SCREENING MAIZE INBRED LINES AND CROSSES FOR RESISTANCE TO AFLATOXIN AND FUMONISIN ACCUMULATION

BY

ABIGAEL ATIENO OUKO BSc. (UON), MSc. (UON)

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN GENETICS AT THE UNIVERSITY OF NAIROBI

APRIL 2018

DECLARATION

I, Abigael Atieno Ouko, registration number, I80/50153/2015, hereby declare that, this is my original work and has never been presented for award of a degree in any other University or Institution.

Signature______________________________Date_____________________________

This thesis has been submitted for examination with our approval as the University Supervisors

1. **Prof. Sheila Okoth**
   School of Biological Sciences
   University of Nairobi

   Signature______________________________Date__________________________

2. **Dr. Nelson Onzere Amugune**
   School of Biological Sciences
   University of Nairobi

   Signature______________________________Date__________________________

3. **Dr. Vesa Joutsjoki**
   MTT Agrifood Research Finland
   Biotechnology and Food Research
   FIN-31600 Jokioinen, FINLAND

   Signature______________________________Date__________________________

DEDICATION

To my father Benson and mother Caren for making me understand importance of education, my brother Herbert for financially supporting my education since childhood, my husband Paul for his endless support, sons Tomhenry, Timhowel and Tamharel for the joy they have always showered me.

ACKNOWLEDGEMENT

During my PhD study period, I received support from different institutions and individuals and I greatly acknowledge their support. I am very grateful to International Maize and Wheat Improvement Centre (CYMMIT) for funding the field work research at Kenya Agricultural and Livestock Research Organisation (KALRO) in Katumani and Kiboko research stations. I am also grateful to German Academic Exchange Service (DAAD) In-Country Scholarship program that financed my studies for two years. I also thank the Office of the Deputy Vice Chancellor Research Production and Extension and The Deans Committee-University of Nairobi for the research grant which supported part of this study.

My sincere gratitude goes to my supervisor Prof. Sheila Okoth for her constant advice, guidance, facilitation of different activities within the project and dedication to the project from the first day of planting to the last day of post-harvest activities. I am also greatly indebted to my other supervisors; Dr. N. Amugune and Dr. J. Vesa for their continuous encouragement and guidance throughout the study period.

Besides my advisors, I am thankful to KALRO team in Katumani and Kiboko. The team includes Mr(s). Henry Nzioki, Peter Simiyu, George Nzioki and Daniel Mutisya for their support and corporation from land ploughing to harvesting period during the field study. Among the CYMMIT team, I give special thanks to Dr. Dan Makumbi for his support during field work. My appreciation also goes to Mr. George Oriyo for assisting in breeding and always being ready to assist whenever necessary.

I am also grateful to the Centre for Biotechnology and Bioinformatics (CEBIB) director and technicians for allowing me to use their laboratories. I greatly thank the CEBIB technologists; Ms. Anne Owiti and Mr. Rono Cheruiyot for their technical support whenever I needed their assistance while using equipment in their laboratory. My sincere appreciation also goes to the Director, School of Biological Sciences, and University of Nairobi for permission, facilitation and guidance whenever necessary. I appreciate assistance and encouragement I received from Mr(s). Patrick Apando, Kelvin Muteru, Boaz Otieno, Victor Kagot, Vincent Ayugi and Ms. Esther Marijani during the study period.

My sincere appreciation also goes to my father Benson and mother Caren for their constant prayers and encouragement, my brother Herbert for financially supporting my education and believing in me. Special thanks to my husband Paul for his support, encouragement, understanding, appreciating my studies, love and always willing and able to take care of the children while I was away. I am also grateful to my three sons Tomhenry, Timhowel and Tamharel for all the time they spent while I was away.

Above all, I thank The Lord Almighty for the far He has brought me. He has provided me with strength and blessed the work of my hands and mind. Glory be to Him.

TABLE OF CONTENTS

DECLARATION ..... **ii**

DEDICATION ..... **iii**

ACKNOWLEDGEMENT ..... **iv**

TABLE OF CONTENTS ..... **vi**

LIST OF TABLES ..... **x**

LIST OF FIGURES ..... **xii**

LIST OF ABBREVIATIONS AND ACRONYMS ..... **xiv**

ABSTRACT ..... **xvi**

CHAPTER ONE **18**

INTRODUCTION **18**

1.1 Introduction 18

1.2 Problem statement 21

1.3 Justification 21

1.4 Objectives 22

1.4.1 Broad objective of the study 22

1.4.1 Specific objectives 22

1.5 Research Hypotheses 22

CHAPTER TWO **24**

LITERATURE REVIEW **24**

2.1 Maize varieties and diseases 24

2.2 Mycotoxins reported in maize 26

2.2.1 Primary mycotoxins in maize 26

2.2.2 *Aspergillus flavus* 27

2.2.2.1 Characterization of *A. flavus* species 28

2.2.3 Fusarium verticilloides 29
2.3 Mode of entry of fungal pathogen into the maize 30
2.4 Toxigenicity of *Aspergillus flavus* and *Fusarium verticilloides* species 30
2.5 Aflatoxin challenge in Africa 32
2.5.1 Aflatoxin exposure and effects 32
2.5.2 Aflatoxin policies in Africa 32
2.5.3 Vulnerability to aflatoxins in Africa 33
2.5.4 Primary interventions to aflatoxins and fumonisin exposure 34
2.6 Pre-harvest aflatoxin accumulation control methods 35
2.7 Post-harvest management strategies 39
2.8 Aflatoxin testing methodology 39
CHAPTER THREE 41
MATERIALS AND METHODS 41
3.1 Study sites 41
3.2 Source of fungal isolates and inoculum 42
3.3 Preservation of fungal cultures for inoculum preparation 44
3.4 Molecular characterisation of *Aspergillus* and *Fusarium* isolates 45
3.4.1 Extraction of DNA 45
3.5 Source of maize inbred lines used in the study 47
3.5.1 Land preparation, planting and management of maize inbred lines 48
3.5.2 Preparation of *Aspergillus* flavus spore's inoculum 48
3.5.3 Preparation of *Fusarium verticilloides* spore's inoculum 49
3.5.3.1 Preparation of Armstrong *Fusarium* medium 49
3.5.3.2 Inoculation of Armstrong liquid medium with *Fusarium verticilloides* spores 50
3.5.4 Inoculation of silked inbred lines with *Aspergillus flavus* and *Fusarium verticilloides* spores 50

3.6 Harvesting and post-harvest practises 51

3.7 Laboratory analysis of aflatoxin and fumonisin levels in maize inbred lines 53

3.7.1 Aflatoxin analysis using ELISA method (Helica Biosystems Inc.) 53

3.8 Hybrid maize seed production 55

3.9 Screening of maize crosses for resistance to aflatoxins and fumonisins 57

3.10 Data analysis 58

CHAPTER FOUR 59

RESULTS 59

4.1 Mating types in *Aspergillus flavus* populations 59

4.2 Agronomic performance of maize inbred lines 62

4.3 Ear rot severity in maize inbred lines 80

4.3.2 *Fusarium* ear rot of maize inbred lines 83

4.4 Aflatoxin levels in maize inbred lines 84

4.5 *F*umonisin in maize inbred lines 86

4.6 Association between ear rots and toxin levels in maize inbred lines 89

4.7 Aflatoxin and fumonisin levels in season I hybrids 90

4.7.1 Aflatoxin in season I crosses 90

4.7.2 Fumonisin levels in season I maize hybrids 91

4.8 Resistance of season II and season III crosses to aflatoxins and fumonisins 92

CHAPTER FIVE 96

DISCUSSION 96

5.1 Mating types in *Aspergillus flavus* populations from Makueni and Nandi 96

5.2 Germination in maize inbred lines 99

5.3 Flowering in maize inbred lines 101

5.4 Blister development in the inbred lines 104

5.5 Ear rots, aflatoxin and fumonisin levels in maize inbred lines 104

5.6 Aflatoxin and fumonisin in maize hybrids 109

5.6.1 Resistance of the hybrids to aflatoxin 109

5.6.2 Resistance of the hybrids to fumonisin 111

CONCLUSION AND RECOMMENDATIONS 115

REFERENCES 117

APPENDICES 126

Appendix 1: Aflatoxin levels (µg/kg) in season II maize hybrids in Katumani Research station 126

Appendix 2: Aflatoxin levels (µg/kg) in season III maize hybrids in Katumani Research station 128

Appendix 3: Fumonisin levels (mg/kg) in Season II and III maize hybrids 130

LIST OF TABLES

Table 2-1: Maize hybrid varieties grown in Kenya 25
Table 2-2: Common mycotoxins associated with maize 26
Table 2-3: Reported human aflatoxicosis outbreaks in East Africa (1981-2016) 33
Table 3-1: *Aspergillus flavus* strains from Makueni county.......................................43
Table 3-2: *Aspergillus flavus* strains from Nandi county 44
Table 3-3: Fungal strains used for inoculum production..44
Table 3-4: Multiplex PCR assay for isolated *Aspergillus flavus* strains......................46
Table 3-5: Planting and harvesting dates at Kiboko and Katumani.............................51
Table 4-1: Germination rate of maize inbred lines at 15 days after planting 63
Table 4-2: ANOVA table; Variation in silking stages in maize inbred lines 68
Table 4-3: ANOVA table; Variation in silking inbred maize lines 68
Table 4-4: Days to silking in maize inbred lines 70
Table 4-5: ANOVA table; Variation in duration to pollen shed in maize inbred lines71
Table 4-6: ANOVA table; Variation in pollen shed in Kenyan and South African maize inbred lines...73
Table 4-7: Variations in time taken to pollen shed in maize inbred lines....................74
Table 4-8: ANOVA table; Variation of blistering stages in maize inbred lines..........75
Table 4-9: ANOVA table; Variation of blistering in Kenyan and South African maize lines...75
Table 4-10: Number of days to blistering in maize inbred lines.................................78
Table 4-11: ANOVA table; *Aspergillus* ear rot severity in maize lines 80
Table 4-12: *Aspergillus* ear rot severity of maize inbred lines 82
Table 4-13: Mean *Fusarium* ear rot % severity in maize inbred lines.........................84
Table 4-14: ANOVA table; Variation in aflatoxin levels in maize inbred lines 85
Table 4-15: Aflatoxin levels (µg/kg) in maize inbred lines..86
Table 4-16: ANOVA table; Fumonisin levels (mg/kg) in inbred maize lines.............86
Table 4-17: Fumonisin levels (mg/kg) in maize inbred lines 88
Table 4-18: *Aspergillus* ear rot versus aflatoxin levels in maize inbred lines 89
Table 4-19: Association between *Fusarium* ear rot and Fumonisin levels in maize inbred lines...89

Table 4-20: Association between *Fusarium* ear rot and *Aspergillus* ear rot in maize inbred lines 90
Table 4-21: Aflatoxin levels (µg/kg) in season I maize hybrids in Katumani Research station 91
Table 4-22: Fumonisin levels (mg/kg) in season I maize hybrids in Katumani Research station 92
Table 5-1: Mating type distribution for *A. flavus* isolates sampled in Makueni..........97
Table 5-2: Mating type distribution for *A. flavus* isolates sampled in Nandi 97

LIST OF FIGURES

Figure 3-1: Map of Kenya showing Katumani and Kiboko Research centres (Source: Abigael Ouko)..42
Figure 3-2: Percentage visual ear rot rating in maize (Source: Pierce, 2016) 52
Figure 4-1: Mating types for *A. flavus* strains from Makueni and Nandi; L-molecular weight marker, Lane 1- IVM201365, 2-IVM100095, 3-IVM100130.........................59
Figure 4-2: Mating types for *A. flavus* strains from Makueni and Nandi; 4-IVM 350G, L5-504820BMIYG, L6-300566, L7-IVM 132G, L8-100957, L9-60756, L10-50400BM2YG, L11-2M1983g, L12-60572BM2Y, L13- 60795BM8BJ, L14-60801BSIYG, L15-40013BM126, L6-300566, L16-IVM131LG, L-molecular weight marker 60
Figure 4-3: Mating types for *A. flavus* strains from Makueni and Nandi; Lane (L)17-3VM 566, L18-40025 BSIYG, L19-IVM 4018, L20-40245 BMIG, L21-2M1983DG, L22- 2VM882G, L23-50444, L24-50536 BMIYG, L25-2M2002LG, L26-40296BSY, L27- 40143BM112G, L28-2VM964, L-molecular weight marker 60
Figure 4-4: Mating types for *A. flavus* strains from Makueni and Nandi; Lane (L)29-40143 BM112G, L30-IVM 130LG, L31-3VM566G, L32-IVM 250WY, L33-40267 BSIYG, L34-IVM 414, L36-2M1002LG, L37-100079, L38-40224 BSIYG, L39-40041BM38Y6, L40-60690BMIYG, L41-IVM201122, L42-IVM 195, L-molecular weight marker 61
Figure 4-5: Mating types for *A. flavus* strains from Makueni and Nandi; Lane (L) 35-50444 BSIYG, L44-60661 BMIYG, L43-201204, L-molecular weight marker.........61
Figure 4-6: Mean germination counts of maize inbred lines in Kiboko......................64
Figure 4-7: Mean germination counts of maize inbred lines in Katumani 65
Figure 4-8: Variation in silking of maize inbred lines...67
Figure 4-9: Variation of pollen shed in maize inbred lines 72
Figure 4-10: Variation in blistering of maize inbred lines..76
Figure 4-11: Average rainfall and temperature at Katumani from 9/12/12 to 30/5/13 79
Figure 4-12: Average rainfall and temperature at Kiboko from 3/1/13 to 13/6/13 79

Figure 4-13: Harvested maize lines A) Clean maize B) *Aspergillus* ear rot maize 80
Figure 4-14: Maize showing *Fusarium* ear rot 83
Figure 4-15: Average temperature and rainfall recorded for growing season II from July to December 2014 95
Figure 4-16: Average rainfall and temperature data at Kiboko for the season III crosses from December 2014 to March 2015 95

LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	:	Analysis of Variance
BSA	:	Bovine Serum Albumin
Bp	:	Base pairs
CAN	:	Calcium Ammonium Nitrate
CDC	:	Centre for Disease Control
CEBIB	:	Centre for Biotechnology and Bioinformatics
CPA	:	Cyclopiazonic acid
CYMMIT	:	International Maize and Wheat Improvement Centre
DAAD	:	German Academic Exchange Service
DAP	:	Diammonium Phosphate
ELISA	:	Enzyme Linked Immunosorbent Assay
FDA	:	Food and Drug Administration
Ha	:	hectares
KALRO	:	Kenya Agricultural and Livestock Research Organisation
KNH		Kenyatta National Hospital
MAT	:	Mating Type
MWM	:	Molecular Weight Marker
OD	:	Optical density
PCR		Polymerase Chain Reaction
PDA	:	Potato Dextrose Agar
Ppb	:	Parts per billion
Ppm	:	Parts per million
QTL	:	Quantitative Trait Locus
TRT	:	Treatment
USA	:	United States of America
VCG	:	Vegetative Compatible Groups
WHO	:	World Health Organisation
Cm	:	Centimetre
Ml	:	millilitre

LIST OF ABBREVIATIONS AND ACRONYMS

Mg	:	milligram
Nm	:	nanometre
µl	:	microliter
ºC	:	centigrade
<	:	less than
>	:	greater than

ABSTRACT

Maize forms a primary diet in Africa and the demand exceeds its local production. In Kenya, it is equally a subsistence and commercial crop, grown by small scale (75%) and large scale farmers (25%). Production of this crop is usually constrained by ear rot fungi in the *Aspergillus* and *Fusarium* genus. In Sub-Saharan Africa, 13-70% of maize yield is lost due to ear rots. Further still, these fungi produce mycotoxins, some of which are poisonous to both humans and animals. Over 300 mycotoxins are known but in this study aflatoxins and fumonisins are reported due to their health concerns in Africa. Outbreaks of aflatoxicosis, resulting into deaths, have occured in Kenya yearly since 1981, in Eastern Kenya, due to consumption of maize contaminated with aflatoxins. *Aspergillus flavus* has been the cause of the contamination. Chronic exposure to aflatoxins is known to cause cancer while consumption at high levels result to death in both humans and other animals. Aflatoxin is classified as class 1 carcinogen by Internationaion Centre for Cancer Research. Among the *Fusarium* toxins, is fumonisin, which is also a commonly reported contaminant in maize in Eastern Kenya and is documented to cause oesophageal cancer in humans. The major cause of fumonisins in maize is *F. verticilloides.* Knowledge and management of these fungi is therefore an important step in control of human exposure to these toxins. This study aimed at determining mating types among *A. flavus* populations and identifying maize inbred lines and hybrids resistant to *Aspergillus* ear rot (AER), *Fusarium* ear rot (FER), aflatoxins and fumonisins accumulation. Forty four *A. flavus* isolates from the University of Nairobi mycology culture collection were grouped by mating types (MAT) using multiplex polymerase chain reaction (PCR) assay. Field trials for screening for resistance were conducted in Kiboko and Katumani during 2013 planting season. Twenty three inbred lines were planted in *Fusarium* and *Aspergillus* blocks. In each block, one seed was sown per hill and a total of 33 seeds planted per row. Inoculation with 2ml of inoculum made from selected three toxigenic species of *A. flavus* and *F. verticilloides* was done respectively in the two blocks when the silks were about an inch long. Hybrids developed from the same inbred lines were planted and inoculated in the same manner. The experimental blocks were managed using common agronomic practices including irrigation as necessary. Timely harvesting was done at four weeks after physiological maturity and ear rot evaluation performed immediately

after harvest. The maize cobs were dried in the sun until about 13% moisture content and the grains screened for aflatoxins and fumonisins accumulation using Enzyme Linked Immunosorbent Assay (ELISA). The isolates with bands corresponding to 270 base pairs (bp) were grouped as *MAT1-2* and those corresponding to 396 bps as *MAT1-1*. *Aspergillus flavus* isolates used for spore inoculation which included strains 201365 had *MAT1-1* and *MAT1-2* at a single MAT locus while 100095 and 100130 belonged to *MAT1-2*. Presence of mating type genes in the isolates was important because through mating type analysis of the isolates, gene flow among the fungi can be traced and appropriate biocontrol strategy incorporating gene flow aspect developed. Lines CML 495 (1.091µg/kg), CB 222 (1.1µg/kg), CML 390 (1.23µg/kg), and CML 444 (1.362µg/kg) had least aflatoxins and can be used for breeding for resistance to aflatoxin. However, lines P502 (38.4µg/kg) and R119W (37.6µg/kg) accrued high aflatoxin levels. Lines CML 390 (1.08mg/kg), CML 247 (1.43mg/kg) and CKL05003 (1.78mg/kg) can be used for breeding for resistance to fumonisin though R2565Y (6.76mg/kg) and VO617Y-2 (6.21mg/kg) accumulated the most fumonisins. Hybrids CKLO5022 (F) x CB 248 (M) (15.877µg/kg) and CB 248 (F) x CKLO5022 (M) (17.545µg/kg) had aflatoxins below 20µg/kg. The other crosses ranged between 22.455-99.263µg/kg. Seventy three percent of the hybrids accumulated fumonisins below 2mg/kg (0.224-1.865). Hybrid CKLO5015 (F) x CML 444 (M) had the least fumonisin (0.224mg/kg) and CML 444(F) x CKLO5015(M), CML 444(F) x CML 495(M), CML 495(F) x CML 444(M), P502(F) x CML 444(M), CKLO5015(F) x R119W(M) and CML 444(F) x R119W(M) have shown resistance to *Fusarium* infection thus recommended for inclusion in hybrid programs.

Key words: *Aspergillus flavus, Fusarium verticilloides*, mating types, aflatoxin, fumonisin, inbred lines, hybrids.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Maize (*Zea mays L.*) is a staple food in most African countries including Kenya. Ninety six percent of the population depends on maize. Food security considerations in Kenya are dominated by maize crop with a per capita consumption of 98 kg per annum and accounts for about 40% of the daily calorie intake. Maize consumption is at a rate of 258g/person/day. In children, maize forms a major portion of the gruel used for weaning. Maize grain is also a crucial component in animal feed and this increases its demand (Okoth, 2016; Kibe, 2015; Mutiga *et al*., 2014; Okoth *et al*, 2012; Felicia *et al.,* 2011; Probst *et al*., 2009). In Kenya, maize farming is dominated by small scale farmers who contribute 75% of the total produce on 2 million hectares with average yield of 1.2-1.6 tons per hectare. In each family there is a garden or a farm where maize is grown for the family consumption and sometimes sold locally in the markets (Kibe, 2015).

Maize is susceptible to aflatoxin contamination and its production lowered by ear rot fungi mostly belonging to the genera of *Aspergillus*. *Aspergillus* species produce secondary metabolites, some of which are poisonous. Aflatoxin is one such metabolite which has been classified as class 1 carcinogen by International Centre for Cancer Research (Okoth, 2016). *Aspergillus flavus* and *A. parasiticus* produce aflatoxins, and are known to be carcinogens, mutagenic and immunosuppressive to humans and animals. Ingestion of aflatoxins at high levels (> 6000 mg) causes liver failure and death within 1-2 weeks of exposure (Okoth, 2016; Obura, 2013). However, prolonged low levels of aflatoxin consumption result in liver cancer. In children, chronic exposures to aflatoxins result in stunted growth, immunosuppression and increases neonatal susceptibility to infections and jaundice. Furthermore, etiology and pathogenesis of malnutrition diseases such as kwashiorkor have been implicated to chronic aflatoxin exposures (Okoth, 2016; Obura, 2013).

In Kenya, since 1981, cases of aflatoxicosis have been reported every year following consumption of maize contaminated with *A. flavus* and aflatoxins. However, aflatoxicosis in 2004 was the severe episode of human aflatoxin poisoning with 317 cases and 125 deaths reported (Okoth, 2016; Okoth *et al*., 2012). It was also reported in 2010 that 2.3 million bags (estimated at $69 million) of corn harvested in Kenya had aflatoxin contamination thus unfit for human or livestock consumption (Okoth *et al*., 2012; Schmidt, 2013).

Other African countries have reported aflatoxicoses in humans (Mwihia *et al*., 2008; Felicia *et al.,* 2011; Okoth, 2016). Tanzania is a key producer of maize in the Sub–Saharan Africa and is among top 25 maize producers in the world. However, contamination of maize with aflatoxins has been commonly reported in the Eastern (Morogoro) and Western (Shinyanga) zones of Tanzania (Suleiman and Rosentrater, 2015; Okoth, 2016). In Nigeria, aflatoxin contaminations are as high as 1000-5000µg/kg in maize, groundnuts, rice and millet. Increased liver cancer cases in Uganda are accredited to ingestion of maize and peanuts contaminated with aflatoxins (Okoth, 2016).

