$ | $266.1^{b}$ | $194.8^{c}$ | $70.6^{ab}$ |
| SYA-072 | $167.1^{f}$ | $2.8^{b}$ | $5.5^{c}$ | $96.7^{a}$ |
| SYA-073 | $765.4^{a}$ | $1482.6^{a}$ | $2026.0^{a}$ | $-164.7^{e}$ |
| KKA-128 | $258.2^{d}$ | $103.1^{b}$ | $102.0^{c}$ | $60.5^{abc}$ |
| KKA-130 | $374.6^{c}$ | $106.2^{b}$ | $588.6^{b}$ | $-57.0^{cde}$ |
| KSM-142 | $145.4^{g}$ | $262.5^{b}$ | $25.1^{c}$ | $82.7^{ab}$ |
| KSM-158 | $118.4^{h}$ | $383.9^{b}$ | $164.1^{c}$ | $-38.2^{bcd}$ |
| BMT-187 | $58.2^{j}$ | $169.5^{b}$ | $117.3^{c}$ | $-102.3^{de}$ |
| BMT-190 | $65.4^{i}$ | $68.9^{b}$ | $132.4^{c}$ | $-102.8^{de}$ |
| Mean | $286.3^{a}$ | $325.0^{a}$ | $354.0^{a}$ | -12.8 |
| LSD ($p \leq 0.05$) | 0.7 | 451.0 | 216.7 | 71.1 |
| CV % | 0.1 | 62.4 | 27.5 | 248.6 |
| P value | <.001 | 0.001 | <.001 | <.001 |
| LSD.F ($p \leq 0.05$) | 83.7 | | | |

Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different Af B1-Aflatoxin B1, g-grams, ppb-parts per billion, BSA-Busia, SYA- Siaya, KKA-Kakamega, KSM-Kisumu, BMT-Bomet, F-fractions

**Table 4. 3: Fumonisins (ppm) levels in the unsorted and sorted samples and their percentage change after sorting**

| Sample ID | Unsorted | Light | Heavy | %change |
|---|---|---|---|---|
| HBY-005 | 16.3 [a] | 16.9 [a] | 0.6 [a] | 71.5 [a] |
| HBY-011 | 12.5 [f] | 15.9 [a] | 11.4 [a] | 9.3 [a] |
| HBY-025 | 11.9 [g] | 18.8 [a] | 4.7 [a] | 60.9 [a] |
| BSA-033 | 14.0 [c] | 24.8 [a] | 3.6 [a] | 74.6 [a] |
| BSA-052 | 12.5 [f] | 3.1 [a] | 0.8 [a] | 93.8 [a] |
| BSA-055 | 13.1 [e] | 16.7 [a] | 4.7 [a] | 95.4 [a] |
| SYA-075 | 13.8 [d] | 30.3 [a] | 6.5 [a] | 52.8 [a] |
| KKA-126 | 12.6 [f] | 24.0 [a] | 1.9 [a] | 85.0 [a] |
| KSM-143 | 12.6 [f] | 15.4 [a] | 3.1 [a] | 75.7 [a] |
| KSM-163 | 14.7 [b] | 21.0 [a] | 1.8 [a] | 87.8 [a] |
| Mean | 13.4[b] | 18.7[a] | 3.9[c] | 70.7 |
| LSD ($p \leq 0.05$) | 0.1 | 20.1 | 8.1 | 63.8 |
| CV % | 0.3 | 48.2 | 94.0 | 40.5 |
| P value | <.001 | 0.341 | 0.250 | 0.240 |
| LSD F. ($p \leq 0.05$) | 3.9 | | | |

Means were separated by Tukeys Protected Least Significant Difference (LSD) at p=≤0.05. Means followed by same letter(s) within columns are not significantly different ppm-parts per million, g-grams, HBY-Homabay, BSA-Busia, SYA-Siaya, KKA- Kakamega, KSM-Kisumu, F- fractions