In Africa accumulation of aflatoxin after harvest is a challenge though contamination can occur at every stage of the supply chain from pre-production to post harvesting, marketing and distribution. The Kenyan human and animal populations are directly exposed to regular doses of aflatoxin through maize ingestion (Okoth, 2016). Contaminated cereal grains, commercially processed maize products, dairy concentrates made from mould-damaged grains and crop residues for homemade dairy concentrates are primary routes of aflatoxin consumption in man and animals. A large proportion of the Kenyan population is therefore at a risk of aflatoxicosis with children below 15 years of age being at most risk (Okoth, 2016; Obura, 2013, Alakonya *et al*., 2008). Though, regulations on exposure to these toxins exist in most parts of the world, the standards have insufficient significance to the poor, small scale farmers in Africa, who rely on maize for daily nutrition and income (Okoth, 2016; Probst *et al*., 2009; Felicia *et al.,* 2011).

Fumonisins, like aflatoxins, are cancer-causing secondary metabolites produced by *Fusarium* species. The major producer of fumonisin in maize is *F. verticilloides*. The

fungus causes *Fusarium* ear rot in maize affecting crop yield (Guo *et al*., 2016; Small *et al.,* 2012). Contamination of maize by fumonisins reduces grain quality. Maize infection by *F. verticilloides* and contamination with fumonisins are influenced by climate, temperature, humidity, insect infestation, maize handling before and after harvest (Fandohan *et al*., 2013). Infection can occur at all developmental stages and in some cases the infection is symptomless. Nevertheless, the infection intensifies in the field since most Kenyan farmers leave maize in the field to dry beyond physiological maturity and the delayed harvesting often coincides with the rains resulting in increased ear rotting. Chronic exposure to fumonisin is associated with oesophageal cancer, liver cancer, growth retardation and immunosuppression in humans. In animals, it causes diseases likes leukoencephalomalacia in equine, pulmonary oedema in pigs and haemorrhage in the brain of rabbits (Guo *et al*., 2016; Small *et al.,* 2012).

Though fumonisin research is less documented compared to aflatoxin, fumonisin contamination has been reported in some African countries, United States of America and China. Fumonisin research has been extensively done in South Africa (SA) where *F. verticilloides* is the most prevalent ear rot causing fungi. In the Transkei region of South Africa and China, eating of maize contaminated with aflatoxins is associated with increased cases of oesophageal cancer (Nyashadzashe, 2014; Mwalwayo and Thole, 2016). In Malawi, a study conducted to assess fumonisin contamination levels of stored maize consumed in rural households ranged between 1-7mg/kg (Mwalwayo and Thole, 2016). According to Fandohan et al. (2005), a study of *F. verticilloides* in maize in Benin showed the presence of extremely high fumonisin contamination ranging from 8,240 to 16,690 mg/kg.

In Kenya, fumonisin contamination has been reported in the Eastern part in areas where cases of aflatoxin outbreaks have frequently occurred (Mwihia *et al.,* 2008; Bii *et al*., 2012). Cases of oesophageal cancer have been reported in men in the North Rift Valley in Kenya and third most common cancer in women (Wakhisi *et al*., 2005). Though studies have associated *Fusarium* species with deeply invasive infections in immune-compromised patients leading to high incidences of oesophageal and liver cancer, more research studies are needed in order to understand fumonisins since they are less documented (Mwihia *et al*., 2008; Bii *et al*., 2012).

Several management strategies have been employed to control aflatoxin and fumonisin accumulation in maize. These include crop rotation, breeding, fertilizer application, storage systems, bio-control among others but all are still under trials and some strategies are even questionable (Ehrlich *et al*., 2014; Okoth *et al*., 2016). Breeding remains the most environmentally sound and economical hence this study which aims at identifying inbred maize lines and crosses resistant to aflatoxin and fumonisin accumulation that can be included in maize breeding programs.

1.2 Problem statement

In Kenya, deaths and cases of health-related problems due to mycotoxin consumption have been reported to re-occur. New cases of diseases like cancer which are associated with aflatoxin and fumonisin consumption have also arisen. Though the Food and Drug Administration (FDA) have recommended maximum levels of mycotoxins that should not be exceeded, this cannot control mycotoxin consumption in maize in Kenya because it is mostly produced by small scale farmers, consumed locally or sold in the neighbourhoods. There is also continuous food insecurity in the country since maize is a staple food and ear rot fungi lower its production. Studies have shown that toxigenic *Aspergillus* and *Fusarium* strains are the main causes of such mycotoxicosis though in Kenya and other countries, bio-control methods are still under trial. The study therefore aimed to characterize 44 isolates of *A. flavus* in the data bank at University of Nairobi mycology laboratory by mating types. Grouping the isolates by mating types was a critical step in determining genetic distribution of mating type genes in Nandi and Makueni counties. The study also aimed at quantifying the amounts of aflatoxin and fumonisin accumulated by selected maize inbred lines and developed hybrids grown and infected with isolated mycotoxigenic fungus from the aflatoxin hot zones in Kenya. This is a mycotoxin control strategy aimed at identifying resistant inbred lines and developing hybrids resistant to aflatoxin and fumonisin accumulation hence lowering amount of mycotoxin consumed in maize.

1.3 Justification

Maize production is often lowered by ear rot fungi belonging to the genera *Aspergillus* and *Fusarium.* They cause economic loss to maize producers and consumers. *Fusarium* and *Aspergillus* ear rots cause production of aflatoxins and fumonisins respectively,

which have been associated with mycotoxicoses and immune suppression in humans and animals. In Kenya, aflatoxins, produced by *A. flavus* and *A. parasiticus*, have caused severe cases of aflatoxicosis since early 1980's, with some outbreaks resulting in human deaths. Fumonisin contamination of maize has also been reported in the aflatoxin "hot zones" that is, the Eastern part of Kenya with the most common species being *F. verticilloides*. Consumption of fumonisin contaminated food is associated with oesophageal cancer and neural tube defects. Despite repeated aflatoxin outbreaks in Kenya and records of high fumonisin exposure, human capacity and expertise to reduce mycotoxin impact has remained limited. Further, no single technology has been identified that can be applied to reduce mycotoxin accumulation in maize. Therefore, developing maize hybrids resistant to aflatoxin, and fumonisin accumulation is an affordable and environmentally friendly means of preventing infection of maize with ear rot pathogens and their mycotoxins for both commercial and subsistence farmers.

1.4 Objectives

1.4.1 Broad objective of the study

To characterize *A. flavus* isolates recovered from Rift valley and Eastern Kenya and identify resistant maize germplasms to aflatoxins and fumonisins accumulation.

1.4.1 Specific objectives

i. To characterize *A. flavus* isolates by mating types

ii. To determine performance of maize inbred lines during germination, silking, pollen shed and blistering stages

iii. To screen maize inbred lines for aflatoxin and fumonisin accumulation levels

iv. To develop hybrids and screen the crosses for aflatoxin and fumonisin accumulation levels

1.5 Research Hypotheses

i. *Aspergillus flavus* isolates previously isolated from Rift valley and Eastern province belong to different mating types.

ii. Performance among maize inbred lines does not differ at germination, silking, pollen shed and blistering stage.

iii. Maize inbred lines and developed crosses are not resistant to infection by *A. flavus* and *F. verticilloides.*

iv. Maize inbred lines and crosses are not resistant to aflatoxin and fumonisin accumulation.

CHAPTER TWO

LITERATURE REVIEW

2.1Maize varieties and diseases

In Kenya, maize farming is the backbone of food security though the produce can barely keep up with the demand. The Kenya seed company limited has competently enlarged creation of certified seeds appropriate for different agro-ecological zones in East Africa. Maize varieties have been bred and preferred for a broad sphere of altitudes above the sea level, rainfall, temperature, soil type and other climatic conditions. Table 2-1 is a summary of maize varieties in Kenya and farmers need to buy seeds that can do well in their geographical regions (www. Kenyaseed.com).

However, various diseases lower maize production in Kenya and the main causal agents of maize diseases include fungi (ear rots, foliar diseases, smuts, stalk rots among others), bacteria (Stewart's wilt, stalk rot, bacterial leaf stripe among others), viruses (maize lethal necrosis, maize stripe virus, maize steak virus, maize chlorotic dwarf virus among others) and mollicutes (maize bushy stunt phytoplasma, corn stunt spiroplasma among others) (MacRobert *et al*., 2014). Maize Lethal Necrosis (MLN) greatly limits maize production in Kenya and farmers loose more than 60% of their usual yields. The disease began in the Rift Valley county of Bomet in September 2011 (National Farmers Information Service, 2012). In July 2012, up to 80% of the planted maize crop was ruined, and in 2015, farmers incurred losses of Kenya shillings 4.1 billion. This was because the disease destroyed crops across the affected areas exposing the country to food insecurity. Up to date, Kenya faces loses as the deadly maize disease ravages crops (Business daily newspaper, Thursday 28th April 2016). Aflatoxin and fumonisin accumulation in maize due to infection by *A. flavus* and *F. verticilloides* respectively also greatly affect agricultural productivity, health and trade in Kenya (Okoth, 2016).

Table 2-1: Maize hybrid varieties grown in Kenya

Variety	Yield per acre (bags)	Altitude range (M)	Days to maturity
H6213	52 x 90kg	1700-2100	160 – 190
H6212	52 x 90kg	1700-2100	160 – 190
H6210	50 x 90kg	1700-2100	160 – 190
H9401	48 x 90kg	1700-2100	160 – 190
H629	48 x 90kg	1700-2400	160 – 190
H628	46 x 90kg	1500-2100	150 – 180
H627	44 x 90kg	1500-2100	150 – 180
H626	42 x 90kg	1500-2100	150 – 180
H625	40 x 90kg	1500-2100	150 – 180
H614	38 x 90kg	1500-2100	160 – 190
H624	32 x 90kg	1000-1800	140 – 180
H623	28 x 90kg	1000-1800	140 – 190
H516	28 x 90kg	1000-1800	100 – 140
H515	26 x 90kg	1000-1800	120 – 150
H513	24 x 90kg	1000-1800	100 – 150
H511	23 x 90kg	1000-1800	100 – 150
PH4	20 x 90kg	0 - 1200	90 – 120
PH1	15 x 90kg	0 - 1200	75 – 120
DH01	15 x 90kg	800-1200	100 – 120
DH02	15 x 90kg	800 – 1200	100 – 120
DH03	15 x 90kg	800 – 1200	100 – 120
DH04	19 x 90kg	800 - 1200	100 – 120
Katumani Composite	15 x 90 kg	1000-1900	75 – 120
DLC1	13 x 90kg	1000-1900	75 – 120

Source: www.kenyaseed.com

2.2 Mycotoxins reported in maize

Mycotoxins are toxic metabolic by-products of fungi and cause health problems in animals (Table 2-2). Low weight gain, capillary brittleness, infertility, reduced immunity and even death have been associated with mycotoxins. No animal is known to be resistant to mycotoxins though younger animals are more susceptible than the older ones (Donald, 1999). In maize, aflatoxins, fumonisins, deoxyrivelenon, trichothecenes, ochratoxins, citrinin, cyclopiazonic acid, and sterigmatocystin have been reported (Donald, 1999). In developing countries, contamination of food supplies by these and other naturally occurring toxins is of concern in the rural communities (Bhat *et al*., 1997).

Table 2-2: Common mycotoxins associated with maize

Mycotoxin	Fungi associated	Symptom/toxicology
Aflatoxin	*Aspergillus flavus*, *A. parasiticus*	liver necrosis, liver cancer, low growth rate, low immunity, carcinogen
Fumonisin	*Fusarium moniliforme*, *F. proliferatum*	leukoencephalomalacia in horses, porcine pulmonary edema
Deoxynivalenol	*Fusarium graminearum*	feed refusal, reduced weight gain, diarrhea, vomiting
Trichothecenes	*Fusarium graminearum, F. culmorum, F. poae*	alimentary toxic aleukia, necrosis, hemorrhages, oral lesion in broiler chickens
Ochratoxins	*Penicillium verrucosum, Aspergillus ochraceus*	porcine nephropathy; various symptoms in poultry
Citrinin	*Penicillium* sp., *Aspergillus* sp.	kidney damage
Cyclopiazonic acid	*Penicillium* sp., *Aspergillus* sp.	Neurotoxin
Sterigmatocystin	*Aspergillus* sp., and others	carcinogen, mutagen

(Source: Donald, 1999)

2.2.1 Primary mycotoxins in maize

The two primary mycotoxins affecting maize are aflatoxin and fumonisin. Aflatoxin is produced by the *Aspergillus* family of moulds and is common in maize, cotton, pea nuts and tree nuts. The fungus growth continues in storage under certain environmental condition. In maize, aflatoxin accumulation is related to drought followed by periods of high humidity (Okoth, 2016; Donald, 1999).

Fusarium verticilloides primarily produce fumonisin as a secondary metabolite which is carcinogenic. Infection of maize by *F. verticilloides* and contamination with

fumonisins are influenced by environmental factors. For example, dry weather early in the season followed by wet weather during silking of the maize plant and insect infestation increases fungal infection of the kernels and production of fumonisin (Payne, 1998). Other factors include insect infestation, pre-harvest and post-harvest handling. Though aflatoxins are common in maize and groundnuts, occurrence of fumonisins in maize is 100% or close to as observed in different parts of Africa (Nyashadzashe, 2014; Fandohan *et al*., 2013).

Management practices such as crop rotation, irrigation, good insect control, timely fertilization, proper drying and removal of damaged kernels reduce stress to maize plants hence lowering accumulated aflatoxin and fumonisin levels. These toxins are, however, relatively stable and long term control strategies like breeding for resistance to toxigenic fungi are promising (Guo *et al*., 2017; Okoth, 2016; Wagacha and Muthomi, 2008).

2.2.2 *Aspergillus flavus*

The fungus is filamentous and grows by producing thread-like branching filaments called hyphae. A network of hyphae called mycelium secretes enzymes that digest complex food sources. The mycelium then absorbs the small molecules that fuel additional fungal growth. *Aspergillus flavus* grows on many nutrient sources (Grubisha and Cotty, 2010), and can also be pathogenic on several plant and animal species, including humans and domestic animals. It infects seeds of maize, peanuts, cotton, and nut trees (Okoth, 2016), sporulates on injured seeds such as maize kernels and usually the infection is asymptomatic. The *A. flavus* is yellow green in appearance when growing on maize kernels and is common in nature though its population increases during hot dry weather. The growth of the fungus is usually favoured by hot and dry conditions with optimum temperature of 37 ºC (98.6 F), but the fungus readily grows between the temperatures of 25-42 ºC (77-108 F) (Felicia *et al*., 2011). It contaminates food with aflatoxins which is a class I carcinogen (Payne, 1998; Grubisha and Cotty, 2010; Okoth, 2016).

Toxigenic strains of *A. flavus* produce aflatoxin B_1 and lesser amounts of B_2. Aflatoxin B_1 is the most toxic of the naturally occurring aflatoxins. In the developed countries like United States of America (USA), aflatoxins consumption in humans and animals

are highly regulated by the Food and Drug Administration (FDA) which has set action levels of total aflatoxins allowed. Commodities with aflatoxin concentrations higher than established limits are either reprocessed or destroyed (Felicia *et al*., 2011; Azziz-Baumgartner *et al*., 2005). However, in developing countries where aflatoxin levels are not regulated, outbreaks of aflatoxicosis and associated deaths in human populations occur periodically (Okoth, 2016; Azziz-Baumgartner *et al*., 2005). In addition to aflatoxins, *A. flavus* produces cyclopiazonic acid (CPA) which contaminates agricultural products and affects the liver, kidney and gut in animals (Hedayati *et al*., 2007). Further still, the fungus causes aspergillosis involving skin, oral mucosa and subcutaneous tissue (Hedayati *et al*., 2007).

Contamination of crops by aflatoxin can occur when the crops are growing in the field, during harvest, or after harvest. However, contamination mostly occurs due to poor handling of the crops after harvest which increases survival rates of the fungal species. In Kenya, aflatoxicosis outbreaks have been experienced in Makueni and Kitui counties. Both areas are prone to food shortage due to very high temperatures, poor and unreliable rainfall (Okoth, 2016; Mwihia *et al*., 2008).

Currently, maize production practices that substantially reduce aflatoxin contamination have not been achieved. Though cultural, chemical, physical, biological and breeding methods have been employed, developing maize hybrids resistance to *A. flavus* infection and successive aflatoxin contamination is considered one of the best and cheapest strategies currently available to reduce aflatoxin losses though resistant commercial hybrids are still not available (Williams *et al*., 2006; Abbas *et al*., 2009; Ehrlich *et al*., 2014; Okoth, 2016; Guo *et al*., 2017).

2.2.2.1 Characterization of *A. flavus* species

Aspergillus spp. belongs to Ascomycota phylum and Trichomaceae family. In the genera, *A. flavus* and *A. parasiticus* are key aflatoxin producers which contaminate food worldwide (Rodrigues *et al*., 2007). The two species can be characterized based on types of toxins they produce and size of sclerotia. Their characterization can also be based on vegetative compatible groups (VCG) and mating types (MAT) comprised of *MAT1-1* and *MAT1-2* (Rodriguez *et al*., 2007; Horn *et al.,* 2009). Isolates in different

VCGs exhibit different sizes of sclerotia, MAT genes, aflatoxin production and aflatoxin inhibition. However, isolates in the same VCG tend to produce similar mycotoxins, sclerotial size and MAT genes (Rodriguez *et al*., 2007; Sweany *et al*., 2011). *Aspergillus flavus* exist in two morphotypes either as S or L strains. The S strain produces much higher aflatoxin concentrations than the L strain. However, some isolates produce no aflatoxins at all and are called atoxigenic strains. Several VCGs occur within a morphotype though gene flow does not occur between individuals with different VCGs (Ehrlich and Cotty 2004; Okoth *et al.,* 2012).

2.2.3 Fusarium verticilloides

Fusarium is a filamentous fungus common in soil and infects plants. The fungus produces mainly fumonisins and trichothecenes (Howard, 2003). Fumonisins are a group of mycotoxins derived from *Fusarium*, Liseola section and more specifically it refers to Fumonisin B1, Fumonisin B2 and Fumonisin B3. In contaminated maize, fumonisin B1 is the most prevalent and the most toxic. *F. verticilloides* belongs to; Class: Sordariomycetes and Family: Nectriiaceae (Howard, 2003).

Environmental factors such as temperature, humidity and rainfall during pre-harvest and harvest periods affect the extent of contamination with fumonisin. Hot and dry weather followed by periods of high humidity are associated with high levels of fumonisin accumulation. Fumonisin accumulation in raw corn can also be high under improper storage conditions for example when the moisture content of the harvested raw corn during storage is 18-23%. Other factors include agronomic and storage practices, vulnerability of the plants to fungal invasion during all phases of growth, storage and processing. (FDA, 2001, Felicia *et al*., 2011; Bii *et al*., 2012).

Acceptable maximum limits of fumonisins in human food and animal feeds have been set by FDA. The body believes that controlling fumonisins to recommended levels can reduce exposure to fumonisins that may be found in maize products intended for human and animal consumption (Food and Drug Administration, 2001). In degermed dry milled maize products, such as flaking grits, maize grits, maize meal, maize flour, with fat content of < 2.25%, dry weight basis, recommended maximum level for fumonisin is 2mg/kg. In cleaned corn intended for popcorn production, recommended maximum

level for fumonisin is 3 mg/kg (Food and Drug Administration, 2001). In Kenya, 2mg/kg is the maximum acceptable fumonisin limit in human food and feed. Contamination of maize by fumonisin has been reported in the eastern part where aflatoxicosis has repeatedly occurred. In these areas, *F. verticilloides* was the most common species isolated (Bii *et al*., 2012).

2.3 Mode of entry of fungal pathogen into the maize

The way fungus gets into the host maize plant is important for the triumphant creation of a parasitic association between the pathogen and the host. There are three main routes for the infection to occur. These include silk route, entry through wounds and systematic entry (Paulina, 2015). The fungus can get into the maize plant via silk route which occurs through growth of mycelium. The mycelia are produced by germinated spores down the silks to the kernels and the cob. The fungus can also get into the maize plant through wound created by a spore-carrying insect and bird (Paulina, 2015). Fungus entry through systematic growth of the pathogen via the stalk occurs through the soil borne hyphae which germinate infecting the germinating seed and its roots. The fungus then moves up the plant through systematic growth (Paulina, 2015).

Susceptibility to fungal infection through silk route is known to be highest during the first 2 - 6 days after silk emergence and studies support that the primary infection pathway in maize kernels is through silk channel and silk route (Paulina, 2015). In this study, infection was induced through the silk route when silking was about an inch (2 - 4 days after silk emergence). Maize lines and hybrids were therefore artificially infected by injecting the silks when about an inch long with inoculum containing *A. flavus or F. verticilloides* spores.

2.4 Toxigenicity of *Aspergillus flavus* and *Fusarium verticilloides* species

Aspergillus flavus and *A. parasiticus* are great producers of aflatoxins and cause contamination of food worldwide (Krishnan *et al.,* 2008; Grubisha and Cotty, 2010; Felicia *et al.,* 2011). They produce aflatoxins B1, B2, G1 and G2 (Krishnan *et al*., 2008; Felicia *et al.,* 2011). Various studies link aflatoxin levels in food samples to the incidences of liver cancer (Krishnan *et al*., 2008; Abdin *et al.,* 2010).

Maize is cultivated worldwide and contamination of maize with aflatoxin is of concern because it is a staple food in many countries. In Kenya, 40% diets both in urban and rural areas are comprised of maize and maize products. Though Kenya has adopted the World Health Organization (WHO) (1969) aflatoxin limits of 20µg/kg for humans, enforcing this is difficult. Maize is produced majorly by small scale farmers, consumed by the subsistence farm household or sold in the neighbourhoods. The produce therefore does not appear typically in the national commercial markets where aflatoxin testing is routinely performed (Mwihia *et al*., 2008).

Fusarium species produce fumonisin and consumption of fumonisin is associated with oesophageal cancer in humans. *Fusarium verticilloides* produces Fumonisin B_1 and B_2. The pathogen is associated with deeply invasive infections in immuno-compromised patients. They usually grow without causing visible symptoms of the disease (Bii *et al*., 2012). Though studies have associated *Fusarium* species with deeply invasive infections in immune-compromised patients leading to high incidences of oesophageal and liver cancer, more research studies are needed in order to understand fumonisins since they are less documented (Mwihia *et al*., 2008; Bii *et al*., 2012).

Eating of grains and cereals contaminated with mycotoxins is a major health problem in the developing world and the population is usually exposed to these mycotoxins primarily by eating contaminated food. Poor storage conditions of grains and cereals in the presence of high humidity and temperature also provides a good environment for fungal growth in contaminated storage facilities (Krishnan *et al*., 2008; Felicia *et al.,* 2011, Bii *et al*., 2012). One of the most severe episodes of aflatoxicosis in Kenya was in 2004. Repeated cases of aflatoxin outbreak in Makueni, Kitui and Machakos have been reported though no acute fumonisin outbreak has been confirmed to occur (Bii *et al*., 2004). However, co-occurrence of fumonisin with aflatoxin is known to play a crucial role in the promotion of carcinogenesis. It is therefore important to study both fumonisin and aflatoxin producing strains and their contamination of maize grains in areas that have experienced repeated aflatoxin outbreaks in Kenya (Bii *et al.,* 2012).

2.5 Aflatoxin challenge in Africa

2.5.1 Aflatoxin exposure and effects

Aspergillus species infect cereals including maize, millet, rice, sorghum, wheat, legumes, including groundnuts, roots and tubers. The crops are contaminated by aflatoxins either in the field or during storage. These contaminated crops are used in the industries for production of processed foods and animal feeds resulting in contaminated processed foods (Okoth, 2016). Though aflatoxin contamination of food and feeds in Africa is a challenge, unawareness on aflatoxin contamination, the health risks and potential mitigation measures exist. Additionally, due to low coverage of vaccination against hepatitis B and absence of vaccination against hepatitis A, the risk of developing liver cancer is enhanced with consumption of contaminated food and food products (Okoth, 2016).

Exposure to aflatoxin poisoning can occur through ingesting aflatoxins, through the skin (dermal) and inhalation routes. The age of the victim, aflatoxin dose and duration of exposure to aflatoxin majorly affect the toxicology leading to a range of consequences. According to FDA (1969), the recommended aflatoxin level in maize is 20µg/kg. When taken in high doses, aflatoxin can cause acute toxicity and death in mammals, birds and fish. However chronic lower doses cause liver cancer, and chronic immunosuppression. In young children, aflatoxin consumption is associated with kwashiorkor and poor growth. All aflatoxin doses have a cumulative effect on the risk of cancer (Bankole and Adebanjo, 2003; Okoth *et al*., 2012; Okoth, 2016).

2.5.2 Aflatoxin policies in Africa

In Africa, guidelines on the tolerable aflatoxin levels in food differ widely in countries ranging from 0-50µg/kg. Research studies done on the condition of food and safety legislation in Africa have found out important gaps, improper connections between strategies, out-dated laws that could not address a whole range of food safety concerns (Okoth, 2016). African countries have failed to meet regulations for aflatoxin levels in their exports and this has caused a negative impact on the African trade with a loss estimated at US$750 million yearly. African countries therefore need to implement regulations on aflatoxin levels and maintain larger trading blocks. This will ensure

negotiations occur with importing countries and larger bodies like the World Trade Organization (Okoth, 2016). Table 2-3 summarizes aflatoxicosis outbreaks in East Africa.

Table 2-3: Reported human aflatoxicosis outbreaks in East Africa (1981-2016)

Year	Numbers affected	Country and county/district	Sources of aflatoxin	Number of deaths
1981	12	Kenya- Machakos	Maize	12
1988	3	Kenya- Meru North	Maize	3
2001	29	Kenya- 3 Meru North, 26 Maua	Maize	16
2003	6	Kenya-Thika	Maize	6
2004	317	Kenya-Eastern Central	Maize	125
2005	75	Kenya-Machakos, Makueni, Kitui	Maize	32
2006	20	Kenya-Machakos, Makueni, Kitui	Maize	10
2007	4	Kenya- Kibwezi, Makueni	Maize	2
2008	5	Kibwezi, Kajiado, Mutomo	Maize	2
2016	67	Tanzania, Dodoma,Manyara, Chemba and Kondoa district	Cereals	14

(Source: Okoth, 2016)

Aflatoxin consumption has negative impacts on health, food security and trade. In Africa, most aflatoxin susceptible commodities produced do not meet internationally accepted standards. This is evidenced by the continuous rejection of the produce by major buyers, processors, traders and international regulatory agencies preventing the produce from entering key export markets. However, the rejected produce enters the African food and feed value chains increasing the risk of exposure to aflatoxins (Okoth, 2016).

2.5.3 Vulnerability to aflatoxins in Africa

In Africa, there is poor awareness about aflatoxins, appropriate aflatoxin control measures in the fields and in storage making most African countries report negative health effects of aflatoxin consumption (Okoth, 2016). Therefore, aflatoxin occurrence in Africa has grown to be a great challenge to agriculture, health and trade (Hell *et al.,* 2008; Okoth, 2016).

In Kenya, Uganda, Nigeria, Ghana, Togo and Benin, age, sex, socioeconomic status, agro-ecological zones and weaning status have been found to have a great influence on aflatoxin poisoning. In Nigeria, low income mothers and the rural populations were vulnerable to aflatoxins (Gong *et al*., 2002; Jolly *et al.,* 2006; Ezekiel *et al.,* 2014; Leroy *et al*., 2015). A positive correlation between socio-economic status and exposure to aflatoxins was reported from a longitudinal evaluation of two cohorts from South Western Uganda (Kang *et al.,* 2015). Further still, reported cases of liver cancer in Uganda are attributed to consumption of contaminated maize and peanuts (Okoth 2016). In Sub-Saharan Africa, Tanzania is a major maize producer ranked among the top 25 maize producers in the world. However, the production is lowered by mycotoxins majorly aflatoxins and fumonisins. Post-harvest losses of maize are significant, up to 30 - 40% in some rural areas (Suleiman and Rosentrater, 2015). In Africa as a whole, aflatoxins have been frequently detected at high levels in sera and liver of children thus implicating aflatoxins in the pathogenesis of kwashiorkor (Okoth *et al.,* 2012; Onyewelukwe *et al.,* 2012; Castelino *et al.,* 2015).

Studies on aflatoxin levels in Kenya on market maize within the period 2006-2009 indicated widespread contamination in both maize grain and its products. This involved random environmental sampling of maize and maize products for food and feed from retail shops within Nairobi county. Of the 144 food samples collected, 120 (83%) had levels greater than the regulatory limit of 20µg/kg. The aflatoxin levels ranged from 0.11µg/kg to values as high as 4,593.93µg/kg (Okoth *et al.,* 2012).

2.5.4 Primary interventions to aflatoxins and fumonisin exposure

Factors that influence fungus infection process and toxin formation include soil type, soil condition and availability of viable spores. *Aspergillus flavus* infection is favoured by high soil and air temperatures, drought, nitrogen crowding of plants and conditions that help in conidia dispersal during silking. Presence of invertebrate vectors, grain damage, oxygen and carbon dioxide levels in the stores, inoculum load, substrate composition, fungal infection levels, prevalence of toxigenic strains and microbiological interactions are the factors that influence the rate of fungal infection. However, control of *A. flavus* in the field can be achieved through crop rotation and management of crop residues (Okoth, 2016).

In order to alleviate the effects of mycotoxin producing fungi, maize varieties that are less susceptible to fungal growth need to be planted, harvesting be done during dry weather when the husks are mature, and any damaged maize kernels or cobs be removed early (Hell *et al.,* 2008). Traditional post-harvest methods such as manual sorting of seeds, winnowing to get rid of lighter grains, which were assumed to be lighter because of insect or mould damage, washing, dehulling contribute up to 40-80% reduction in aflatoxin levels in grains. In Kenya, ash is traditionally used to reduce insect pests that have been documented to increase the effects of fungi in maize kernels. Smoking is useful not only in reducing moisture content in grains but also in reduction of the effects of insects and act as a fungicide. Local maize varieties in Benin have been found to accumulate lower aflatoxin levels than the imported varieties (Okoth, 2016). Therefore, planting of resistant maize varieties developed through crossing in addition to the traditional control measures can highly reduce aflatoxicosis in Africa.

2.6 Pre-harvest aflatoxin accumulation control methods

The pre-harvest aflatoxin control strategies are considered the most promising, cheap and easy to use plan for farmers. Adequate combination of approach is required for prevention of mycotoxin contamination in the field and development of plants resistant to fungal infection. Plants that reduce the toxic effects of the mycotoxins themselves or interrupt mycotoxin biosynthesis can as well be developed. The pre-harvest management strategies include breeding, bio-control and good agricultural practices (Okoth, 2016).

2.6.1 Breeding

Maize hybrids are produced by crossing two inbred lines. An inbred line is a plant that only has the traits of the self- pollinated plant. Self- pollination occurs when the pollen from a maize plant fertilizes its own ear. The hybrids and inbred lines are both homogeneous that is, every plant of the same hybrid or inbred line is identical genetically. Development of inbred lines with desirable characteristics is the researcher's first step of field breeding. Therefore, parent lines must be properly selected in order to develop top performing hybrids (Mandal, 2014; Dhillon and Prasanna, 2001).

Production of hybrid maize seed involves deliberately crossing a female parent with a male parent in isolated fields. Therefore, for successful crossing to occur, the identity and arrangement of the two parent populations must be considered. This is because maize hybrids provide farmers with varieties containing improved genetics such as high yield potential and unique trait combinations to counter diseases and adverse growing conditions (Mandal, 2014; Dhillon and Prasanna, 2001). Therefore, through hybrid maize creation, the farmer's productivity is improved, and a reliable, sustainable food supply ensured. High yields, increased value and reduced production costs is generated through hybrid production. Hybrid vigour or heterosity is established when two genetically unrelated inbred parents are crossed to create a hybrid. The hybrid vigour is important to the high productivity by the maize farmers (Mandal, 2014; Dhillon and Prasanna, 2001).

Plants that emerge from hybrid seeds are bigger, stronger and more vigorous compared to those grown from the inbred lines. This is because maize is a highly cross-pollinated crop and its population is composed of freely interbreeding individuals that are heterogeneous. Inbred lines, however, are self-pollinated hence the inbred lines are affected by inbreeding depression which results in reduction in vigour, size, yield and appearance of lethals and sub-lethals due to the uncovering of recessive genes. Additionally, inbred lines are more sensitive to micronutrient deficiencies than hybrids. This makes inbred lines unsuitable for adoption as commercial products (Mandal, 2014; Dhillon and Prasanna, 2001). Heterozygosity is thus an essential feature of maize cultivars and must be maintained in the end products. Therefore, when two unrelated inbred lines are crossed to form a hybrid, the resultant seed produces plants with restored vigour and the yield are significantly higher than either of the two parents. Hybrid varieties have the advantage of higher yield potential and uniformity and are preferred over open pollinated populations due to the higher level of heterosis. Maize farmers prefer seeds with hybrid vigour which is important for the high productivity. Therefore, development of hybrid maize has the capacity to increase world's agricultural production (Mandal, 2014; Dhillon and Prasanna, 2001).

Classical plant breeding uses deliberate crossing of closely or distantly related individuals to produce new crop varieties or lines with desirable properties. Cross

breeding in plants introduces genes from one variety or line into a genetic background (Okoth, 2016). Generally, plant resistance is a highly beneficial approach to lowering or stopping *A. flavus* infection and succeeding aflatoxin accumulation. Prospective biochemical and genetic resistance markers have been established in crops especially in maize. These are used in maize as selectable markers in breeding for resistance to aflatoxin contamination (Okoth, 2016).

Maize varieties that are resistant to drought and insect infestations have been reported. Such maize varieties have been found to have relatively reduced pre-harvest aflatoxin contamination compared to the check cultivars in the other parts of the world. Breeding for stress tolerant cultivars is thus seen as the most suitable strategy for aflatoxin management (Okoth, 2016).

In a maize plant, male and female reproductive components are present though it is monoecious meaning the male flowers (tassels) are on a separate part of the plant from the female flowers (ear shoots). Therefore, it can fertilize itself and nearby maize plants. Three visible reproductive system components exist in a mature maize plant. These include; the tassel at the top of the plant, the ear which has the embryo and the silks which are found on top of the ear. The tassel produces pollen needed to produce seed and the ears produce silks (Westgate *et al*., 2003). Pollen moves to fertilize seed embryo on the ear through the silk. Under favourable conditions, each fertilized embryo produces a new seed since there is one silk for each embryo on the ear. Wind is the agent for pollen dispersal from the tassels to the silks of ear shoots. Typically, a corn plant produces approximately 4, 5000,000 pollen grains and 300-600 silks (Westgate *et al*., 2003). When seeds develop only at the base of the cob, this is a clear indication that there was early male pollen shed or late female silking. However, when seeds develop at the tip of the cob indicate late male pollen or early female silks. On the other hand, when there are few seeds scattered on the cob, this indicates inadequate pollen supply. Therefore, for crossing to be successful, the timing of pollen shed of the male and silking of the female must coincide.

2.6.2 Bio-control

This involves using living organisms to suppress the fungus growth. One of the registered bio-control products in Kenya and Nigeria is the Aflasafe^{TM} (Okoth, 2016;

Ehrlich, 2014). It uses atoxigenic *A. flavus* that can out-compete closely related toxigenic *A. flavus* in field environments hence lowering levels of aflatoxins in crops (Ehrlich, 2014; Okoth, 2016). The International Institute of Tropical Agriculture (IITA) has published stable reduction of contamination by aflatoxin in maize and groundnuts by 80-90%. The Aflasafe is under trial in Zambia, Ghana, Malawi, Mali, Mozambique, Tanzania and Uganda and is also ready for registration in Burkina Faso and Senegal. Four separate sets of atoxigenic have been isolated from locally-grown maize to form bio-control products called Aflasafe KE01™ and Aflasafe BF01™ in Kenya and Burkina Faso, respectively (Ehrlich, 2014; Ehrlich *et al.*, 2014; Okoth, 2016). Experiments are still on-going on the use of the other organisms like bacteria and yeasts as bio-control agents (Okoth, 2016).

The use of biological control method is presently the most favourable strategy for lowering pre-harvest contamination of cereals, groundnuts and tree nuts with aflatoxin. Non-aflatoxin producing *A. flavus* is introduced into the environment and out-competes the naturally occurring aflatoxin producing strains in the soil (Ehrlich, 2014). The technology is under trial in Kenya in the aflatoxin hot spots (Ogumbayo *et al.,* 2013; Atehnkeng *et al.,* 2008; Hell *et al.,* 2008). Though bio-control method is considered the most promising strategy for aflatoxin control in plants, there are questions that need to be addressed before the bio-control strategy is approved as the most convincing strategy for elimination of pre-harvest aflatoxin contamination (Ehrlich, 2014). These include cost effectiveness of atoxigenic *A. flavus* as bio-control, and their safety on human and animal ingestion because gene flow may make non-aflatoxigenic strains become superior aflatoxin producers in future. This is because the diversification of *A. flavus* is thought to be a result of its potential to outcross by sexual recombination under special conditions in the soil. When such outcrossings occur, non-aflatoxigenic strains could gain ability to become aflatoxigenic strains (Ehrlich *et al.,* 2014). When using bio-control strategies, precaution should also be observed to prevent unnecessary crop destruction or damage to the soil micro-flora (Ehrlich *et al.,* 2014). The long term use of Aflasafe bio-control products to the farmers and the environment is therefore questionable (Ehrlich *et al.,* 2014). Developing ear rot resistant maize varieties to lower aflatoxin and fumonisin build up is a broadly accepted safe and easy to use option (Okoth, 2016).

2.6.3 Good agricultural practices

These include selection of healthy seeds, early planting, mixed cropping, treatment of foliar diseases, application of lime or gypsum, mulching, maintenance of optimal density of plants in the field, irrigating the farm during drought season, removal of dead plants from the field before harvest and crop rotation (Okoth, 2016). These practices lower fungus load in the soil, lower environmental stress on the plants and promote growth of healthy plants in the field. The good agricultural practices (GAPs) are encouraged in order to reduce aflatoxin contamination (Okoth, 2016).

2.7 Post-harvest management strategies

Aflatoxins cause contamination of goods at any point along the value chain. However, high contamination occurs during storage. Proper crop management practices at harvest and postharvest should be observed for they offer effective methods of preventing or at least lowering infection by *A. flavus* causing aflatoxin contamination (Okoth, 2016). The post-harvest management approaches include harvesting at maturity, circumventing kernel damage, fast drying on stands to avoid soil contact, drying seeds to less than 13% moisture level, practising shelling methods that reduce grain damage, sorting, use of clean and aerated storage structures, control of insect damage and avoiding long storage periods (Okoth, 2016). In Africa, there is no single aflatoxin control strategy that has been found effective for wide scale adoption. Each approach has its own advantages and disadvantages (Okoth, 2016).

2.8 Aflatoxin testing methodology

Immunoassays are considered better alternatives during regular or field detection of aflatoxins. The commonly used quick screening methods include Enzyme Linked Immunosorbent Assay (ELISA), fluorometric methods, lateral flow devices and other tests that are either a yes or no results indicating contamination above or below a set control level. The methods are useful for testing the toxin level where fast conclusions are necessary in the granaries, silos and factories (IARC, 2012).

In Africa, regarding cost, speed of analysis, availability of personnel and facilities as well as test characteristics (sensitivity, specificity and reproducibility) Thin Layer chromatography (TLC), High Performance Liquid Chromatography (HPLC), Enzyme

linked immunosorbent assay (ELISA) and other immunoassays are preferable (Okoth, 2016). However, ELISA is favourable because the sample does not have to be purified and many samples can be quantified in just one experiment (Okoth, 2016; Pascale and Visconti, 2008). Direct competitive ELISA is thus the most preferred in Africa for most aflatoxin and fumonisin research studies because it is less expensive, more rapid and has high throughput (Guo *et al*., 2017; Okoth, 2016). Chromatographic methods are expensive and thus the local researchers cannot meet the cost in the few laboratories that conduct aflatoxin testing (Okoth, 2016).

2.8.1 Aflatoxin and fumonisin assay principle using ELISA

The Helica total aflatoxin assay (Helica Biosystems Inc.) is a solid phase competitive inhibition enzyme immunoassay. The assay is antibody specific to aflatoxin and is enhanced to cross react with all four subtypes of aflatoxin coated to a polystyrene microwell. Toxins are obtained from a ground sample with 70% methanol. The extracted sample and horseradish peroxidase (HRP)–conjugated aflatoxin are mixed and put on the antibody-coated microwell. Extracted aflatoxin from the sample and HRP- conjugated aflatoxin compete to bind to the antibody coated microwell. Microwell contents are emptied and non-specific reactants removed by washing. Colour (blue) is obtained when an enzyme substrate tetramethylbenzidine (TMB) is added. The intensity of the colour is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of aflatoxin in the sample or standard. Therefore, as the amount of aflatoxin in the sample or standard escalates the strength of the blue colour decreases. An acidic stop solution is added which changes the chromagen colour from blue to yellow and the microwells are gauged optically by a microplate reader with an absorbance filter of 450nm. The optical densities (ODs) of the samples are compared to the ODs of the kit standards and an interpretative result is obtained (Helica Biosystems total aflatoxin assay).

Fumonisin and aflatoxin assay principles are the same except that in the fumonisin assay principle, fumonisin specific antibody is optimized to cross-react with the three fumonisin subtypes coated to a polystyrene microwell and toxins are obtained from a ground sample with 90% methanol.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

The field trials in this study were done at Kenya Agricultural and Livestock Research Organisation (KALRO) in Katumani and Kiboko. Kiboko is located in Makindu Sub-County of Makueni County, about 160 kilometres (km) South East (SE) of Nairobi, along Mombasa - Nairobi Highway, about 12 km before Makindu Town. It lies between latitude 2° 10' and 2° South and longitude 37° 40' and 37° 55' East. The farm is surrounded by the Kyulu Hills to the South, agro-pastoral Kamba community to the North and East and the pastoral Maasai community to the West. The station is 975 m above sea level and receives between 545 and 629 mm of rainfall coming in two seasons. The long rains season is between April and May while the short rains season is between October and January. Kiboko is a hot dry region with a mean annual temperature of 22.6 ºC, mean annual maximum of 28.6 °C and mean annual minimum of 16.5 °C. The soils are well drained, very deep, dark reddish brown to dark red, friable sandy clay.

Katumani is located in Machakos county, on latitude 1º 34' 60S and longitude 37º 15' 0E, and at an altitude of about 1600m above sea level. The center is located about 80 km south east of Nairobi, and 8km south of Machakos town, along the Machakos – Wote road. Katumani Research center experiences a semi-arid tropical climate described as Agro-ecological zone IV, with a bi-modal pattern of rainfall. The first rains, traditionally known as the long rains, come in the months of March to May, with the peak in April. Following the long rains is an extended dry period to mid-October when the second season (short rains) commences. The short rains season has its peak in November, and begins to taper off towards mid-December. Mean annual rainfall is 655 mm and the average seasonal rainfall for the long rains is 272 mm while that for the short rains is 382 mm. The seasonal and annual rainfall totals exhibit wide variation with mean maximum and minimum temperatures at the center being 24.7 ºC and 13.7 ºC, respectively.

These two sites were selected because of the previous recurrent aflatoxin outbreaks in the area. Mycotoxigenic strains culture collections of *Aspergillus flavus* and *Fusarium verticilloides* in the mycology laboratory used for infecting the inbred lines and crosses were also isolated from these two regions. Figure 3-1 shows locations of the two study sites.