### 4.5.3 Correlations among toxins, bulk density, and populations of *Aspergillus* and *Fusarium* species

The level of aflatoxin B1 in the bulk, heavy and light fractions exhibited a significant positive correlation. *Aspergillus flavus* populations exhibited insignificant positive correlation to the aflatoxin B1 levels in the bulk, heavy and light fractions. However, *Aspergillus* parasiticus populations exhibited an insignificant negative correlation to the aflatoxin B1 levels in the bulk, heavy and light fractions. *Aspergillus flavus* populations exhibited a significant positive correlation to the bulk density in the heavy fractions whereas *Aspergillus parasiticus* exhibited an insignificant negative correlation to the same. In the light fractions, the correlation between the *Aspergillus flavus* populations to the bulk density was positive though insignificant. However, for *Aspergillus parasiticus* populations exhibited insignificant negative correlation to the bulk density in the light fractions.

The fumonisins level in the bulk samples exhibited a positive insignificant correlation to the light fractions and a negative insignificant correlation to the heavy fractions. *Fusarium verticilloides* populations exhibited a positive insignificant correlation to the fumonisins level in the bulk and heavy fractions. However, in the light fractions the *Fusarium verticilloides* populations exhibited

a negative insignificant correlation to the fumonisins level. *Fusarium verticilloides* population exhibited a positive insignificant correlation to the bulk density in the heavy fractions. The *Fusarium verticilloides* populations and the bulk density in the light fractions exhibited a negative insignificant correlation.

**Table 4. 4: Correlation coefficients among aflatoxin B1, bulk density and *Aspergillus* spp.**

| Variable | Bulk aflaB1 | Heavy aflaB1 | Light aflaB1 | B-Asp-F | B-Asp-p | H-Asp-F | H-Asp-P | L-Asp-F | L-Asp-P | BD-H | BD-L |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Bulk_aflaB1 | - | | | | | | | | | | |
| 2. Heavy_aflaB1 | 0.740* | - | | | | | | | | | |
| 3. Light_aflaB1 | 0.682* | 0.917*** | - | | | | | | | | |
| 4. B_Asp_F | 0.545ns | 0.717* | 0.899*** | - | | | | | | | |
| 5. B_Asp_P | -0.170ns | -0.201ns | -0.264ns | -0.476ns | - | | | | | | |
| 6. H_Asp_F | 0.787** | 0.595ns | 0.723* | 0.801** | -0.341ns | - | | | | | |
| 7. H_Asp_P | -0.306ns | -0.349ns | -0.264ns | -0.145ns | -0.302ns | -0.034ns | - | | | | |
| 8. L_Asp_F | 0.820** | 0.506ns | 0.494ns | 0.564ns | -0.290ns | 0.888*** | -0.112ns | - | | | |
| 9. L_Asp_P | -0.454ns | -0.407ns | -0.493ns | -0.727* | 0.768** | -0.658* | -0.039ns | -0.675* | - | | |
| 10. BD_H | 0.557ns | 0.540ns | 0.759* | 0.940*** | -0.409ns | 0.867** | -0.161ns | 0.697* | -0.778** | - | |
| 11. BD_L | 0.527ns | 0.382ns | 0.591ns | 0.695* | -0.071ns | 0.853** | 0.037ns | 0.612ns | -0.347ns | 0.754* | - |

BD- Bulk density, L-Light, H-Heavy, B-Bulk, Asp F-Aspergillus flavus, Asp P-Aspergillus parasiticus, aflaB1- aflatoxin B1, ns- not significant
Significantly correlated * ($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$)