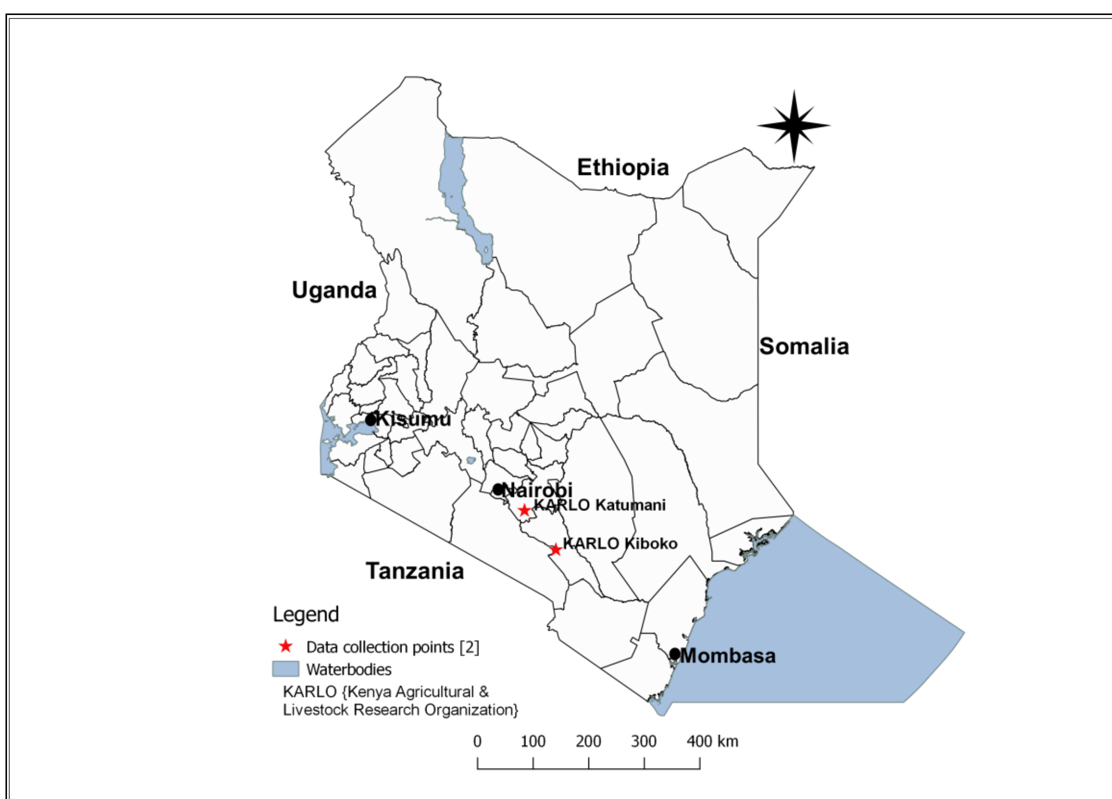

Figure 3-1: Map of Kenya showing Katumani and Kiboko Research centres (Source: Abigael Ouko)

3.2 Source of fungal isolates and inoculum

Forty-four *Aspergillus flavus* isolates previously isolated from Makueni (Table 3-1) and Nandi (Table 3-2) were maintained at the mycology laboratory, University of Nairobi (UoN). The three toxigenic *A. flavus* strains used for inoculating maize inbred lines and the hybrids were the most virulent strains selected among the isolates. The total aflatoxin levels (µg/kg) production was determined in yeast extract with supplements (YES) agar as described in Okoth et al. (2012) and Nyongesa et al. (2015). The fumonisin producing strains of *F. verticilloides* were also previously isolated from

eastern and Rift Valley regions of Kenya and maintained at the mycology laboratory culture collection (Table 3-3).

Table 3-1: *Aspergillus flavus* strains from Makueni county

Aspergillus flavus strain identity	Aflatoxin (µg/kg) produced in yeast extract sucrose
1VM201365	152965
1VM40018	97184
1VM100095	92249
1VM100130	88878
2M2002LG	74367
2M1983G	67350
1VM250WY	47653
2VM964	29484
1VM132G	23069
2VM882G	19038
1VM414	13574
1VM100957	11971
1VM201122	11292
1VM130LG	10199
1VM100079	9089
1VM350G	2912
3VM566G	1850
2M1983DG	1165
2M1002LG	962
1VM195	785
1VM131LG	602
3VM566	332
1VM201204	22
1VM300566	0

(Source: Okoth *et al*., 2012 and Nyongesa *et al*., 2015)

Table 3-2: *Aspergillus flavus* strains from Nandi county

Aspergillus flavus strain identity	Aflatoxin (µg/kg) produced in yeast extract sucrose
40245BMIG	116666
40041BM38Y6	80664
60801BSIYG	23883
40224BSIYG	22203
40296BSY	12803
60756	11292
50400BM2YG	247
40243BMIB	151
40013BM126	145
40267BSIYG	96
60661BMIYG	91
504820BMIYG	77
50536BMIYG	77
60690BMIYG	57
40143BM112G	46
60572 BM2Y	46
60795BM8BJ	22
40025BSIYG	22
50444 BSIYG	0

(Source: Okoth *et al*., 2012 and Nyongesa *et al*., 2015)

Table 3-3: Fungal strains used for inoculum production

Fungal collection code	Species	County of origin
201365	*Aspergillus flavus*	Makueni
100130	*A. flavus*	Makueni
100095	*A. flavus*	Makueni
K38	*Fusarium verticilloides*	Machakos
K48	*F. verticilloides*	Machakos
K54	*F. verticilloides*	Makueni

(Source: Mycology culture collection data base, UoN)

3.3 Preservation of fungal cultures for inoculum preparation

Aspergillus flavus strains (201365, 100095 and 100130) and *F. verticilloides* strains (K38, K48 and K54) previously cultured in soil at 4 ℃ in the fridge were transferred to 5 - 2 salt media (Cotty and Misaghi, 1984) for culture maintenance. Silica vials were prepared by pouring silica granules 3 - 8 mesh (silica gel amorphous-Columbus chemical Industries, Canada) into 14 ml glass vials until about 2/3 full. Uncapped vials were placed in a beaker and covered with aluminium foil, baked in an oven at 180 ℃ for 105 minutes. Vial caps were autoclaved at the same time in a separate beaker for 20 minutes at 120 ℃. After autoclaving, the container of caps was placed in an oven at 60 ℃ to dry.

Milk of 200 ml (3 % w/v skim milk) was placed in 500 ml bottle and autoclaved for 12 minutes at 120 ºC. The bottle was removed immediately after the cycle was complete and cooled to minimize carmelization of the milk. Using a pipette, 500 µl of sterilized milk was transferred into sterile tubes and covered with sterile caps.

Sterile swabs, silica vials and milk tubes were placed in ice and allowed to cool. Once cool, sterile swabs were dipped into milk vials and the spores swabbed from 5 - 10-day old cultures in the 5 - 2 salt media plates. The spores were transferred to milk vial and swabbing in a plate was repeated to capture more spores. Once a very dense spore suspension had been made in the milk vial, the milk was poured into the silica vial. The vial was immediately capped, shaken and the vial vortexed to disperse fungal spores over silica. During vortexing, once dryness was evidenced by dust starting to form in silica vials, the vial was immediately returned to ice to cool. This was ensured because vortexing generate heat and overheating would possibly injure the spores. Each silica vial was labelled appropriately, and each fungus strain preserved in triplicates. The vials were kept in the fridge at 4 ºC.

3.4 Molecular characterisation of *Aspergillus* and *Fusarium* isolates

3.4.1 Extraction of DNA

Forty-four fungal isolates with determined aflatoxin accumulation levels according to Okoth et al. (2012) and Nyongesa et al. (2015) were used. Before culturing each fungus on potato dextrose agar (PDA) medium, the fungi were left to grow at room temperature for at least 1 hour. Silica bottles containing the fungus were placed in a sterile lamina floor one strain at a time. The bottle was shaken for about 1 minute and about 2 - 3 silica gel beads dropped into the prepared PDA plates (39 gm commercial PDA powder (20 gm dextrose, 15 gm agar and 4 gm potato starch) and 1 litre distilled water-Oxoid Microbiology products). Each *A. flavus* strain was cultured in replicates in the labelled petri dishes. The petri dishes were tightly sealed with parafilm and then removed from the lamina floor, put to grow in an incubator for 7 days at 37 ºC.

Fully grown mycelia were harvested after 7 days for use in DNA extraction. The extraction was performed using Zymo Research fungal/Bacterial DNA kit^{TM}. Two hundred milligram wet weight of fungal mycelia was suspended in 750 µl lysis buffer

in a 2 ml Eppendorf tube. A bead beater fitted with a 2 ml Eppendorf tube holder assembly was used to disrupt the fungus cells for 5 minutes at 1500 rpm.

Eppendorf tube containing processed fungal cells was centrifuged in a micro-centrifuge at 10,000 rpm for 1 minute. Up to 400 µl supernatant was transferred to a zymospin^{TM} IV filter in a collection tube and centrifuged at 7000 rpm for 1 minute. To the collection tube containing the collected filtrate, 1,200 µl fungal DNA binding buffer was added and 800 µl of the mixture was transferred to a zymospin^{TM} IIC column in a collection tube then centrifuged at 10,000 rpm for 1 minute. The flow through from the collection tube was discarded. The step above was repeated by again transferring 800 µl of the mixture to the zymospin^{TM} IIC column and centrifuged at 10,000 rpm for 1 minute. The flow through from the collection tube was discarded.

To the zymospin^{TM} IIC column in a new collection tube, 200 µl DNA pre-wash buffer was added and centrifuged at 10,000 rpm for 1 minute. Fungal DNA wash buffer of 500 µl was added and centrifuged at 10,000 rpm for 1 minute. The zymospin^{TM} IIC column was transferred to a clean 1.5 ml micro centrifuge tube and 100 µl DNA elution buffer added directly to the column matrix and centrifuged at 10,000 rpm for 30 seconds to elute the DNA. The ultra-pure DNA was ready for use in polymerase chain reaction (PCR).

3.4.2 Analysis of isolates of *Aspergillus flavus* by mating type

Multiplex PCR assay was performed to determine mating type genes in the 44 *A. flavus* strains mentioned in section 3.4.1 above. The PCR assay included four mating type specific oligonucleotide primers with MIF and MIR which amplified 396 bp region within the α-box of *MAT1-1*, M2F and M2R that amplified a 270 bp segment within *MAT1-2* isolate (Wada *et al*., 2012 and Rodmirez-Prado 2008) (Table 3-4).

Table 3-4: Multiplex PCR assay for isolated *Aspergillus flavus* strains

Primer code	Target gene	Primer sequence	PCR product size
MIF	*MAT1-1*	ATTGCCCATTTGGCCTTGAA	396 base pairs
MIR	*MAT1-1*	TTGATGACCATGCCACCAGA	396 base pairs
M2F	*MAT1-2*	GCATTCATCCTTTATCGTCAGC	270 base pairs
M2R	*MAT1-2*	GCTTCTTTTCGGATGGCTTGCG	270 base pairs

(Source: Wada *et al*., 2012 and Rodmirez-Prado, 2008)

The PCR for the 44 isolates was performed in 20 µl reactions, which included 1µl of a 1:10 or 1:100 DNA dilution, 1 U RedTaq DNA polymerase (Sigma–Aldrich), 2 µl Red Taq buffer supplemented with 1.7 µl of 22 mM $MgCl_2$ for a final concentration of 3.0 mM, 10 mM deoxyribonucleotide triphosphates, 0.5% Bovine Serum Albumin (BSA) and 0.5 µM of each of the 4 primers (M1F, M1R, M2F and M2R). Reactions were run in a master cycler ep gradient (Brinkmann Instruments) with a thermal profile of 5 min at 95 °C followed by 40 cycles of 30s at 95 °C, 60 s at 54 °C and 45s at 72 °C. The amplified DNA was electrophoresed in 1.5% (w/v) Tris–acetate–Ethylenediaminetetraacetic acid (EDTA) agarose gels, and amplicons were designated as *MAT1-1* and *MAT1-2* using a 100 bp DNA ladder (exACTGene, Fisher Scientific International) as a size standard.

3.4.3 Agarose gel electrophoresis

A concentration of 1 % agarose was made. The agarose was boiled at 100 ºC for 5 minutes in a conical flask, left to cool to 55 ºC and 0.3 µl of ethidium bromide added while swirling the flask to enable the gel mix with ethidium bromide. The mixture was poured into a gel tank with the combs on and left to solidify. Molecular marker (2 µl) was added to one well, DNA (4 µl) to the other wells and the arrangements noted. The gel was run for 45 minutes at 80 voltage and viewed under gel doc (BIO-RAD, molecular imager gel doc^{TM} XR-CLASS, imaging system).

3.4.4 DNA data analysis

DNA analysis was based on observed bands on the gel. Genetic interpretation of the bands depended on the gene ruler 1kilo base (kb)-Thermo Fisher Scientific in which 270 bps corresponded to *MAT1-2* and 396 bps corresponded to *MAT1-1*.

3.5 Source of maize inbred lines used in the study

Inbred maize lines used for trials in Kiboko and Katumani were obtained from International Maize and Wheat Improvement Centre (CYMMIT) in Kenya and from South Africa. Maize inbred lines CB 248, CML 390, CB 222, CML 444, R119W, R0549W, I137tnw, VL0617Y-2, CML 182, R2565Y, US2540W were obtained from South Africa while the Kenyan lines included CML 264, CKL05003, CKL05015,

CKL05022, CKL05019, CML 247, CML 495, La Posta, MIRTC5, P502, CML 442 and VL06688.

3.5.1 Land preparation, planting and management of maize inbred lines

One and half hectares piece of land was ploughed in Katumani and Kiboko field stations. In each site, the field was equally divided into two blocks namely: *Aspergillus* block and *Fusarium* block. The inbred maize lines were randomly planted in *Aspergillus* and *Fusarium* blocks in triplicates. In the planting holes, 5g of Diammonium Phosphate (DAP) was put, stirred with a stick to ensure thorough mixing. Each planting row was clearly labelled with a tag on a permanent peg. One seed was hand planted per hill and a total of 33 seeds per row. It was ensured that during planting, no seed was directly in contact with the fertilizer.The seeds were then covered with loose soil and six guard rows were planted around every block. Planting dates in Kiboko and Katumani were recorded.

The farm was irrigated three times a week whenever necessary, early in the morning at 6.00 am and in the evening at 9.00 pm. Germination counts were done after planting on the the 9th, 12th and 15th day after planting. The number of germinated seeds per row was recorded per seedline and the farm was under close watch for 2 weeks to keep away squirrels and field mice. Calcium Ammonium Nitrate (CAN) was applied between rows when the plants were about knee-height. The field was kept clean by weeding, checked daily and any insect infestation or diseases noted and controlled appropriatelly. Off-types were also checked daily and uprooted.

Silking, pollen shed and blister development dates were recorded for each line. Silking and pollen shed data of each line was recorded at first silk emergence/pollen shed, 25% and 50%. Blister development in each inbred line was recorded at first blister development stage, 50% and 100% blister stage.

3.5.2 Preparation of *Aspergillus* flavus spore's inoculum

Toxigenic *A. flavus* strains 201365,100095 and 100130 isolates were used to prepare inoculum. The inoculum was prepared by weighing out 50g of maize kernels into 250 ml conical flasks. Into the flask, 25 ml of water was added and covered with aluminium

foil for at least 6 hours. The seeds were autoclaved at 120 ℃ for 15 minutes and allowed to cool then placed in a biosafety cabinet.

Inoculum transfer loop was sterilized by immersing in absolute ethanol and flamed to red hot then allowed to cool in air within the cabinet. Some conidia were picked from 5 days old *A. flavus* (201365, 100095 and 100130) Potato Dextrose Agar (PDA) labelled petri-dishes and transferred to their respective labelled flasks containing autoclaved maize. One strain was inoculated at a time into the flasks and the loop sterilized. The flask top was completely covered with aluminium foil, shaken to ensure contact between conidia and the seeds, then the flasks incubated at 30 ^{o}C for 7 - 14 days.

Every day, the flasks were thoroughly shaken to maximize conidia formation. After 7-14 days, conidia were washed from the grains using a soap solution (20 ml H_2O:40µl Tween 20) and the suspension sieved using a fine sieve into beaker. By using a pipette, a drop of inoculum was added onto a hemacytometer and conidia counted. The concentration of conidia in the inoculum was made to10^8 spores/ml. The quantity of inoculum made depended on the estimated number of cobs that were just about to silk.

3.5.3 Preparation of *Fusarium verticilloides* spore's inoculum

3.5.3.1 Preparation of Armstrong *Fusarium* medium

The following were weighed in order to make Armstrong medium (Booth, 1971) :20g sucrose, 0.4g magnesium sulphate ($MgSO_4.7H_2O$), 1.6g potassium chloride (KCl), 1.1g potassium dihydrogen phosphate (KH_2PO_4), 5.9g Calcium nitrate ($Ca(NO_3)_2$, 20 µl Ferric Chloride ($FeCl_3$) [stock @ 10 mg/ml], 20 µl Manganese sulphate ($MnSO_4$)[stock @ 10 mg/ml], 20 µl Zinc sulphate ($ZnSO_4$)[stock @ 10 mg/ml]. All the reagents were put in a media bottle and made up to 1litre (L) with sterile deionized water then autoclaved at 120 ℃ for 15 minutes. The following were weighed; 0.1g of $FeCl_3$, $MnSO_4$ and $ZnSO_4$ and 10ml of autoclaved deionized water was added. Final concentration was then 10 mg/ml and 20 µl of each solution was used in 1L of Armstrong Medium.

3.5.3.2 Inoculation of Armstrong liquid medium with *Fusarium verticilloides* spores

Three toxigenic *F. verticilloides* isolates K38, K48 and K58 preserved in silica gel were used for inoculum preparation. The isolates were obtained from the data bank at the mycology laboratory isolated according to Okoth *et al*., 2012. Into each autoclaved 250 ml Erlenmeyer flask, 100 ml Armstrong media was poured, and a sterile scalpel was used to scrape some hyphae from K38, K48 and K54 which were selected plates of freshly grown *F. verticilloides* on PDA placed in their respective flask. The number of inoculated Armstrong flasks made depended on the estimated number of cobs that were just about to silk. The flasks were incubated in an incubator shaker at 25 ^{o}C at 100 rpm for 4 - 5 days.

The 4 – 5-day old liquid cultures were removed from the incubator-shaker and fungal spores filtered by pouring through two layers of sterile cheese cloth (maximum pore size 0.7 mm) into a 50 ml falcon tube. The filtrate was centrifuged at 3500 rpm for 5 minutes to remove the supernatant. Conidia were washed twice with de-ionized, autoclaved water of volumes equivalent to that of the original suspension. The tubes were centrifuged between washes and the spores suspended in 250 – 500 ml sterile distilled water. A hemacytometer was used to determine spore concentration and adjusted to a final concentration of 1×10^6 conidia $^{ml-1}$ (Booth, 1971). Tween 20 surfactant (polyoxyethylene 20-sorbitan monolaurate) was added to the conidial suspension before inoculation of maize ears, at a rate of three drops per litre.

3.5.4 Inoculation of silked inbred lines with *Aspergillus flavus* and *Fusarium verticilloides* spores

Prepared *Aspergillus* spores and *Fusarium* spores' inoculum were transported in a cool box to Katumani and Kiboko research stations. It was ensured that the prepared spores were used within 24 hours after preparation. In the *Aspergillus* block, only *Aspergillu*s inoculum was used to infect the silked maize and in the *Fusarium* block only *Fusarium* inoculum was injected into the silked maize. Inoculation was done by injecting 2 ml of the inoculum into the silks (when the silk was about an inch long) using 18-gauge needle and 10 ml syringe. Before every injection, the inoculum was shaken to ensure

even distribution of conidia. During infection, the inoculum was wrapped with aluminium foil to protect conidia from ultra violet light from the sun. Each infected ear was tagged and the cob left to grow to maturity waiting for harvesting.

3.6 Harvesting and post-harvest practises

In the inoculated block, one block was harvested at a time, that is, for example, all the tagged cobs in *Fusarium* block were harvested first and all the cobs from one row were placed in the same jute bag which was then labelled with the inbred line, treatment and plot number. Gloves were changed and then the next plot was harvested in the same manner. The same procedure was repeated in the *Aspergillus* block. Since each row in the block was made of the same female plant, all the tagged cobs in the row were harvested and placed in a labelled jute bag. Planting and harvesting dates of the inbred lines and crosses were recorded throughout (Table 3-5).

Table 3-5: Planting and harvesting dates at Kiboko and Katumani

Planted seeds	Location	Planting dates	Harvesting Dates
	Katumani	9/12/12	30/5/13
Inbred Lines	Kiboko	3/1/13	13/6/13
Season I Crosses	Katumani	9/12/12	30/5/13
Season II Crosses	Kiboko	3/7/14	2/12/2014
Season III Crosses	Kiboko	6/12/14	2/5/2015

3.6.1 Quantification of ear rots by percentage visual ear rot rating

The level of ear rot damage was estimated by determining the % area of the ear affected (severity) in the harvested cobs of the inbred lines. This was done by placing all the harvested ears of a labelled row on a clean table previously disinfected with 70% ethanol. Supposing a row had 50 harvested ears, the full length of each of the ears per row was inspected. The examination included those without visual symptoms as well. The rating scale (Figure 3-2) was used to approximate the % severity for each ear and the average across all the examined ears obtained. For example, if a row had 50 ears, the average was obtained by adding up the percentages then divided by 50. The same procedure was repeated for the other rows. The average % visual ear rot severity data

was obtained for each line treated with *Aspergillus* and *Fusarium* spores. The % visual rating procedure is supported by Pierce, 2016.

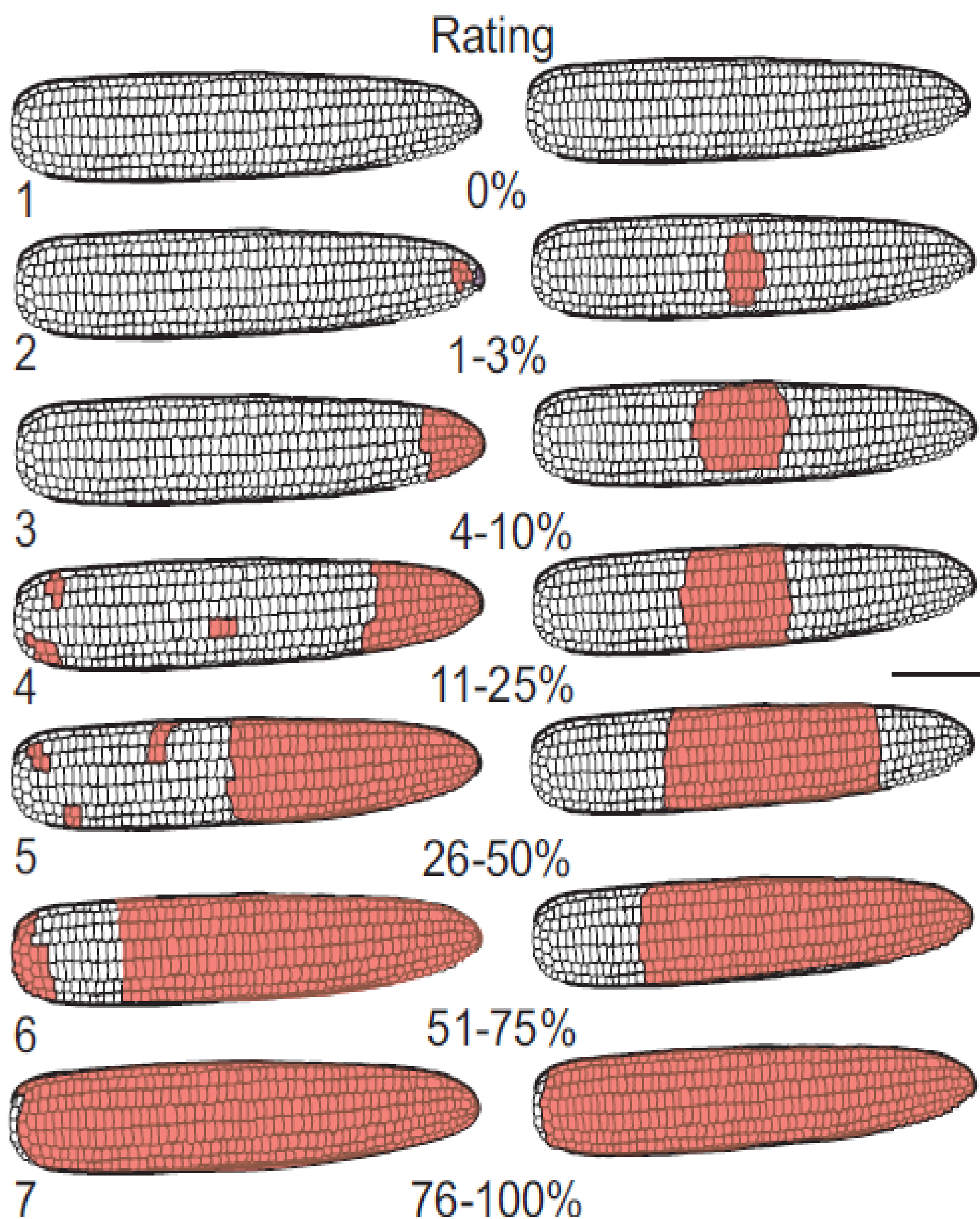

Figure 3-2: Percentage visual ear rot rating in maize (Source: Pierce, 2016)

3.6.2 Drying of the harvested maize inbred lines

Drying of the maize cobs in the sun continued after harvesting for 1 week. The cobs were left in their respective jute bags in a ventilated laboratory on the benches for 1 week with the room ensured cool and dry in order to continue the drying process. During the drying period, identity of each seed line was maintained by ensuring no mixing of seeds occurred.

The dried cobs were hand shelled. Seed airing continued until the grain moisture content was less than 13 %. A moisture reader (Superpro Moisture Analyzer) was used to determine the moisture content of the seeds. The seeds were kept in their respective clearly labelled brown paper bags. Seed cleaning was manually done through picking out bits of cobs, husks, broken kernels, stones, weed seeds among others. Actellic dust which is an organophosphate composed of 500g/l pirimiphos-methyl (Syngenta Crop Protection Postfach, Switzerland) was sprinkled on kernels (about 15g per 1kg of seeds) in the labelled brown paper bags containing seeds. Actellic dust is a broad-spectrum insecticide which gives both knock-down activity and long lasting protection against invasion of seeds by a range of insect pests in stored grain. The paper bags containing seeds were stapled then kept in a dry cool place waiting for grinding.

3.6.3 Grinding of maize inbred lines

The shelled inoculated kernels were milled in the mycology laboratory grinder at UoN. Each treatment was milled at a time, that is, maize inbred lines infected with *Aspergillus* spores' inoculum were all ground first then all maize lines treated with *Fusarium* spores' inoculum followed. Every time a treated maize inbred line was ground, the grinder was opened and sterilized with 70% alcohol before a different maize inbred line was milled. The flour was packed separately into a labelled zip locked bag and kept in the fridge at 4 ºC.

3.7 Laboratory analysis of aflatoxin and fumonisin levels in maize inbred lines

3.7.1 Aflatoxin analysis using ELISA method (Helica Biosystems Inc.)

Ground portion of the maize sample weighing 2g was added to 10 ml of 70 % methanol and mixed by shaking in a sealed container for a minimum of 2 minutes. The particulate

matter was allowed to settle then 5 - 10mls of the extract allowed to filter through a Whatman No. 1 filter paper and the filtrate collected. All the reagents were brought to room temperature before use and a multi-channel pipette was used to perform the assay. Dilution wells were placed in the microwell holder for each standard and sample to be tested. Equal numbers of antibody coated microtiter wells were placed in another microwell holder. Two hundred microliters of conjugate were dispensed into the wells.

Using a new pipette tip for each, 100 µl of each standard and sample was added to appropriate dilution well containing conjugate and mixing was done by priming pipette at least three times. Using a new pipette tip for each, 100 µl of content from each dilution well was transferred to a corresponding antibody coated microtiter well and incubated at room temperature for 15 minutes. Contents from the microwells were decanted into a discard basin and the microwells washed by filling each with deionized water, then the water decanted into a discard basin. The wash was repeated for a total of five washes. The microwells were then tapped on a layer of absorbent towels to remove residual water. Required volume of substrate reagent was measured (120 µl/well) and placed in a separate container. Substrate reagent volume of 100 µl was added in the same sequence and at the same pace as the substrate was added. Optical density (OD) of each microwell was read on an ELISA reader and recorded using a microtiter plate reader using a 450 nm filter.

3.7.2 Fumonisin analysis using ELISA method (Helica Biosystems Inc.)

Ground portion of the sample weighing 2g was added to 4ml of the extraction solvent (90 % methanol). The mixture was shaken in a sealed bottle for 1 minute and the particulate matter allowed to settle, then 5-10 ml of the extract was filtered through a Whatman filter paper No.1. The filtrate to be tested was collected in a sterile 50 ml falcon tube. The sample extract was diluted 1:20 in distilled water. All the reagents were then brought to room temperature before use and a multichannel pipette was used to perform the assay.

Phosphate buffer solution (PBS) tween packet was reconstituted by washing out the contents in a gentle stream of distilled water into a 1L container. One dilution well was placed in a microwell holder for each standard and sample to be tested. An equal

number of antibody coated microtiter wells were placed in another microwell holder. Into the dilution wells, 100 µl of conjugate solution A was dispensed followed by 100 µl of conjugate B. Using a new pipette tip for each, 100 µl of each standard and sample were separately added to appropriate dilution well containing conjugate. Mixing was done by priming pipette three times. Using a new pipette tip for each, 100 µl of the contents from each dilution well was transferred to a corresponding antibody coated microtiter well and incubated at room temperature for 10 minutes. Contents from the microwells were then decanted into a discard basin and the microwells washed by filling each with PBS Tween wash buffer then the water decanted into a discard basin.

The wash was repeated for a total of three washes and the microwells tapped on a layer of absorbent towels to remove residual water. Using a new clean container, 120 µl/well of substrate reagent was measured and placed into the container. Into each microwell, 100 µl of the substrate was added, covered with aluminium foil and incubated at room temperature for 10 minutes. A separate container was used to place measured 120 µl/well of stop solution and 100 µl of the stop solution added in the same sequence and the same pace as the substrate was added. Optical density of each microwell was read and recorded using a microtiter plate reader using a 450 nm filter.

3.8 Hybrid maize seed production

Planting holes were made 30 cm apart in a row and 90 cm between rows in a 1 ha farm. Five grams of Diammonium Phosphate fertilizer was applied in each planting hole mixed with the soil and during planting it was ensured no seed was in contact with the fertilizer.

Male and female plants were planted in sequential rows and planting was done three times the number of female rows to a single male row. The ratio of the number of female rows to male rows in the field was 3:1. Planting of the male and female plants was done depending on the collected previous data on germination, pollen shed, and silk emergence obtained from the inbred lines trials since during the trials growth, randomized crosses were also developed in the crossing block. In some parents, pollen shedding and silk emergence would not coincide even if the male and the female parents were planted on the same date. For example, if the female parent developed 50 %

silking 63 days after planting and the male plant reached 50% pollen shed 70 days after planting, then to achieve a perfect nick/male-female flowering synchrony in the crossing field, the male was planted 5 - 7 days earlier than the female.

Additionally, in a case where a male parent recorded short pollen shedding period or limited amount of pollen, the male parent was sowed on two or three consecutive rows apart in order to get enough pollen for the silk emergence period. The split-planting of the male in such parents was done in two adjacent rows. Each plot was permanently labelled with a peg. On the label, were the parent names, date of planting and source of the maize lines. Data was consistently recorded from day 1 of planting at different stages of maize growth from germination to harvesting stage. The crops in the field were observed daily and keen observation on performance of the lines was enhanced especially at flowering stage. The field was irrigated as required and inspected regularly for pests, weeds and diseases which were controlled appropriately. Calcium ammonium Nitrate (CAN) was applied at 4 -5 leaf stage and at 8 -10 leaf stage. Growth of the inbred lines was observed, and nutrient deficiency symptoms corrected immediately when observed. For example, the inbred lines had foliar spray of nitrogen and phosphorous just before flowering.

During vegetative growth of the seed crops, off-types in the field were uprooted (rouging) from the 6 -12 leaf stage and before tassels emerged. Rouging was usually done early in the morning or late in the afternoon. On male plants, rouging was completed before pollen shedding begun while rouging on female plants was completed before silk emergence to prevent female self-pollination.

At flowering time, the silks were constantly checked for stalk borer and bollworm since the pests could feed on the developing kernels and reduce seed quality and yield. Detasseling of the female plant was done before pollen shedding began, that was when the top 3 - 4cm of the tassel was visible above the leaf whorl. Close supervision of the field continued daily, and it was ensured all the female parents were detasselled. Early detasselling was observed for this is known to quicken silk emergence by one or two days. After emergence of the ear just a few days before silk emergence, the ear shoots were covered by stapling with small glycine/shoot bags to prevent the silk from receiving pollen from unwanted source. Shoot bagging was done just before silk

emergence and any cob whose silk emerged before shoot bagging was done was excluded from breeding.

Pollen was tapped in a pollination bag in the evening between 3-5 pm and the tassel remained tightly covered overnight so that the following day in the morning only pollen from the targeted male flower remained viable ready for use in fertilizing the female plant. During pollen tapping, the mature male flower (tassel) intended for fertilizing the female flower was tightly stapled with a labelled pollination bag immediately the onset of silking was observed. Pollination was done by carefully transferring collected pollen in the pollination bag onto the silk then the pollination bag stapled until harvesting date. Several hundred pollinations could be made in a day. The pollen from the male flower sourcing the pollen and the female plant it was to fertilize was clearly labelled on the pollination bag and date on which pollination was done. Crossing was done between 8 – 11 am in the morning. The males were removed from the field after pollination. This was done by cutting the male plant at the base and left to rot in the row; an important process as it ensured no mixture of the male and female seed at harvest time. Removal of the male plant is also known to improve yield of the female parent because more light penetration into the female rows is allowed and competition for moisture reduced.

Harvesting was done four weeks after physiological maturity. This was because by reducing the time mature seeds remain in the field, seed quality was improved by minimizing pest and disease infestation. Since the ratio during planting was 3:1 female: male ratio, the three rows of the same female plant were placed in the same labelled jute bag. However, each cob was left in its respective pollination bag during harvesting.

3.9 Screening of maize crosses for resistance to aflatoxins and fumonisins

Field preparation, planting and agronomic practices including fertilizer application, weed and pest control, irrigation was done as described in section 3.5.1. Preparation of *Aspergillus* and *Fusarium* spore's inoculum and artificial inoculation of the hybrids was performed as described in section 3.5.2, 3.5.3 and 3.5.4. The crosses were harvested four weeks after physiological maturity, dried and ground according to the procedure followed in section 3.6. Accumulated aflatoxin and fumonisin levels in the crosses were

determined using Enzyme Linked Immunosorbent Assay (ELISA) as described in section 3.7.

3.10 Data analysis

Logarithmic transformation (log 10(y+1)) was used on the data to stabilize the variance. Where there was significant difference, Tukey's High Significant Difference (HSD) in the agricolae package (Felipe de Mendiburu, 2017) was used to differentiate the means. All statistical analyses were performed in R 3.2.2 statistical environment (R Core Team, 2015). Agronomic performance data including germination, silking, pollen shed, blistering, differences in aflatoxin and fumonisin concentrations were compared using analysis of variance (ANOVA). Pearson correlation was used for the different correlation analysis in the study.

CHAPTER FOUR

RESULTS

4.1 Mating types in *Aspergillus flavus* populations

Among the 44 isolates of *A. flavus* strains tested for mating types include those with at least fragments of both MAT genes, either *MAT1-1* (396 bp) or *MAT1-2* (270 bp) genes. Nine strains had *MAT1-1* and *MAT1-2* (Figures 4-1, 4-2, 4-3, 4-4 and 4-5), 6 strains were *MAT1-1* (Figures 4-1, 4-2, 4-4 and 4-5) while 29 strains had *MAT 1-2* (Figures 4-2, 4-3, 4-4 and 4-5). Of the three isolates used for silk inoculation of the inbred lines and crosses, isolate 201365 was in *MAT1-1* and *MAT1-2* while isolate 100095 and 100130 fell in *MAT1-1* (Figure 4-1).

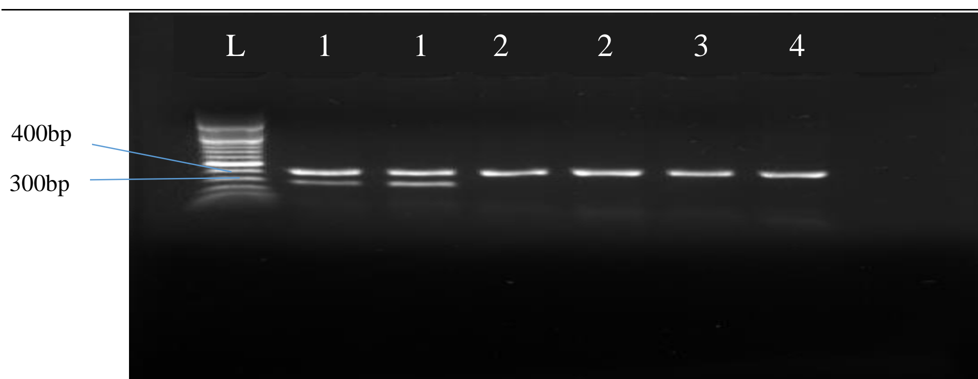

Figure 4-1**:** Mating types for *A. flavus* strains from Makueni and Nandi; L-molecular weight marker, Lane 1**-** IVM201365, 2-IVM100095, 3-IVM100130

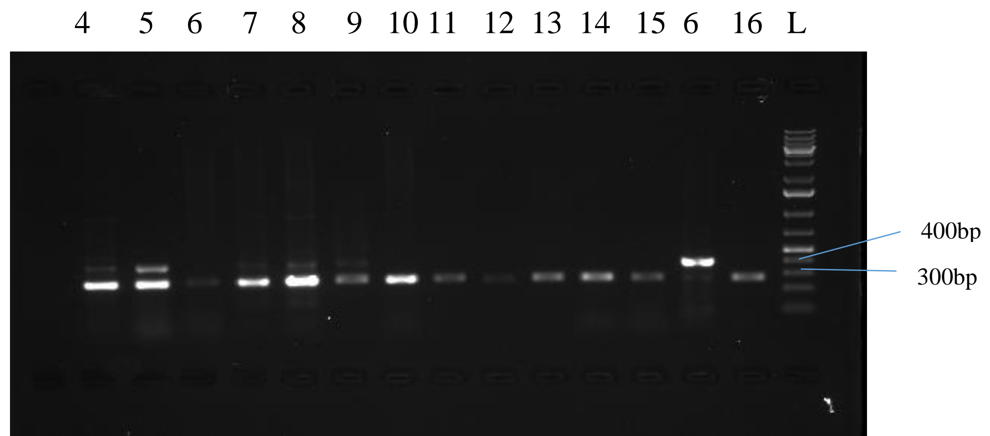

Figure 4-2**:** Mating types for *A. flavus* strains from Makueni and Nandi; 4-IVM 350G, L5-504820BMIYG, L6-300566, L7-IVM 132G, L8-100957, L9-60756, L10-50400BM2YG, L11-2M1983g, L12-60572BM2Y, L13- 60795BM8BJ, L14-60801BSIYG, L15-40013BM126, L6-300566, L16-IVM131LG, L-molecular weight marker

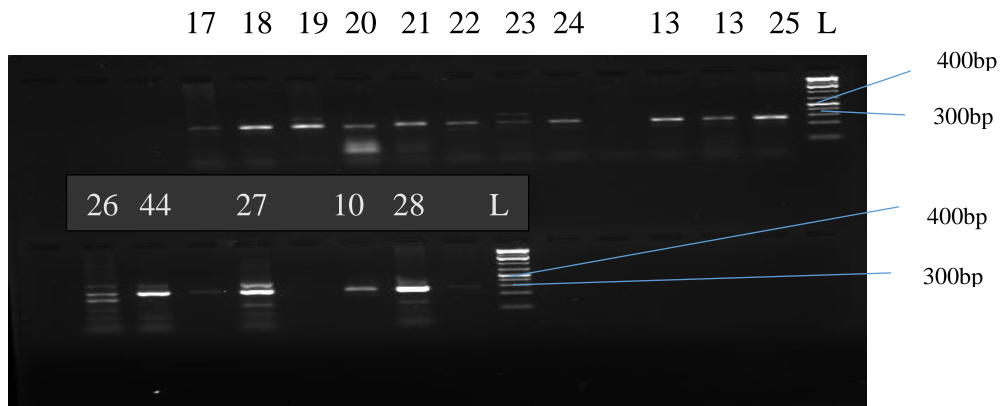

Figure 4-3**:** Mating types for *A. flavus* strains from Makueni and Nandi**;** Lane (L)17-3VM 566, L18-40025 BSIYG, L19-IVM 4018, L20-40245 BMIG, L21-2M1983DG, L22-2VM882G, L23-50444, L24-50536 BMIYG, L25-2M2002LG, L26-40296BSY, L27-40143BM112G, L28-2VM964, L-molecular weight marker

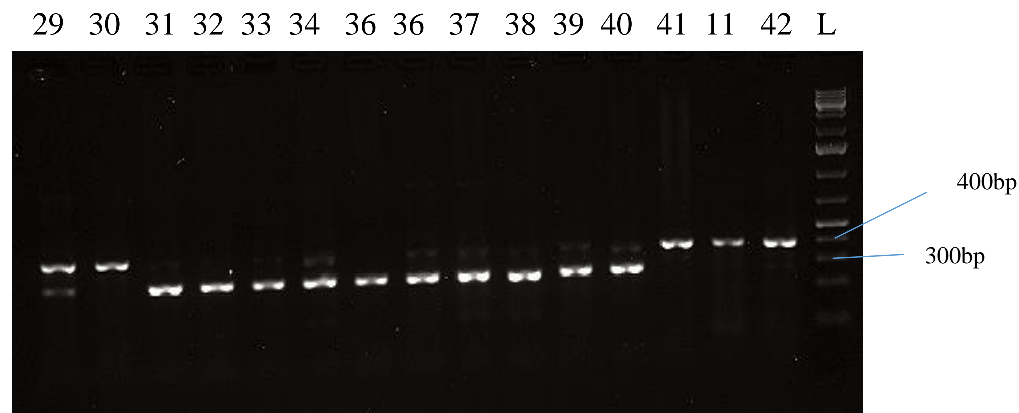

Figure 4-4**:** Mating types for *A. flavus* strains from Makueni and Nandi; Lane (L)29-40143 BM112G, L30-IVM 130LG, L31-3VM566G, L32-IVM 250WY, L33-40267 BSIYG, L34-IVM 414, L36-2M1002LG, L37-100079, L38-40224 BSIYG, L39-40041BM38Y6, L40-60690BMIYG, L41-IVM201122, L42-IVM 195, L-molecular weight marker

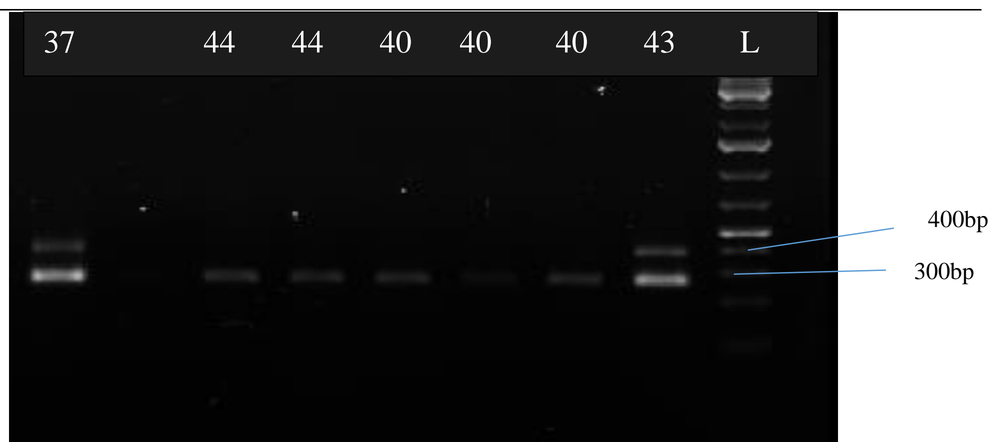

Figure 4-5**:** Mating types for *A. flavus* strains from Makueni and Nandi; Lane (L) 35-50444 BSIYG, L44-60661 BMIYG, L43-201204, L-molecular weight marker

Sampled *A. flavus* from Makueni and Nandi include those that contain at least fragments of both MAT genes and those that contain a single MAT gene. There were 20.45% isolates with fragments of both MAT genes though Nandi had 11.36% while in Makueni, the percentage was lower (9.09%). In both regions, *MAT1-2* isolates frequency was dominant (61.36%), although the frequency in Nandi was higher (75%)

than in Makueni (54.17%). The isolates from Nandi had no *MAT1-1* genotypes, while in Makueni their percentage was 15.91%.