**Table 4. 5: Correlation coefficients among fumonisins, bulk density and *Fusarium verticilloides***

| Variable | Bulk-FUM | Heavy-FUM | Light-FUM | B-Fus-V | H-Fus-V | L-Fus-V | BD-H | BD-L |
|---|---|---|---|---|---|---|---|---|
| 1. Bulk_FUM | - | | | | | | | |
| 2. Heavy_FUM | -0.375ns | - | | | | | | |
| 3. Light_Fum | 0.247ns | 0.226ns | - | | | | | |
| 4. B_Fus_V | 0.009ns | 0.084ns | 0.458ns | - | | | | |
| 5. H_Fus_V | -0.112ns | 0.332ns | 0.246ns | 0.328ns | - | | | |
| 6. L_Fus_V | 0.529ns | -0.562ns | -0.289ns | 0.332ns | 0.031ns | - | | |
| 7. BD_H | 0.344ns | 0.133ns | 0.973*** | 0.417ns | 0.304ns | -0.196ns | - | |
| 8. BD_L | 0.162ns | -0.180ns | 0.706* | 0.334ns | 0.351ns | -0.227ns | 0.733* | - |

BD- Bulk density, L-Light, H-Heavy, B-Bulk, Fus v-Fusarium verticilloides, FUM-fumonisins, ns-not significant
Significantly correlated * ($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$)

## 4.6 Discussion

### 4.6.1 Levels of Aflatoxin B1 and fumonisins in unsorted samples and their reduction after sorting

Density sorting had an effect on the level of fumonisins and aflatoxin B1 in the heavy fractions compared to the unsorted samples with a greater effect on the fumonisins. Heavy fractions exhibited highest percentage reduction in fumonisins levels. However, the level of reduction of aflatoxin B1 in the heavy fractions varied among the samples. The average level of fumonisins detected in Homabay, Kakamega, Siaya, Kisumu, Kericho, Bomet and Busia were beyond the acceptable limit of fumonisins which is at 1ppm in Kenya. Homabay had the highest level of fumonisins detected while Vihiga had the lowest level of fumonisins being the only county whose prevalence was below the regulatory limit. The percentage of samples that exhibited co-existence of the two toxins was high in all the counties.

Fumonisins reduction was more effective than aflatoxin B1 which concurs with (Stasiewicz *et al*., 2017) who found toxin enrichment in the reject fractions to be higher for fumonisins than for aflatoxin. Reduction of fumonisins in the heavy fractions is in line with the findings of (Kang'ethe *et al*., 2017; Mutiga *et al*., 2014) who noted a reduction in fumonisins after sorting grains visually. Stasiewicz *et al*. (2017) and Pearson *et al*. (2010) reported an effective reduction of fumonisins to below the acceptable limits in some samples after sorting maize kernels using optical sorting techniques and Near Infrared sorting technology. Density sorting reduced fumonisins levels to below acceptable limit in only two samples. Sorting highly contaminated samples to below acceptable limit could require more grains to be rejected. According to Pearson *et al*. (2010) passing grains through the sorter twice led to higher percentage reduction of the toxin and a larger amount of grain lost. In this study, we focused on sorting up to 35% of the kernels from the bulk sample and retained the rest in the heavy fraction. Mycotoxin contamination is highly skewed (Stasiewicz *et al*., 2017) thus some highly infected kernels could have been retained in the heavy fractions limiting the effectiveness in reducing the fumonisins to acceptable limits.

The findings on aflatoxin B1 reduction agree to those of Mutiga *et al*. (2014) who did not find any significant differences on aflatoxin levels between visually sorted and unsorted kernels. However, the findings in this study contradict those of Kang'ethe *et al*. (2017) who reported effective reduction of aflatoxins levels after visual sorting of maize kernels by farmers in Nandi and