4.2 Agronomic performance of maize inbred lines

Germination counts per line was recorded in the two research stations at 9th, 12th and 15th days after planting with fast germinators attaining and maintaining high seed count (≥30 seed counts) within 9 - 12 days. In Kiboko at 9th to 15th days after planting, inbred lines that had best germination rates included CKLO5015 with germination rates of 91.92%, 92.42%, 94.44% respectively and VLO6688 with 91.92%, 92.42%, 93.94% germination rates respectively. The poorest germinator was R2565Y with germination rates of 17.68%, 21.21% and 21.72% respectively. In Katumani, CML 495 was the best germinator throughout the germination period with germination rate of 91.41% at the 9th day, 93.94% at the 12th day and 93.94% at the 15th day. Line R2565Y recorded the lowest germination rate of 50% at the 9th and 12th day and increased to 53.03% on the 15th day.

The maize inbred lines differed significantly in germination rate in the two research stations (Katumani and Kiboko) at 15 days after planting (F (22, 237 = 7.1927, p = < 2.2e-16)). Inbred line CML 390 had the highest percentage germination rate (89.39%). Other inbred lines that recorded high germination rates included; CKLO5015 (88.89%), CML 495 (85.1%), CKLO5019 (84.34%) and CKLO5022 (82.83%). Line R2565Y was the poorest germinator (37.12%) (Table 4-1). Significant differences in germination were observed in Kiboko with p value of 1.46e-16, F = 14.21 and df = 22. In Kiboko, line R2565Y was the poorest germinator with germination rate of 21.21%. However, the best germinating line was VL06688 with a germination rate of 92.43% (Figure 4-6). In Katumani, germination rates varied across the lines (F (22, 105 = 2.7513, p = 0.0003034)). Line CML 495 recorded the highest germination rate of 93.94% (Table 4-1) with mean germination counts of 30, 31 and 32 at 9, 12 and 15 days after planting respectively. However, CML 442 and VL06688 were the least germinators compared to any other line grown in Katumani with germination counts of 23, 24 and 24 at 9, 12 and 15 days after planting, respectively (Figure 4-7).

Table 4-1: Germination rate of maize inbred lines at 15 days after planting

	Percentage Germination		
Inbred Line	Katumani and Kiboko	Katumani	Kiboko
CB 222	77.78	76.79	78.79
CB 248	78.54	77.78	79.29
CML 182	73.99	85.86	60.61
CML 390	89.39	93.43	85.35
CML 444	74.24	70.71	77.78
I137tnw	76.01	68.18	83.84
R0549W	80.3	72.73	87.88
R119W	56.81	50.03	60.61
R2565Y	37.12	53.03	21.21
US2540W	74.24	62.12	86.36
V0617Y-2	81.81	81.31	82.32
CKL05003	80.81	77.27	84.34
CKL05015	88.89	83.33	94.44
CKL05019	84.34	90.91	77.78
CKL05022	82.83	83.88	76.26
CML 247	77.53	81.31	73.74
CML 264	61.87	68.18	55.56
CML 442	76.26	72.73	81.31
CML 495	85.1	93.94	76.26
La Posta	75.76	81.82	69.7
MIRTC5	80.3	77.27	83.33
P502	76.01	80.81	71.21
VL06688	75.51	72.73	92.43

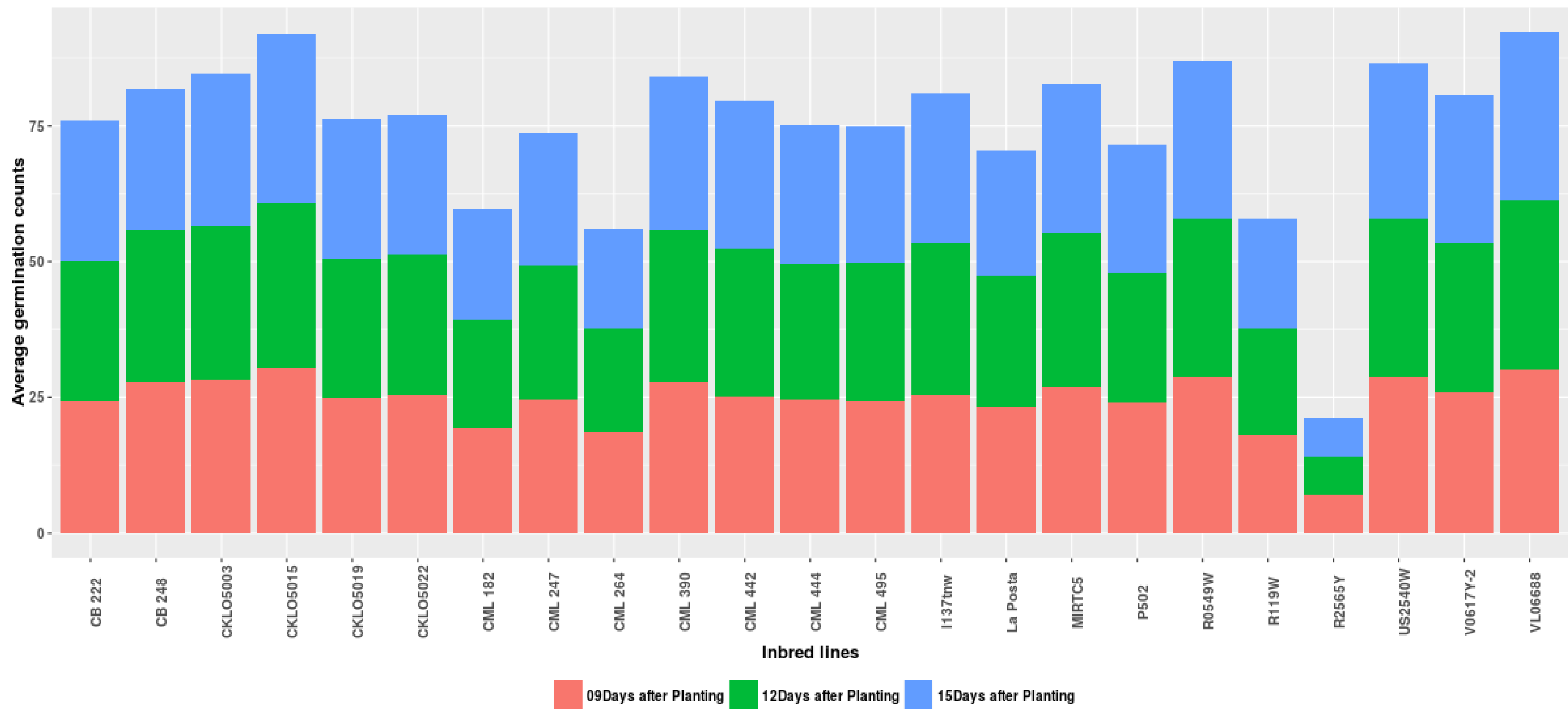

Figure 4-6: Mean germination counts of maize inbred lines in Kiboko

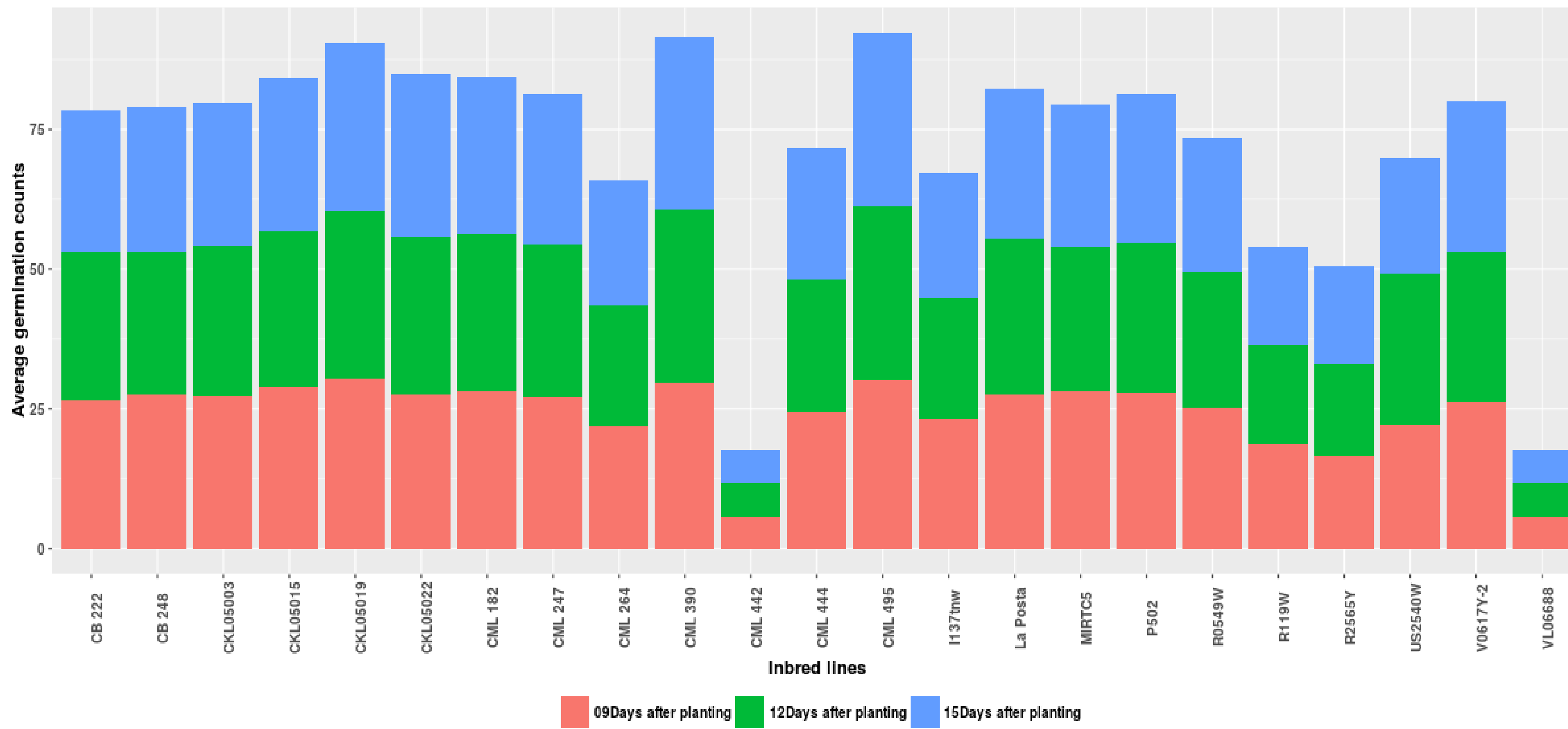

Figure 4-7: Mean germination counts of maize inbred lines in Katumani

Silking data was recorded at different stages which included days to first silk emergence, days to 25% and days to 50% emergence of silk. Silking began at the 51st DAP and spread through to the 68th DAP. Line VLO6688 (51st day) was the first line to silk. Among all the lines, South African lines silked earlier than the Kenyan lines in all the stages. Line VLO6688 silked earliest among the South African inbred lines while CML 390 (58th day), R119W (59th day) and R2565Y (59th) silked latest. Inbred line CKLO5022 was the Kenyan inbred line earliest silker (61st day) while CML 264, CML 247 and CML 442 silked on the 63rd day (Figure 4-8).

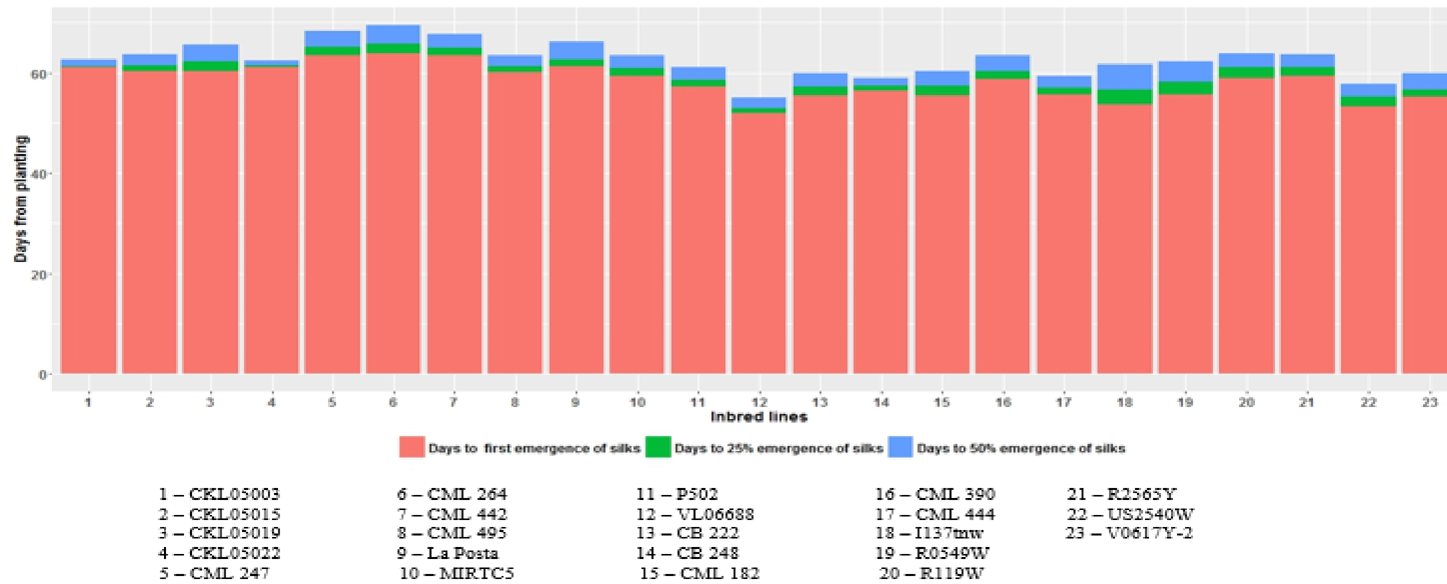

Figure 4-8: Variation in silking of maize inbred lines

The inbred lines considerably had a wide-range in the different silking stages ($p < 0.05$) at first, 25% and 50% silking. Variation was highest at first silking F (42.669) and lowest at 25% silking F (29.629) (Table 4-2).

Table 4-2: ANOVA table; Variation in silking stages in maize inbred lines

Silking Stages	F value	df	p value
First silking	42.669	22	< 2.2e-16
25% Silking	29.629	22	< 2.2e-16
50% Silking	35.36	22	< 2.2e-16

*Significant differences ($p < 0.05$) in silking

Significant variations occurred in silking between the Kenyan and South African inbred lines (Table 4-3). The variation was highest among Kenyan inbred lines at $p < 2.2e-16$, F (45.768). High variation in silking was also observed at 50% silking among the Kenyan inbred lines at $p < 2.2e-16$, F (37.411). However, South African inbred lines silking at 50% recorded the lowest variation (p (1.9e-10), F (12.237) (Table 4-3).

Table 4-3: ANOVA table; Variation in silking inbred maize lines

Days to Silking	Country of origin	df	F	p
First Silking	South Africa	10	12.848	8.287e-11
	Kenya	10	45.768	< 2.2e-16
25% Silking	South Africa	10	16.723	7.335e-13
	Kenya	10	24.172	< 2.2e-16
50% Silking	South Africa	10	12.237	1.9e-10
	Kenya	10	37.411	< 2.2e-16

In terms of the duration taken to first silking, the lines that silked at the same time included CKLO5015, CML 495 and CKLO5019 (c); CKLO5003 and CKLO5022 (bc); CML 247 and CML 442(ab); CB 222, CML 182, CML 444, RO549W, and VO617Y (fgh). Lines CML 264, La Posta, MIRTC5, P502, VLO6688, CB 248, CML 390, I137tnw, R119W, R2565Y and US2540W silked at different times (Table 4-4).

Lines CKLO5003, CKLO5015, and CML 495 (cd); CKLO5019 and La Posta (bc); CML 247 and CML 442 (ab); CB 222, CML 444 and 137tnw (ghi); CB 248 and CML

182 (fghi); R119W and R2565Y (cde) silked at the same time regarding the number of days to 25% silking. However, CKLO5022, CML 264, CML 495, MIRTC5, P502, VLO6688, CML 390, RO549W, US2540W and VLO617Y contrasted with any other inbred line in the number of days to 25% silking (Table 4-4). At 50% silking, CKLO5003, CKLO5015 and CML 495 (cde); CKLO5022 and MIRTC5 (cdef); CML 247and CML 264 (a); CB 222, CB 248, CML 182, CML 444 and VO617Y (gh); R119W and R2565Y (cdef) silked within the same number of days. However, lines CKLO5019, CML 442, CML 495, La Posta, MIRTC5, P502, VLO6688, CML 390, I137tnw, RO549W, US2540W differed with each other in time of silking (Table 4-4). South African lines silked at different times with Kenyan lines in their days to first, 25% and 50% silking. None of the South African inbred line was similar with any Kenyan line in their silking rates (Table 4-4).

Table 4-4: Days to silking in maize inbred lines

Inbred line	Days to first silking	Days to 25% silking	Days to 50% silking
CKL05003	61.00^{bc}	61.33^{cd}	62.33^{cde}
CKL05015	60.33^{c}	61.50^{cd}	62.50^{cde}
CKL05019	60.33^{c}	62.33^{bc}	63.50^{bcd}
CKL05022	61.00^{bc}	59.83^{cdefg}	62.00^{cdef}
CML 247	63.33^{ab}	65.17^{ab}	66.50^{a}
CML 264	63.83^{a}	65.83^{a}	67.50^{a}
CML 442	63.50^{ab}	65.00^{ab}	66.25^{ab}
CML 495	60.17^{c}	61.33^{cd}	62.17^{cde}
La Posta	61.20^{abc}	62.60^{bc}	64.80^{abc}
MIRTC5	59.33^{cd}	60.83^{cde}	61.83^{cdef}
P502	57.17^{def}	58.50^{defgh}	59.83^{efg}
VL06688	52.00^{i}	52.80^{j}	54.20^{i}
CB 222	55.33^{fgh}	57.17^{ghi}	58.00^{gh}
CB 248	56.33^{efg}	57.33^{fghi}	58.00^{gh}
CML 182	55.33^{fgh}	57.33^{fghi}	58.33^{gh}
CML 390	58.67^{cde}	60.33^{cdef}	61.67^{def}
CML 444	55.67^{fgh}	57.00^{ghi}	58.00^{gh}
I137tnw	53.67^{ghi}	56.67^{ghi}	58.67^{fgh}
R0549W	55.67^{fgh}	58.17^{efghi}	59.83^{efg}
R119W	59.00^{cde}	61.00^{cde}	61.80^{cdef}
R2565Y	59.33^{cd}	61.00^{cde}	62.00^{cdef}
US2540W	53.33^{hi}	55.17^{ij}	56.00^{hi}
V0617Y-2	55.17^{fgh}	56.67^{hi}	58.33^{gh}

*Means in a column followed by the same superscript alphabetical letter do not differ at $p \leq 0.05$ confidence level Tukey HSD.

Pollen shedding in the inbred lines commenced at 51st day and stretched to the 68th day. Line I137tmw was the first to shed pollen (51st day) followed by US2540W (53rd). However, La Posta and CML 247 were the last (68th and 67th respectively) in pollen shed (Figure 4-9). Differences ($p = < 2.2e\text{-}16$) were observed in pollen shed among the inbred lines across the three stages (days to first, 25% and 50% pollen shed) (Table 4-5). Variation was highest at first pollen shed $p < 2.2e\text{-}16$, F (29.725) and lowest at 25% pollen shed ($p < 2.2e\text{-}16$, F (20.984)) (Table 4-5).

Table 4-5: ANOVA table; Variation in duration to pollen shed in maize inbred lines

Pollen shed	F	df	p
First Pollen Shed	29.725	22	$< 2.2e-16$
25% Pollen Shed	20.984	22	$< 2.2e-16$
50% Pollen shed	26.550	22	$< 2.2e-16$

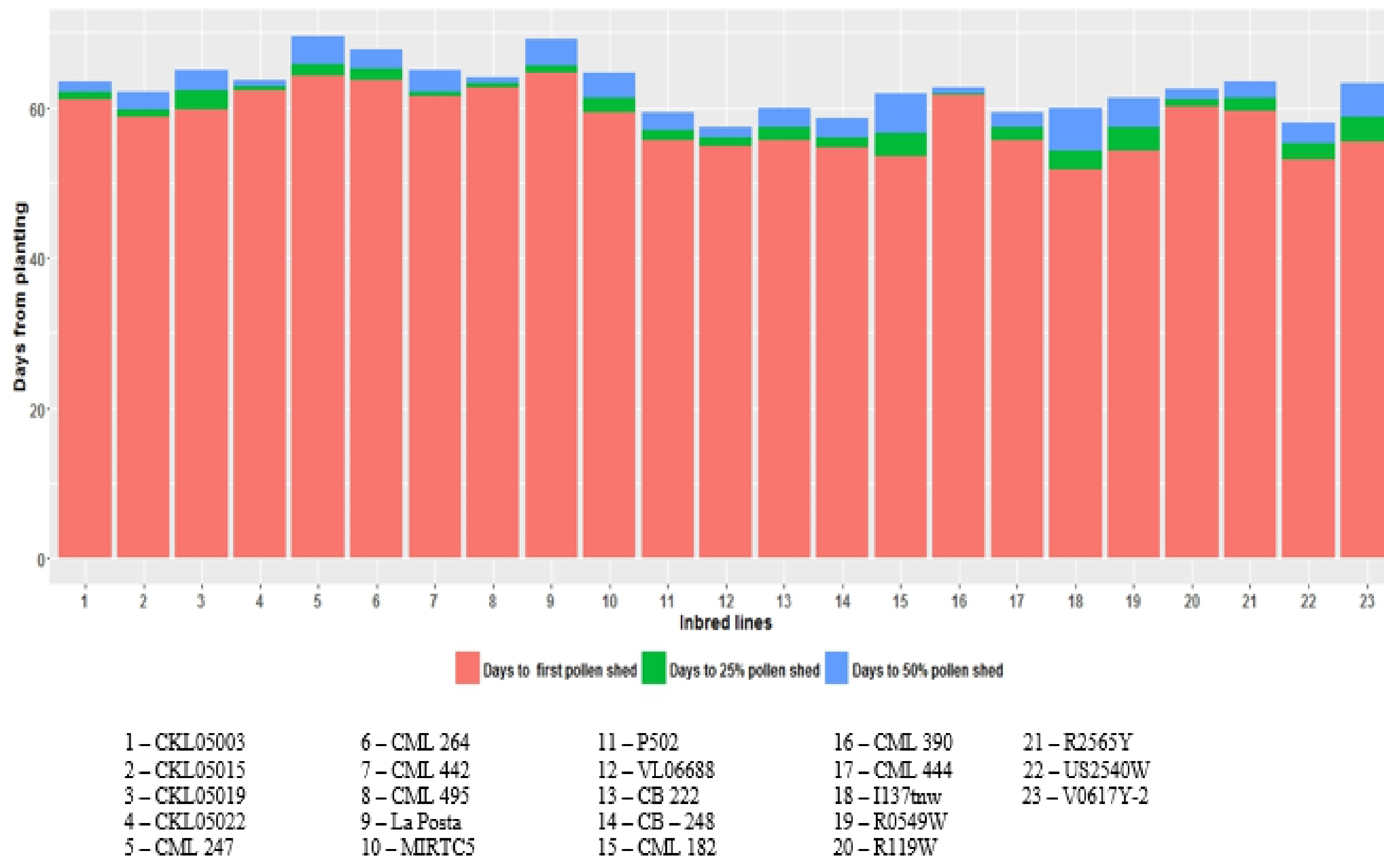

Figure 4-9: Variation of pollen shed in maize inbred lines

Variations occurred in number of days to pollen shed at the three pollen shed stages among the Kenyan and South African inbred lines as shown in Table 4-6.