Machakos counties. Additionally, Shi *et al*. (2014) showed that gravity table sorting of kernels and removing those with lower density effectively reduced the aflatoxin levels in all the samples that were retained. Stasiewicz *et al*. (2017) and Pearson *et al*. (2010) found that in all the reject fractions, aflatoxins were enriched and significantly reduced in the accept fractions after sorting maize kernels using the NIR machine and optical sorting techniques. As earlier mentioned, density sorting still retained a larger percentage of kernels in the heavy fractions and aflatoxin B1 being highly skewed further explains the high levels of aflatoxin B1 and an increase in some of the heavy fractions. The variation could be attributed to the fact that a highly contaminated kernel in a whole kilogram of maize can lead to a large increase in the level of aflatoxin B1 (Shi *et al*., 2014). Other factors such as kernel size in same samples that were sorted could have contributed to this anomaly since aflatoxin accumulation is higher in large kernels and lower in smaller kernels according to (Mutiga *et al*, 2018). Larger kernels offer more substrate for fungal contamination and colonization consequently leading to higher aflatoxin accumulation and in this case, some smaller kernels could have been sorted into the light fractions but not due to toxin levels. Density sorting was carried out on highly contaminated samples which could pose challenges at sorting levels as this may only be effective if a higher percentage of grains are rejected (Pearson *et al*., 2010).

Warm and dry conditions are prevalent in all the counties in this study apart from Vihiga and Kericho counties which reported relatively low levels of fumonisins on average. The levels of fumonisins in individual counties did not tally with the temperature and rainfall conditions reported. This anomaly could be attributed to the fact that general climatic characteristics cannot satisfactorily explain the differences in fumonisins contamination due to other factors such as socio-economic factors, agronomic factors and dramatic changes during kernel development, filling and drying in line with the findings of (Santiago *et al.,* 2015; Njeru *et al*., 2019). Impact of the external variables on the external processes of maize determines the fumonisins levels in the kernels at harvest. Aspects such as meteorological patterns, cultural practices and fungal diversity have been identified as major determinants in the final levels of fumonisins in maize kernels (Santiago *et al*., 2015; Njeru *et al*., 2019). The high prevalence in fumonisins in Western and Nyanza regions were in agreement with the findings of (Mutiga *et al.,* 2015) who reported high levels of fumonisins. The high prevalence of fumonisins in this region and exposure above the acceptable limits is of great concern especially with an increase in reported cases of esophageal cancer in this region (Mutiga *et al*., 2015). The differences in toxin levels of the sorted and unsorted

samples could be attributed to the origin of the samples, climatic conditions, cultural practices, socio-economic factors, agronomic factors, variety of the samples, storage period and conditions before analysis for aflatoxin B1 which is in line with the findings of (Santiago *et al.,* 2015;Njeru *et al*., 2019).

Co-existence levels reported in this study were higher than those of (Njeru *et al*., 2019). This could be attributed to the fact that samples analyzed in this study focused on samples from markets which had also been in storage for a period of time whereas (Njeru *et al*., 2019) focused on samples from standing crops in the field. Presence of the two toxins in the samples was not correlated which is in line with the findings of (Mutiga *et al*., 2015). In most samples that had co-existence, those with very high aflatoxin B1 levels had very low levels of fumonisins and vice versa. Co-exposure of the two toxins to humans is highly detrimental. Recent statistics have shown that Kenya ranks 8$^{th}$ in esophageal cancer and 76$^{th}$ in liver cancer (Chai and Jamal, 2012). Co-exposure of these two mycotoxins has been shown to increase human morbidity and stunted growth in children (Smith *et al*., 2012). Density sorting had an effect on fumonisins levels in the heavy fraction and partially on the aflatoxin B1 levels. Therefore, the technique can be used to reduce fumonisins levels in maize grain and with further adjustments it can reduce aflatoxin B1.

### 4.6.2 Correlations among toxins, bulk density, and populations of *Aspergillus* and *Fusarium* species

Samples sorted based on their aflatoxin B1 and fumonisins levels exhibited a positive relationship between bulk density and toxin levels. The findings in this study contradict those of Mutiga *et al*, (2017; Shi *et al*. (2014); Stasiewicz *et al*. (2017) whose findings showed a negative relationship between bulk density and aflatoxins. However, Mutiga *et al*. (2017) highlighted that a negative correlation was observed in the intermediate and late maturity groups but not in the early maturity group further stating that ear rot decreased with maturity being approximately two times more in the early lines than those with late maturity. Mutiga *et al*, (2017) in their work with different lines of maize varieties found that soil nitrogen levels affect kernel bulk density which was seen to be higher in those grown under high nitrogen levels. According to Mutiga *et al*. (2017) aflatoxin contamination was higher in dent than flint maize. Samples selected for sorting in this study based on their aflatoxin B1 levels had both dent and flint grains due to non-pure varieties from where they were collected. This explains the findings in the relationship between bulk density and

aflatoxin B1 levels as some grains could have been sorted into their fractions not necessarily BecAuse of the toxin levels but BecAuse of grain type differences which have different bulk densities.

Fumonisins levels and bulk density findings contradict those of Morales *et al.* (2019) who in their work tried to establish bulk density as a proxy for fumonisins. They established that kernels with lower density were more likely to have high fumonisins levels as compared to those with higher bulk densities. Kernels with lower density had higher levels of fumonisins than those with higher density but when compared within fractions the fumonisins levels were positively correlated to the bulk density which could be attributed to the varietal differences owing to the mixed grain types in each sample. An increase in fumonisins in the light fractions led to a slight increase in fumonisins in the heavy fractions which could be due to the one sample in which fumonisins were not enriched. Density sorting is effective but could be more effective if pure varieties are sorted so that kernels are purely sorted based on bulk density as opposed to other aspects such as varietal differences which contribute to kernel characteristics.

The *Aspergillus flavus* populations and the level of aflatoxin B1 being positively correlated corresponds to the findings of (Mutiga *et al*., 2018; Kang'ethe *et al*., 2017; Martins *et al*., 2000) who highlighted that sporulation can be used as a proxy for aflatoxin accumulation. Mutiga *et al*. (2018) further established that larger kernels are more susceptible to *Aspergillus flavus* colonization and toxin accumulation even within a sample where kernels could have been obtained from different parts of the same ear. Stasiewicz *et al*. (2017) also reported significant positive correlation to *Aspergillus flavus* S-strain which agrees to the findings in this study. Kang'ethe *et al*. (2017) highlighted that, farmers who sorted maize kernels and removed those which were moldy reduced aflatoxin to a great level. This indicated a direct relationship between aflatoxin B1 and *Aspergillus* sp. However, the findings in this study contradict those of Mutiga *et al*. (2014) who in their work in trying to establish the key drivers of mycotoxin contamination reported that apparent moldiness in the kernels was not a key pointer to aflatoxin contamination. *Aspergillus flavus* tends to colonize kernels internally hindering the effectiveness of visual sorting (Mutiga *et al*., 2014). They further established that, maize which seemed to be highly damaged by ear rot at pre-harvest, if it is sorted before shelling the detected aflatoxin levels could be highly reduced. However, if the sorting is done after shelling, effect on aflatoxin levels may not be evident. In this study, maize grains were sorted way after shelling and also after a short period of storage. This

could have led to the observed results because some kernels may have been harvested while looking very clean thus no need for visual sorting at harvest leading to fungal sporulation and aflatoxin accumulation in storage.

The findings on *Fusarium verticilloides* population having a negative relationship with fumonisins agree to those of (Morales *et al.,* 2019) whose work established that presence of high *Fusarium verticilloides* populations is not a proxy for fumonisins as in their study, asymptomatic plots had high levels of fumonisins compared to the symptomatic ones. They further established that internal infection severity is a better indicator to fumonisins contamination than external symptom severity. Mutiga et al. (2015) established that very clean looking kernels could be highly contaminated with mycotoxins. Rosa Junior *et al*. (2019) in their work on trying to establish fumonisins production in different maize genotypes in different environments found similar findings. They highlighted that disease severity or apparent moldiness is not a surety of fumonisins presence.