Table 4-6: ANOVA table; Variation in pollen shed in Kenyan and South African maize inbred lines

Days to pollen shed	Country	df	F	p
First Pollen Shed	South Africa	10	17.859	1.567e-13
	Kenya	10	21.290	2.573e-16
25% Pollen shed	South Africa	10	16.266	9.276e-13
	Kenya	10	13.879	1.952e-12
50% Pollen shed	South Africa	10	7.670	2.519e-07
	Kenya	10	22.127	< 2.2e-16

The lines that shed their pollen within the same time at first pollen shed included CKLO5022 and CML 495 (abc); CKLO5003, CML 442 and CML 390 (abcd); CKLO5019, MIRTC5 and R2565Y (cd); CML 247 and La Posta (a); P502, CB 222, CML 444 and VLO617Y-2 (ef); VLO6688, CB 248, CML 182, I137tnw, RO549W and US2540W (f). However, CKLO5015, CML 264 and R119W took different days to shed their first pollen (Table 4-7).

At 25% pollen shed, CML 247, CML 264, CML 495 and La Posta shed their pollen at different times compared with any other inbred line. Conversely, CKLO5003, CML 442 and R2565Y (bcde); CKLO5019, CKLO5022, MIRTC5 and CML 390 (abcde); CML 442 and R2565Y (bcde); P502 and CML 182 (ghi); VLO6688 and CB 248 (hi); CB 222, CML 444 and RO549W (fghi) shed their pollen at the same time (Table 4-7). Lines CML 264, CML 442, P502, VLO6688, CML 390 and R2565Y had significant differences in the time taken to attain 50% pollen shed. However, CKLO5003 and CKLO5019(c); CKLO5022 and CML 495 (bc); CML 247 and La Posta (a), CML 444 and I137tnw (ghi); CB 222 and RO549W (efgh); MIRTC5 and R119W (cdef); CB 248 and US2540W (hi); CML 182 and VO617Y-2 (defgh) shed their pollen at similar time (Table 4-7).

Table 4-7: Variations in time taken to pollen shed in maize inbred lines

Inbred line	First Pollen shed	25% pollen shed	50% pollen shed
CKL05003	61.00^{abcd}	62.00^{bcde}	62.33^{c}
CKL05015	58.67^{de}	59.67^{efgh}	61.17^{cdefg}
CKL05019	59.67^{cd}	62.17^{abcde}	62.50^{c}
CKL05022	62.33^{abc}	63.17^{abcde}	62.83^{bc}
CML 247	64.17^{a}	65.83^{a}	67.83^{a}
CML 264	63.67^{ab}	65.17^{ab}	66.17^{ab}
CML 442	61.50^{abcd}	62.00^{bcde}	64.50^{abc}
CML 495	62.67^{abc}	63.67^{abcd}	63.50^{bc}
La Posta	64.50^{a}	64.33^{abc}	68.00^{a}
MIRTC5	59.33^{cd}	62.50^{abcde}	61.33^{cdef}
P502	55.67^{ef}	57.00^{ghi}	58.00^{fghi}
VL06688	54.80^{f}	56.00^{hi}	54.60^{i}
CB 222	55.67^{ef}	57.33^{fghi}	58.33^{efgh}
CB 248	54.67^{f}	56.00^{hi}	57.17^{hi}
CML 182	53.50^{f}	56.67^{ghi}	58.67^{defgh}
CML 390	61.67^{abcd}	62.50^{abcde}	61.83^{cde}
CML 444	55.67^{ef}	57.33^{fghi}	57.67^{ghi}
I137tnw	51.67^{f}	54.33^{i}	57.33^{ghi}
R0549W	54.33^{f}	57.33^{fghi}	58.33^{efgh}
R119W	60.17^{bcd}	61.00^{cdef}	61.60^{cdef}
R2565Y	59.50^{cd}	61.67^{bcde}	62.17^{cd}
US2540W	53.00^{f}	55.00^{i}	56.00^{hi}
V0617Y-2	55.50^{ef}	60.00^{defg}	58.67^{defgh}

*Means in a column followed by the same superscript do not differ at $p \leq 0.05$ Tukey HSD test.

Blister development in each inbred line was recorded when the lines were at first blister stage, 50% and 100% blistering stage. Blistering in the inbred lines started at the 65th

day and continued till 109th day. Line VLO6688 blistered earliest (65th day) followed by US2540W (66th day) while line CML 442 (88th day) blistered latest (Figure 4-10). Significant variations occurred in the three blistering stages and the difference was highest at 100% blistering stage (p = 1.381e-09, F (5.3837)) and lowest at 50% blistering stage (p = 8.956e-05, F (2.9946)) (Table 4-8).

Table 4-8: ANOVA table; Variation of blistering stages in maize inbred lines

Days to blistering	F	df	p
First blistering	3.288e-15	22	3.288e-15
50% blistering	2.995	22	8.956e-05
100% blistering	5.384	22	1.381e-09

Kenyan inbred lines recorded the highest variation in blistering at 25% blistering stage (p = 9.176-14, F (16.385)) compared to the South African lines (p = 4.031e-06, F (6.2044)). However, Kenyan lines had the lowest deviation at 50% (p = 4.42e-3, F (2.8931)) while the differences in South African lines was highest (p = 4.871e-3, F (2.9885)) (Table 4-9).

Table 4-9: ANOVA table; Variation of blistering in Kenyan and South African maize lines

Days to blistering	Country	df	F	p
25% Blistering	South Africa	10	6.204	4.031e-06
	Kenya	10	16.395	9.176-14
50% Blistering	South Africa	10	2.989	4.871e-3
	Kenya	10	2.893	4.42e-3
100% Blistering	South Africa	10	3.351	1.383e-3
	Kenya	10	4.755	8.287e-5

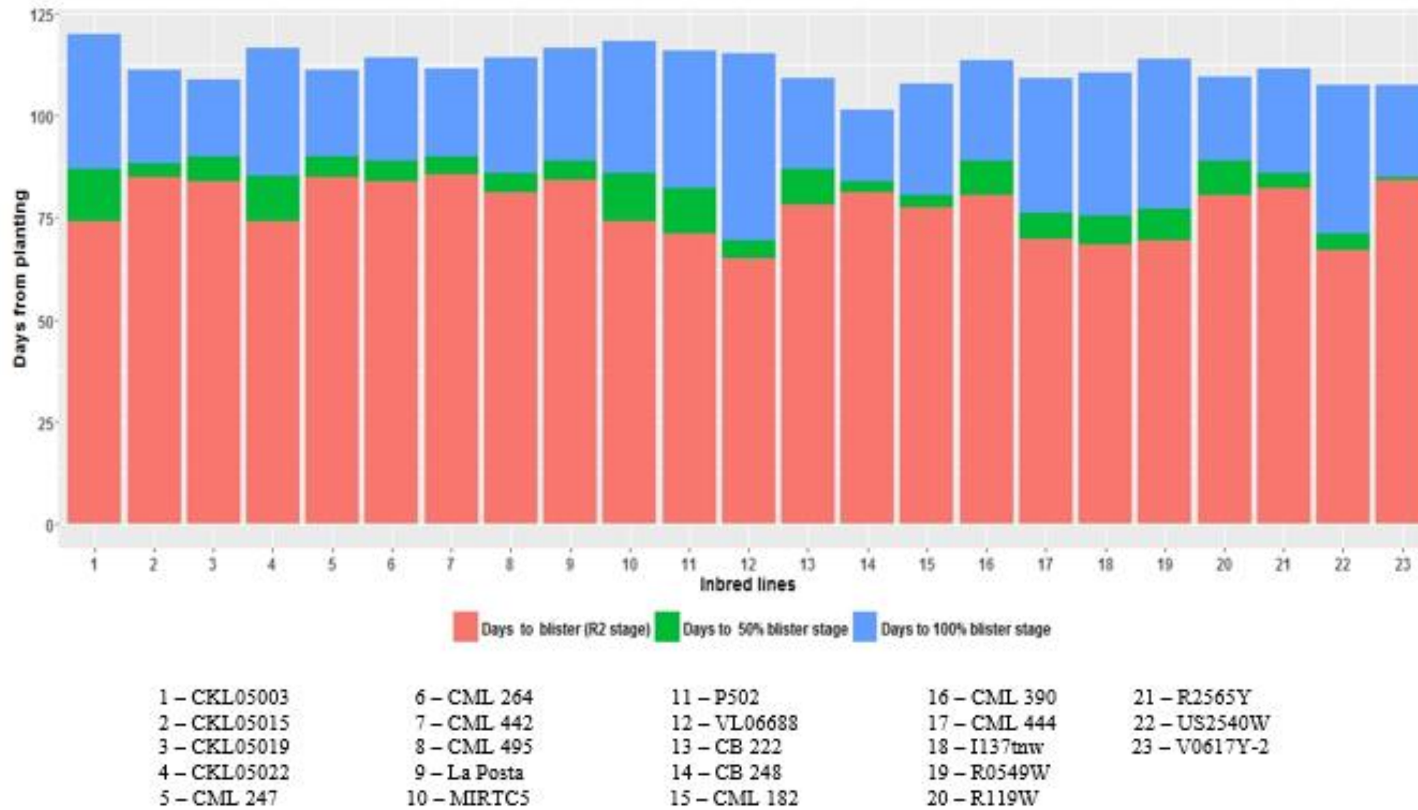

Figure 4-10: Variation in blistering of maize inbred lines

Lines CKLO5015, CKLO5019, CML 247, CML 264, CML 442, La Posta, and VO6017Y (a); CKLO50003, CKLO5022 and MIRTC5 (abcdef), CML 495, and CB 248; CB 222, R119W and R2565Y (abcd); I137tnw and RO549W(def) blistered within the same time. However, line P502, VLO6688, CML 182, CML 390, CML 444 and US2540W differed with any other inbred line in their blistering period (Table 4-10). Lines CKLO5003, CKLO5015, CKLO5022, CML 247, CML 442, CML 495, La Posta, MIRTC5, P502, CB 248, CML 182, CML 444, I137tnw, RO549W, R119W, R2565Y and VO617Y-2 (abc); CML 264 and CML 390(ab) attained their 50% blistering within the same time (Table 4-10). However, CKLO5019, VLO6688, US2540W and CML 264 differed significantly with any other inbred line in their number of days to 50% blistering. At 100% blistering, CKLO5003, CML 247, CML 442, MIRTC5, RO549W and VO617Y-2 (abcde); CKLO5022 and CML 390 (abcdef); CKLO5019 and CML 444 (cdef); CML 264 and CML 495 (abc); P502, CML 182, I137tnw and US2540W (bcdef) were similar in their days to blister. However, La Posta, VLO6688, CB 222, CB 248, R119W, R2565Y and CKLO5015 blistered at different times (Table 4-10).

Table 4-10: Number of days to blistering in maize inbred lines

Inbred line	Days to 1st blistering	Days to 50% blistering	Days to 100% blistering
CKL05003	74.00^{abcdef}	87.00^{abc}	107.00^{abcde}
CKL05015	85.00^{a}	88.33^{abc}	107.83^{abcd}
CKL05019	84.00^{a}	90.00^{a}	102.83^{cdef}
CKL05022	74.16^{abcdef}	85.33^{abc}	105.33^{abcdef}
CML 247	85.00^{a}	84.83^{abc}	106.16^{abcde}
CML 264	83.83^{a}	89.00^{ab}	109.00^{abc}
CML 442	85.50^{a}	74.50^{abc}	107.00^{abcde}
CML 495	81.16^{ab}	77.66^{abc}	109.50^{abc}
La Posta	84.20^{a}	82.60^{abc}	112.00^{a}
MIRTC5	75.66^{abcdef}	86.00^{abc}	106.16^{abcde}
P502	71.00^{bcdef}	82.33^{abc}	104.50^{bcdef}
VL06688	65.20^{f}	69.40^{c}	111.00^{ab}
CB 222	78.33^{abcd}	87.00^{abc}	100.33^{ef}
CB 248	81.16^{ab}	83.83^{abc}	98.66^{f}
CML 182	77.33^{abcde}	75.33^{abc}	104.50^{bcdef}
CML 390	80.66^{abc}	89.00^{ab}	105.33^{abcdef}
CML 444	69.66^{cdef}	76.00^{abc}	102.83^{cdef}
I137tnw	68.33^{def}	75.33^{abc}	103.66^{bcdef}
R0549W	69.50^{def}	77.16^{abc}	106.16^{abcde}
R119W	80.60^{abcd}	89.00^{abc}	101.00^{def}
R2565Y	79.00^{abcd}	75.50^{abc}	107.83^{abcd}
US2540W	66.50^{ef}	71.00^{bc}	103.66^{bcdef}
V0617Y-2	84.33^{a}	84.83^{abc}	107.00^{abcde}

Rainfall and temperature data in Katumani and Kiboko during the experimental period were as shown in Figure 4-11 and Figure 4-12.

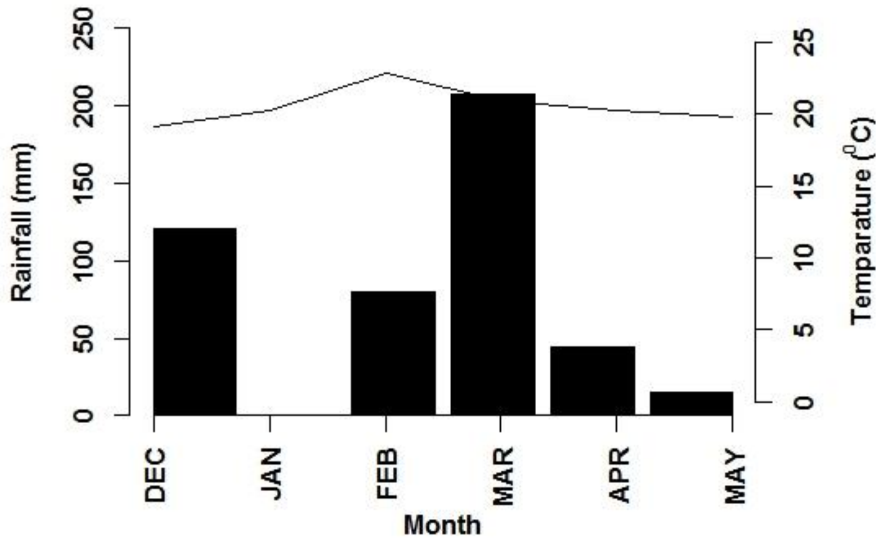

Figure 4-11: Average rainfall and temperature at Katumani from 9/12/12 to 30/5/13

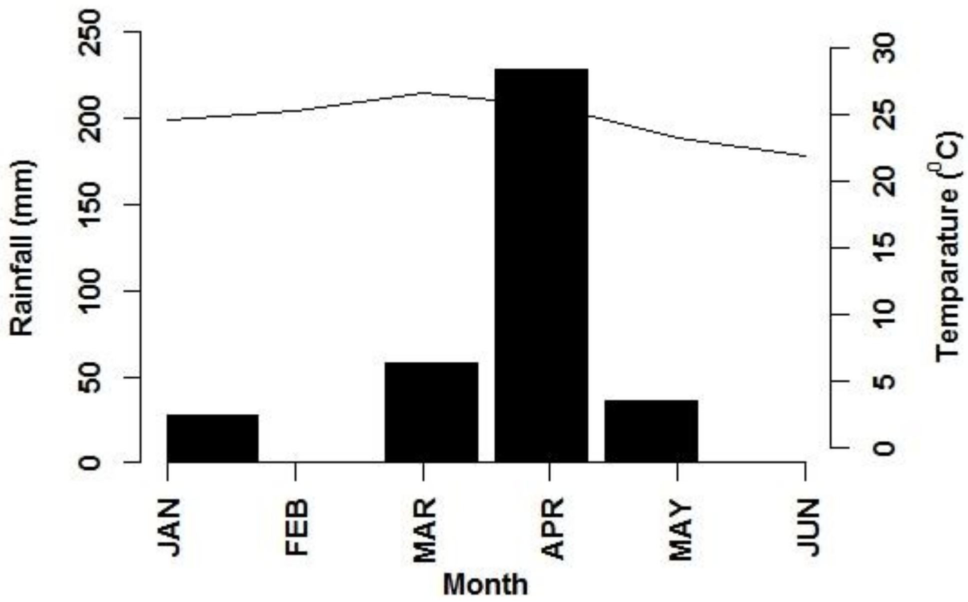

Figure 4-12: Average rainfall and temperature at Kiboko from 3/1/13 to 13/6/13

4.3 Ear rot severity in maize inbred lines

4.3.1 *Aspergillus* ear rot of maize inbred lines

Ear rot severity in Katumani and Kiboko differed among the lines (*F* (22, 112 = 4.34, p = 1.154e-07)). Kiboko lines showed significant variations (α = 0.05) in AER severity (*F* (22, 46 = 3.45, p = 0.0001). However, in Katumani no variations (α = 0.01) were recorded (*F* (22, 43 = 2.1325, p = 0.0167)) (Table 4-11). Figure 4-13 shows an inbred line with no ear rot and maize line with *Aspergillus* ear rot.

Table 4-11: ANOVA table; *Aspergillus* ear rot severity in maize lines

	F	df	p
Kiboko + Katumani	4.34	22	1.154e-07
Katumani	2.13	22	0.0167
Kiboko	3.45	22	0.0001

Figure 4-13: Harvested maize lines A) Clean maize B) *Aspergillus* ear rot maize

In the *Aspergillus* block, the lines had variations in AER severity (*F* (22, 112 = 4.337, p = 1.154e-07)) (Table 4-12). Among the lines, VLO6688 (66%) had the highest severity. Other lines that recorded high AER severity included CB 248 (26.3%), CML 442 (18.9%) and CML 264 (17.8%) (Table 4.12). Lines CKLO5022 (< 0.2%) and CML 390 (1.367%) had the lowest AER severity among all the inbred lines. South African lines that recorded high AER severity were CB 248 (26.3%), I137 tnw (14.5%),

RO549W (10.2%) and CM 182 (9.4%). Kenyan lines with high AER severity included VLO6688 (66%), CML 442 (18.9%), CML 264 (17.8%) and CKLO5003 (11.9%). Among the Kenyan lines, CKLO5022 (< 0.2%) and CML 247 (1%) recorded the least AER severity. South African lines with low AER severity included CML 390 (1.367%), CML 442 (1.9%) and VLO617Y (2.1%) (Table 4-12).

Table 4-12: *Aspergillus* ear rot severity of maize inbred lines

Inbred line	AER severity (%) Kiboko + Katumani	Inbred line	AER severity in Kiboko
CKL05003	11.90^{b}	VLO6688	1.93^{a}
CKL05015	7.38^{b}	CB 248	1.53^{a}
CKL05019	8.36^{b}	CML 264	1.50^{a}
CKL05022	0.13^{b}	I137tnw	1.25^{ab}
CML 247	0.95^{b}	US2540W	1.21^{ab}
CML 264	17.83^{b}	CKL05019	1.18^{ab}
CML 442	18.90^{b}	CML 442	1.17^{ab}
CML 495	3.10^{b}	CKL05003	1.09^{ab}
La Posta	1.81^{b}	RO549W	0.92^{ab}
MIRTC5	2.95^{b}	CKL05015	0.81^{ab}
P502	1.40^{b}	CML 444	0.35^{ab}
VL06688	65.97^{a}	MIRTC5	0.22^{ab}
CB 222	6.28^{b}	CML 182	-0.79^{ab}
CB 248	26.26^{b}	R119W	-0.85^{ab}
CML 182	9.45^{b}	CML 495	-1.02^{ab}
CML 390	1.36^{b}	CB 222	-1.06^{ab}
CML 444	1.68^{b}	La Posta	-1.19^{ab}
I137tnw	14.5^{b}	V0617Y-2	-1.27^{ab}
R119W	5.5^{b}	R2565Y	-2.90^{ab}
R2565Y	4.3333333^{b}	P502	-3.09^{ab}
R0549W	10.1833333^{b}	CKLO5022	-5.00^{b}
US2540W	8.4666667^{b}	CML 247	-5.00^{b}
V0617Y-2	2.1666667^{b}	VLO6688	1.93^{a}

* Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test ($p \leq 0.05$).

4.3.2 *Fusarium* ear rot of maize inbred lines

In Kiboko and Katumani, line VL06688 (87.25%) had the highest FER severity followed by R119W (49.83%). However, CML 442 (4.15%) recorded the least ear rot severity compared to any other line. Among the lines, substantial variances (F (22, 115 = 6.466, p = < 26.15e-12)) in FER occurred. Figure 4-12 shows maize line with *Fusarium* ear rot.

Figure 4-14: Maize showing *Fusarium* ear rot

In Katumani, there was uniformity ($p \leq 0.05$) in FER severity among the lines (F (22, 46 = 1.897, p = 0.0337)). However, in Kiboko, variations occurred among the lines (F (22, 46 = 2.5661, p = 0.003522)). Line VL06688 recorded the highest severity (88.67%) followed by I137tnw (85.63%). Line CML 442 on the other hand had the lowest FER. However, 82.6% of the lines had severity levels within the same range denoted as ab except lines I137tnw, VL06688, R119W and as shown in Table 4-13.