However, the findings in this study contradict those of Mutiga *et al*. (2014) who highlighted that visual sorting worked in reducing fumonisins since apparent moldiness and kernel ear rot were highly correlated to fumonisins accumulation. Afolabi *et al*. (2006) highlighted that sorting grains that are rotten, moldy or discolored greatly reduced fumonisins levels. In their study, it was further established that visual sorting works perfectly if the *Fusarium verticilloides* colonizing the kernels is the fumonisins producing type. Afolabi *et al*. (2006) highlighted that visual sorting may not have an effect on fumonisins levels if non-producing *Fusarium verticilloides* are high in numbers. This further explains possible causes of *Fusarium verticilloides* populations being poorly correlated to fumonisins levels. The findings in this study could be attributed to the variability among samples tested for *Fusarium verticilloides* and the correspondent fumonisins levels. The grains were of different varieties grown in different environments and according to Rosa Junior *et al*. (2019) high disease severity may not result to mycotoxin indices but the two aspects could be as a result of intrinsic characteristics of each hybrid and prevailing environmental conditions.

Bulk density being positively correlated to *Aspergillus flavus* population contradicts those of Morales *et al.* (2019) and Mutiga *et al*. (2017) whose work established that bulk density has a negative relationship with the toxigenic fungi populations. Morales *et al*. (2019) established that lower density kernels are likely to have higher fumonisins levels despite having little or no external symptoms of molding, blush and starbust which are a result of *Fusarium verticilloides*

colonization. As is evident, the kernels with lower density (light fractions) had higher levels of fumonisins and aflatoxin B1 than those with higher density (the heavy fractions). The light fractions had higher levels of *Fusarium vertcilloides* and *Aspergillus flavus* populations as compared to the heavy fractions. Morales *et al.* (2019) highlighted that kernels with lower density are likely to be more colonized by *Fusarium verticilloides*. However, correlating bulk density within fractions in this study did not show this negative correlation which could be attributed to the varietal differences owing to the mixed grain types in each sample. Mutiga *et al*. (2017) in their work on association between agronomic traits and aflatoxins found that maize kernels with higher bulk density were less contaminated by the toxigenic fungi and had less aflatoxins. They found that other factors contribute to the kernel density including the nitrogen levels in the soil as well as genotypic characteristics such as maturity period. Mutiga *et al*. 2017 established that late maturity groups are likely to have increased fungal colonization contributing to a reduction in kernel density. Flint maize is likely to have reduced fungal colonization as compared to the dent maize due to the presence of a hard outer endosperm layer that encloses the softer endosperm making it difficult for fungi to colonize them thus having higher kernel density than the dent (Czembor and Piotr, 2009; Mutiga *et al*., 2017). Varietal differences and farmer practices could have contributed to the relationship observed in this study. Apparent fungal infection symptoms on maize kernels are not a perfect indicator of the mycotoxin levels. Bulk density can be used as a proxy for fumonisins levels but not aflatoxin B1.

# CHAPTER FIVE

# GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 5.1 General discussion

Density sorting had no effect on the fungal populations in different fractions, however *Aspergillus* and *Fusarium* populations were concentrated in the light fractions. *Aspergillus* and *Fusarium* populations were reduced in at least 35% of the samples comparing the while comparing the unsorted samples to the heavy fractions. The technique was identified and set based on the different densities of the clean and contaminated kernels (Nelson, 2016). As such, kernels already contaminated by the fungi causing mycotoxins are lighter and the clean ones heavier. It is possible that the fungi remain in the kernels forming sclerotia that enable them to survive saprophytically for extended periods of time in soil and crop debris (Muthomi and Wagacha, 2008; Staciewicz *et al*., 2017). This makes it difficult to alter their populations once already in the kernels. Stasiewicz *et al*. (2017); Pearson *et al.* (2004); Shi *et al*. (2014) in their sorting work aimed at reducing mycotoxins reported no effect of sorting on fungal contamination. Further improvements may be required to enhance effectiveness of this technique in reducing mycotoxin-producing fungi.

Density sorting had an effect on the two toxins with a greater effect on the fumonisins reduction exhibiting a 100% effectiveness and 50% in aflatoxin B1. Previous studies on attempted sorting of maize grains using different techniques have reported higher effectiveness in reducing fumonisins as compared to aflatoxins. Visual sorting has effectively reduced fumonisins better than aflatoxin according to (Mutiga *et al*., 2014). Kang'ethe *et al*., (2017) established that visual sorting can lower the level of aflatoxins by 40%-80%. Sorting contaminated maize grains using NIR by Stasiewicz *et al*. (2017) showed higher effectiveness in lowering fumonisins as compared to aflatoxins with a mean reduction of 83% for each toxin. High speed sorting of wheat grains based on color or texture using a high speed image based sorting device has been attempted with an accuracy of 96% in separating red wheat  from white wheat (Pearson, 2010). The high speed sorting technology also achieved a 92% of accuracy in separating brown flax from yellow flax and 93% accuracy in separating durum from barley (Pearson, 2010). Gravity sorting has also been used in the seed industry where samples are drawn three times from four discharge fractions of heavy, heavy or medium, medium and light in an attempt to sort corn seed for quality (Krueger *et al*., 2007). Quality of the heavy fractions was the highest with the light fractions exhibiting the lowest

quality. Krueger *et al*. (2007) established that removal of the light fraction from commodity corn lots resulted in reduced broken corn and foreign material.

Density sorting has been opted, aiming at separating maize kernels based on the bulk density of each kernel using a pair of filters A750 and A1200 (Leslie and Logrieco, 2014) as well as using a gravity table (Shi *et al*., 2014). The method uses the concept of free fatty acid content of the grain as the index of incipient grain deterioration. Density sorting of kernels with above 100 ppb of aflatoxin has been attempted using this method and 98% accuracy has been achieved (Leslie and Logrieco, 2014). High speed sorting using this method sorted out 5% of the grains that had above 10 ppb and this removed 82% of aflatoxin contamination. High speed sorting using a dual wavelength sorter for fumonisins achieved 88% reduction in fumonisin levels and this applied even for lowly contaminated samples (Leslie and Logrieco, 2014). More developments are underway to modify this sorting technique and enhance its effectiveness.

*Aspergillus flavus* and *Fusarium verticilloides* populations had a positive relationship with their respective toxins with an exception in *Fusarium verticilloides* in the light fractions. Correlating bulk density to *Aspergillus* sp. and *Fusarium* sp., only the light fractions and *Fusarium* sp. exhibited a negative relationship. Stasiewicz *et al*. (2017) and Mutiga *et al*. (2017) highlighted that total aflatoxins in their study was positively correlated to the *Aspergillus flavus* colony count. Morales *et al*., (2019) in their work established that external severity symptoms are not a proxy for fumonisins levels since those with least severity symptoms had high fumonisins levels. Morales *et al.* (2019) also found that bulk density can be used as a proxy for fumonisins with lower density kernels expected to have higher fumonisins. Mutiga *et al*. (2017) established a negative relationship in bulk density and aflatoxins with an exception in early maturity groups. Varietal differences could have been a major contributor to the findings in this study.

### 5.2 Conclusions

Density sorting concentrated *Aspergillus* and *Fusarium* populations in the light fractions but did not effectively reduce the populations in the heavy fractions. Density sorting effectively reduced fumonisins in the heavy fractions but aflatoxin B1 was not effectively reduced. However, reducing the two toxins to below the acceptable limit was not effectively achieved, but can be achieved with greater modification to the sorting technique. In the case of aflatoxin B1, fungal populations were correlated to the toxin levels though it wasn't the case for fumonisins. Mycotoxin-producing fungal

populations can be better reduced through integrated approaches coupled with density sorting. Density sorting as a technique is effective but could be more effective if pure varieties are used so that kernels are purely sorted based on bulk density as opposed to other aspects such as varietal differences which contribute to kernel characteristics. With further adjustments, density sorting is a promising technique for reduction of mycotoxins especially the fumonisins which are highly prevalent in the Western region of Kenya. The throughput of the density sorter was approximately 1kg per hour although with further improvement and calibration, the sorting capacity can be improved. The density sorter is currently estimated at 10,000 Kenya shillings as compared to the LED- based sorter previously used in Kenya, estimated at 90,000 Kenyan shillings. The training needs are not much and one density sorter can be used at community level.

## 5.3 Recommendations

i. Farmers and millers should be encouraged to adopt density sorting technique prior to milling and consuming their maize.
ii. Development of an improved density sorting machine with high thorough-put and increased efficiency for commercial use.
iii. Density sorting can be used to sort consignments with very high levels of mycotoxins to safer levels and the reject grain can be used as industrial raw materials.

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# APPENDICES

## APPENDIX 1: HELICA AFLATOXIN B1 TESTING PROTOCOL

### Materials Required But Not Provided

### Extraction Procedure

Grinder sufficient to render sample to particle size of fine instant coffee

Collection Container: Minimum 125ml capacity

Balance: 20g measuring capability

Graduated cylinder: 100ml

Methanol: 70ml reagent grade per sample

Distilled or deionized water: 30ml per sample

Filter Paper: Whatman #1 or equivalent

Filter Funnel

### Assay Procedure

Pipettor with tips: 100µl and 200µl

Timer

Wash bottle

Absorbent paper towels

Microplate reader with 450nm filter

### PRECAUTIONS

1. Bring all reagents to room temperature (19º - 27ºC) before use.
2. Store reagents at 2 to 8°C, and do not use beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents back into their original bottles. The assay procedure details volumes required.
4. Adhere to all time and temperature conditions stated in the procedure.
5. Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may effect the test results.
6. Never pipette reagents or samples by mouth.
7. Standards are flammable. Caution should be taken in the use and storage of these reagents.
8. The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.

9. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.
10. Dispose of all materials, containers and devices in the appropriate receptacle after use.

**EXTRACTION PROCEDURE**

Note: The sample must be collected according to established sampling techniques
1. Prepare the Extraction Solution (70% Methanol) by adding 30ml of distilled or deionized water to 70ml of methanol (reagent grade) for each sample to be tested.
2. Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
3. Weigh out a 20g ground portion of the sample and add 100ml of the Extraction Solvent (70% methanol).
Note: The ratio of sample to extraction solvent is 1:5 (w/v).
4. Mix by shaking in a sealed container or in a blender for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5 - 10ml of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested. The sample is now ready for testing.

**ASSAY PROCEDURE**

Note: It is recommended that a multi-channel pipettor 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.
1. Bring all the reagents to room temperature before use.
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.
3. Dispense 200µl of the Conjugate into each Dilution Well.
4. Using a new pipette tip for each, add 100µl 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.
5. Using a new pipette tip for each, transfer 100µl of contents from each Dilution Well to a corresponding Antibody Coated Microtiter Well. Incubate at room temperature for 15 minutes.

6. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with distilled or deionized water, then decanting the water into a discard basin. Repeat wash for a total of 5 washes.
7. Tap the microwells (face down) on a layer of absorbent towels to remove residual water.
8. Measure the required volume of Substrate Reagent (1 ml/strip or 120 µl/well) and place in a separate container. Add 100µl to each microwell. Incubate at room temperature for 5 minutes.
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.

10. Read the optical density (OD) of each microwell with a microtiter plate reader using a 450nm filter. Record the optical density (OD) of each microwell.

**INTERPRETATION OF RESULTS**

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero (0.0) standard against the aflatoxin content of the standard. Unknowns are measured by interpolation from the standard curve. The 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 B1 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.