Table 4-13: Mean *Fusarium* ear rot % severity in maize inbred lines

Inbred Line	Kiboko + Katumani	Katumani	Kiboko
CKL05003	39.72[abcd]	1.36[a]	1.35[ab]
CKL05015	0.64[d]	-3.24[a]	-3.23[ab]
CKL05019	6.66[cd]	-2.90[a]	-2.89[ab]
CKL05022	11.86[cd]	-1.04[a]	-1.04[ab]
CML 247	3.25[cd]	-1.21[a]	-1.21[ab]
CML 264	11.11[cd]	0.95[a]	0.95[ab]
CML 442	0.00[d]	-5.00[a]	-5.00[b]
CML 495	3.57[cd]	-2.99[a]	-2.99[ab]
La Posta	28.48[bcd]	1.36[a]	1.36[ab]
MIRTC5	11.26[cd]	0.89[a]	0.81[ab]
P502	37.99[abcd]	1.35[a]	1.34[ab]
VL06688	72.42[a]	1.93[a]	1.93[a]
CB 222	20.00[bcd]	1.26[a]	1.25[ab]
CB 248	44.55[abc]	1.64[a]	1.64[ab]
CML 182	33.04[abcd]	1.51[a]	1.50[ab]
CML 390	5.09[cd]	-1.08[a]	-1.07[ab]
CML 444	3.29[cd]	-1.32[a]	-1.31[ab]
I137tnw	57.70[ab]	1.94[a]	1.93[a]
R0549W	31.96[abcd]	1.46[a]	1.45[ab]
R119W	58.88[ab]	1.76[a]	1.76[a]
R2565Y	20.83[bcd]	-0.73[a]	-0.73[ab]
US2540W	7.08[cd]	-0.99[a]	-0.98[ab]
V0617Y	24.39[bcd]	1.23[a]	1.23[ab]

*Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test ($p \leq 0.05$). Any mean below zero becomes negative after log transformation

4.4 Aflatoxin levels in maize inbred lines

Line P502 (38.37µg/kg) accumulated the highest aflatoxin levels followed by I137tnw (37.6µg/kg), R119W (37.5µg/kg), while CML 495 (1.091µg/kg) and CB 222 (1.1 µg/kg) had the least aflatoxin levels compared to any other evaluated inbred line. The other lines that accumulated low aflatoxin were CML 390 (1.23µg/kg) and CML 444 (1.362µg/kg) (Table 4-14). Aflatoxin susceptible South African inbred lines included R119W (37.6µg/kg), I137tnw (37.5µg/kg), R2565Y (31.3µg/kg), US2545W (29.2 µg/kg), RO549W (25.3µg/kg), VO617Y-2 (32.8µg/kg). Line CB 222 (1.1µg/kg) had the lowest aflatoxin levels among all the South African inbred lines. Other South African inbred lines that accumulated low aflatoxin levels were CML 390 (1.2µg/kg) and CB 248 (2.5µg/kg) (Table 4-14). Among the Kenyan inbred lines, P502 (38.4 µg/kg), VLO6688 (30.8µg/kg), MIRTC5 (25.8µg/kg) and CKLO5003 (24µg/kg) accumulated high amounts of aflatoxin levels. Inbred lines CML 495 (1.091µg/kg), CML 247 (1.61µg/kg) and La Posta (2.03µg/kg) were the Kenyan lines that had low aflatoxin levels (Table 4-14).

Table 4-14: ANOVA table; Variation in aflatoxin levels in maize inbred lines

	F	Df	p
Kiboko+ Katumani	76.93	22	< 2.2e-16
Katumani	47.30	22	< 2.2e-16
Kiboko	45.02	22	< 2.2e-16

Lines R119W (ab) and I137 tnw (ab); R2565Y (abcd) and VLO6688 (abcd); RO549W (cd) and MIRTC5 (cd) accumulated aflatoxin levels did not vary from each other. Lines CKLO5019 (h), CKLO5022(h), CML 247 (h), CML 495 (h), La Posta (h), CB 222 (h), CB 248 (h), CML 390(h) and CML 444 (h) were the inbred lines that had least aflatoxin levels (Table 4.15). However, CKLO5003 (de), CKLO5015 (fgh), CML 264 (gh), CML 442 (ef), P502 (a), CML 182 (fg), US2540W (bcd) and VO617Y-2 (abc) differed significantly with the other inbred lines in the amounts of accumulated aflatoxin levels at $p \leq 0.05$ Tukey HSD test (Table 4-15).

Table 4-15: Aflatoxin levels (µg/kg) in maize inbred lines

Inbred line	Katumani + Kiboko	Kiboko	Katumani
CKL05003	23.99[de]	1.39[abc]	1.36[ab]
CKL05015	8.87[fgh]	1.04[cd]	0.81[bcde]
CKL05019	4.28[h]	0.73[def]	0.45[defg]
CKL05022	4.49[h]	0.66[defg]	0.62[cdef]
CML 247	1.61[h]	0.42[efgh]	-0.33[hi]
CML 264	6.96[gh]	0.73[de]	0.90[bcd]
CML 442	16.32[ef]	1.24[abc]	1.15[abc]
CML 495	1.09[h]	-0.14[i]	0.03[gh]
La Posta	2.03[h]	0.32[efghi]	0.27[efg]
MIRTC5	25.84[cd]	1.44[abc]	1.36[ab]
P502	38.36[a]	1.63[a]	1.49[ab]
VL06688	30.79[abcd]	1.46[abc]	1.53[ab]
CB 222	1.09[h]	0.25[fghi]	-0.80[i]
CB 248	2.47[h]	0.45[efgh]	0.27[efg]
CML 182	13.35[fg]	1.11[bcd]	1.13[abc]
CML 390	1.22[h]	-0.01[hi]	0.15[fgh]
CML 444	1.36[h]	0.20[ghi]	0.01[gh]
I137tnw	37.49[ab]	1.57[ab]	1.56[a]
R119W	37.60[ab]	1.39[abc]	1.39[ab]
R2565Y	31.27[abcd]	1.62[a]	1.51[ab]
R0549W	25.26[cd]	1.54[ab]	1.43[ab]
US2540W	29.25[bcd]	1.53[ab]	1.38[ab]
V0617Y-2	32.75[abc]	1.53[ab]	1.49[ab]

* Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test ($p \leq 0.05$).

4.5 *F*umonisin in maize inbred lines

Among the lines, R2565Y and VO617Y-2 had the highest levels of fumonisin (6.76mg/kg and 6.21mg/kg respectively). Line CML 390 (1.08mg/kg) accumulated the least fumonisin compared to any other inbred line. Other inbred lines with low fumonisin levels included CML 247 (1.43mg/kg) and CKLO5003 (1.78mg/kg) as shown in Table 4.16. Among the lines, significant differences in fumonisin levels occurred in Kiboko + Katumani ((F (22, 117 = 2.9311, p = 0.0001001) (Table 4-16).

Table 4-16: ANOVA table; Fumonisin levels (mg/kg) in inbred maize lines

Location	F	df	p
Kiboko+ Katumani	2.931	22	0.0001001
Katumani	3.952	22	0.00003.816
Kiboko	11.232	22	4.033e-12

Lines CKLO5015 (abcd), CKLO5019 (abcd), CKLO5022 (abcd), CML 264 (abcd), CML 442 (abcd), CML 495 (abcd), La Posta (abcd), P502 (abcd), VLO6688 (abcd),

CB 222 (abcd), CML 182 (abcd), CML 444 (abcd), I137tnw (abcd), R119W (abcd) and US2540W (abcd); CKLO5003 (bcd) and CB 248 (bcd); MIRTC5 (abc) and RO549W (abc) were similar in their accumulated fumonisin levels (Table 4-17). However, CML 247 (cd), CML 390 (d), R2565Y (a) and VO617Y-2 (ab) varied with other inbred lines in their accumulated fumonisin levels (Table 4-17).

Fumonisin levels in the inbred lines grown in Kiboko ((F (22, 47 = 11.232, p = 4.033e-12)) and Katumani ((*F* (22, 47 = 3.9519, p = 3.816e-05)) varied significantly with CML 390 accumulating the lowest fumonisin in Katumani and Kiboko (0.115 mg/kg and 0.076mg/kg respectively). In Katumani, CML 442 and R119W recorded the highest fumonisin levels (4.0068mg/kg and 3.8556mg/kg respectively). However, V0617Y-2 had the highest amounts of fumonisin in Kiboko (9.65mg/kg) followed by R119W (5.64mg/kg) (Table 4-17).

Table 4-17: Fumonisin levels (mg/kg) in maize inbred lines

Inbred Line	Kiboko+ Katumani	Katumani	Kiboko
CKL05003	1.78^{bcd}	0.38^{abcd}	0.03^{de}
CKL05015	2.74^{abcd}	0.20^{cd}	0.55^{abc}
CKL05019	3.29^{abcd}	0.31^{abcd}	0.65^{ab}
CKL05022	3.74^{abcd}	0.32^{abcd}	0.72^{ab}
CML 247	1.43^{cd}	0.22^{bcd}	0.06^{de}
CML 264	4.12^{abcd}	0.57^{ab}	0.62^{ab}
CML 442	4.33^{abcd}	0.60^{a}	0.66^{ab}
CML 495	3.48^{abcd}	0.43^{abcd}	0.61^{ab}
La Posta	4.52^{abcd}	0.48^{abc}	0.76^{ab}
MIRTC5	5.76^{abc}	0.46^{abcd}	0.90^{ab}
P502	3.50^{abcd}	0.59^{a}	0.48^{bcd}
VL06688	3.86^{abcd}	0.48^{abc}	0.63^{ab}
CB 222	3.81^{abcd}	0.36^{abcd}	0.72^{ab}
CB 248	2.07^{bcd}	0.39^{abcd}	0.09^{cde}
CML 182	2.88^{abcd}	0.44^{abcd}	0.46^{bcd}
CML 390	1.07^{d}	0.11^{d}	-0.07^{e}
CML 444	3.12^{abcd}	0.32^{abcd}	0.59^{ab}
I137tnw	3.19^{abcd}	0.53^{abc}	0.47^{bcd}
R0549W	5.69^{abc}	0.49^{abc}	0.74^{ab}
R119W	4.74^{abcd}	0.58^{ab}	0.97^{a}
R2565Y	6.75^{a}	0.561^{abc}	0.89^{ab}
US2540W	4.80^{abcd}	0.41^{abcd}	0.79^{ab}
V0617Y-2	6.20^{ab}	0.43^{abcd}	0.90^{ab}

* Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test ($p \leq 0.05$).

4.6 Association between ear rots and toxin levels in maize inbred lines

4.6.1 Relationship between *Aspergillus* ear rot and aflatoxin

In the sites where the study was conducted, no relations occurred between *Aspergillus* ear rot (AER) severity and aflatoxin accumulation levels in the inbred lines as summarized in Table 4-18 below;

Table 4-18: *Aspergillus* ear rot versus aflatoxin levels in maize inbred lines

Location	r value	P value
Combined Katumani +Kiboko	0.127	> 0.05
Kiboko	0.044	> 0.05
Katumani	0.11	> 0.05

4.6.2 Relationship between *Fusarium* ear rot and fumonisin

Significant correlations occurred between FER (%) severity and fumonisin concentration levels in the inbred lines in the two sites combined. However, there were no significant connections between the two variables in either of the sites (Table 4-19).

Table 4-19: Association between *Fusarium* ear rot and Fumonisin levels in maize inbred lines

Location	r value	P value
Combined Katumani +Kiboko	0.300	< 0.05
Kiboko	0.023	> 0.05
Katumani	0.032	>0.05

4.6.3 Connection between *Fusarium* and *Aspergillus* ear rots

Among the inbred lines grown in Katumani + Kiboko combined, Kiboko and Katumani, there were no significant relationships between FER and AER (Table 4-20).

Table 4-20: Association between *Fusarium* ear rot and *Aspergillus* ear rot in maize inbred lines

Location	r value	p value
Combined Katumani +Kiboko	-0.116	> 0.05
Kiboko	-0.045	> 0.05
Katumani	-0.129	> 0.05

4.7 Aflatoxin and fumonisin levels in season I hybrids

4.7.1 Aflatoxin in season I crosses

Season I maize hybrids accumulated aflatoxin levels ranging from 15.88 to 163.36 µg/kg. Among the crosses, only two crosses accumulated aflatoxin levels below the regulatory action level of 20µg/kg. These included CKLO5022 (F) x CB 248 (M) (15.877µg/kg) and CB 248 (F) x CKLO5022 (M) (17.545µg/kg) (Table 4-22).

Though there were substantial variances in accumulated aflatoxin levels (*F* (21, 43 = 25.183, p = < 2.2e-16)), some crosses had no differences in their aflatoxin levels. These included group ab - 2 (137.185µg/kg), 3 (131.428µg/kg) and 4 (122.677µg/kg); group abc - 5 (115.488µg/kg), 6 (114.287µg/kg) and 7 (112.738µg/kg); group abcde - 9 (78.782µg/kg) and 10 (78.78µg/kg); group bcdefg - 12 (61.295µg/kg) and 13 (60.73µg/kg); group hi - 19 (20.537µg/kg) and 20 (18.503µg/kg); group i - 21 (17.545µg/kg) and 22 (15.877µg/kg) (Table 4.21). Among the crosses, group b (2, 3 and 4) accumulated the highest amounts of aflatoxin while group i (22 and 21) and hi (20 and 19) accumulated the least aflatoxins (Table 4-21).

Crosses that had different aflatoxin levels were 1 (a), 8 (abcd), 11 (bcdef), 14 (cdefg), 15 (defg), 16 (efgh), 17 (fghi) and 18 (ghi). Reciprocal crosses i (21 and 22) accumulated almost equal amounts of aflatoxin levels while 16 and 17 reciprocal crosses differed in their aflatoxin levels hence grouped as efgh and fghi respectively (Table 4-21).

Table 4-21: Aflatoxin levels (µg/kg) in season I maize hybrids in Katumani Research station

Season I maize hybrids	Aflatoxin levels
CKL05015 (m) x R119W (F)	2.21^{a}
CKL05022 (m) x VO617Y 2 (F)	2.13^{ab}
CKL05022 (F) x V0617Y 2 (m)	2.11^{ab}
P502 (m) x CML 390 (F)	2.08^{ab}
CML 390 (m) x P502 (F)	2.05^{abc}
P502 (F) x CB 248 (m)	2.05^{abc}
P502 (m) x CB 248 (F)	2.04^{abc}
P502 (m) x CB 222 (F)	2.01^{abcd}
P502 (F) x CB 222 (m)	1.89^{abcde}
P502 (F) x CB 222(m)	1.88^{abcde}
CKL05019 (F) x CML 444 (m)	1.84^{bcdef}
CML 444 (F) x CKL05022 (m)	1.78^{bcdefg}
CB 248 (m) x CML 390 (F)	1.78^{bcdefg}
CML 390 (m) x CB 248 (F)	1.71^{cdefg}
La Posta (F) x CB 248 (m)	1.68^{defg}
La Posta (F) x CB 222(m)	1.58^{efgh}
CB 222 (F) x La Posta(m)	1.52^{fghi}
La Posta (F) x CML 444 (m)	1.46^{ghi}
CML 247 (F) x CML 444 (m)	1.28^{hi}
CML 247(F) x CB 222 (m)	1.23^{hi}
CKL05022 (m) x CB 248 (F)	1.23^{hi}
CKL05022 (F) x CB 248 (m)	1.18^{i}

*Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test ($p \leq 0.05$).

4.7.2 Fumonisin levels in season I maize hybrids

Two reciprocal crosses had fumonisin above 2 mg/kg and they included MIRTC5 (F) x R119W (M) (2.793mg/kg) and R119W (F) x MIRTC5 (M) (2.983mg/kg). However, CML 444 (F) x CKLO5015 (M) (0.50227mg/kg) accumulated the lowest fumonisin level compared to any other hybrid. The other crosses CML 444 (F) x CML 495 (M), CML 495 (F) x CML 444 (M) and P502 (F) x CML 444 (M) recorded fumonisin levels below 2mg/kg ranging between 0.8211 to 0.90468mg/kg (Table 4-23). Significant

variations occurred in fumonisin concentrations among the season I crosses (F (5, 12 = 44.344, p = 2.543e-07)).

Table 4-22: Fumonisin levels (mg/kg) in season I maize hybrids in Katumani Research station

Season I maize hybrids	Fumonisin concentration
MIRTC5 (m) x R119W (F)	0.45^{a}
MIRTC5 (F) x R119W (m)	0.43^{a}
P502 (F) x CML 444 (m)	-0.05^{b}
CML 444 (F) x CML 495 (m)	-0.05^{b}
CML 495 (F) x CML 444 (m)	-0.08^{bc}
CML 444 (F) x CKL0 5015 (m)	-0.30^{c}

*Means followed by the same letter do not differ significantly according to Tukey's HSD (honest significant difference) test (p ≤ 0.05).

4.8 Resistance of season II and season III crosses to aflatoxins and fumonisins

All season II crosses accumulated aflatoxin levels above 20µg/kg with aflatoxin levels ranging between 22.455-95.295µg/kg. The crosses differed significantly in their aflatoxin levels (F (39, 59 = 7.6655, p = 1.373e-14)). However, some crosses had similar concentrations of aflatoxin and they included group a 1 (95.295µg/kg) and 2(92.633µg/kg); abcdefg 8(47.94µg/kg), 9(45.013µg/kg), 10 (45.013µg/kg), 11 (41.267µg/kg), 12 (39.965µg/kg) and 13 (39.583µg/kg); bcdefg 15(37.64µg/kg), 16 (36.563µg/kg), 17 (36.047µg/kg) and 18 (35.922µg/kg); cdefg 19 (35.482µg/kg), 20 (33.393µg/kg), 21 (33.2µg/kg), 22 (32.643µg/kg), 23 (32.43µg/kg), 29 (29.505µg/kg), 30 (29.32µg/kg), 31 (29.23µg/kg) and 32 (28.868µg/kg); efg 33 (27.53µg/kg), 34 (27.075µg/kg) and 35 (27.03µg/kg); g 37 (25.515µg/kg), 38 (25.22 µg/kg), 39 (24.355µg/kg) and 40 (22.455µg/kg) (Table 33) . However, 6 crosses; ab 3 (79.14µg/kg), abc 4 (68.927µg/kg), abcd 5 (65.125µg/kg), abcde 6 (60.872µg/kg), abcdef 7 (60.108µg/kg) and group fg 36 (26.042µg/kg) aflatoxin levels were different from any other crosses.

Season III crosses had variations in aflatoxin levels (F (39, 79 = 9.1518, p = < 2.2e-16)). Some crosses had same aflatoxin levels and they included group abcdef 6(61.648µg/kg) and 7 (60.233µg/kg); abcdefg 8(58.158µg/kg), 9(56.893µg/kg), 10(56.617µg/kg), 11(55.21µg/kg); abcdefgh 12 (52.435µg/kg), 13 (49.43µg/kg), 14

(48.903µg/kg), 15 (47.817µg/kg), 16 (47.726µg/kg); abcdefghi 17 (47.545µg/kg), 18 (46.447µg/kg), 19 (45.199µg/kg), 20 (45.987µg/kg); defghij 23 (39.343µg/kg), 24 (38.873µg/kg), 25 (37.995µg/kg), 26 (37.228µg/kg), 27 (36.46µg/kg), 28 (35.873µg/kg), 29 (35.343µg/kg), 30 (34.88µg/kg), 31(34.397µg/kg), 32 (33.985µg/kg); ghij 35 (28.573µg/kg), 36 (28.19µg/kg); hij 37 (26.195µg/kg) and 38 (26.018µg/kg) (Table 34). The crosses that accumulated different aflatoxin levels from any other included group a 1 (99.263µg/kg), ab 2 (91.703µg/kg), abc 3 (83.068µg/kg), abcd 4 (69.004µg/kg), abcde 5 (64.373µg/kg), bcdefghi 21 (42.08µg/kg), cdefghij 22 (40.077µg/kg), efghij 33 (31.168µg/kg), fghij 34 (29.955µg/kg) ij 39 (22.637µg/kg) and j 40 (22.8µg/kg) (Table 34). All the hybrids in season III had aflatoxin levels above 20µg/kg.

In seasons II and III, La Posta (F) x R119W (M) accumulated the highest levels of aflatoxin though season III (99.263µg/kg) was higher than in season II (95.295µg/kg). In season II crosses, CKLO5022 (F) x CML 444 (M) recorded the lowest aflatoxin level (22.455µg/kg) (Table 33) while CML 444 (F) x CKLO5022 (M) had the lowest aflatoxin level in season III (22.18µg/kg).

Fumonisin levels in season II hybrids varied among the crosses and MIRTC5 (F) x CML 444 (M) had the highest amount of fumonisin (2.714mg/kg) compared to any other cross. However, CKLO5015 (F) x CML 444 (M) had the least fumonisin level (0.224mg/kg). Among the crosses, 80% accumulated fumonisin levels below 2mg/kg (Table 35). The hybrids varied significantly in their fumonisin concentrations (F (29, 59 = 7.4248, p = 4.803e-11)), though some hybrids had similar amounts of fumonisin; ab 2 (2.462mg/kg) and 3(2.304mg/kg); abcd 5 (2.116mg/kg), 6 (2.034 mg/kg) and 7 (1.993 mg/kg); abcde 9 (1.713 mg/kg), 10 (1.544 mg/kg), 11 (1.347 mg/kg), 12 (1.338 mg/kg), 13 (1.329 mg/kg), 14 (1.225 mg/kg), 15 (1.279 mg/kg), 16 (1.205mg/kg), 17 (1.229mg/kg) and 18 (1.092mg/kg); bcdefg 22 (0.811mg/kg), 23 (0.812mg/kg), 22 (0.811mg/kg), 23 (0.812mg/kg), 24 (0.784mg/kg) and 25 (0.779 mg/kg) (Table 35). However, some hybrids accrued fumonisin levels different from other crosses and they included; a 1(2.714mg/kg), abc 4(2.112mg/kg), abcdefg 21 (0.878mg/kg), cdefg (0.668mg/kg), defg (0.674mg/kg), efg (0.522mg/kg), fg (0.377mg/kg) and g (0.224mg/kg).

Among season III crosses, 73.33% of the crosses accrued fumonisin levels below 2mg/kg ranging between 0.037-1.865mg/kg. Hybrid CKLO5015 (F) x CML 444 (M) accumulated the least amount of fumonisin (0.03735mg/kg). Other crosses that recorded very low fumonisin levels included CML 444 (F) x P502 (M) (0.3894mg/kg), CKLO5015 (F) x R119W (M) (0.51436mg/kg) and CML 444 (F) x R119W (M) (0.62668 mg/kg). Hybrids MIRTC5 (F) x CML 444 (M), MIRTC5 (F) x CKLO5015 (M) and CML 495 (F) x R119W (M), had high fumonisin levels (3.0453mg/kg, 3.26557mg/kg and 3.25955mg/kg respectively). The hybrids differed significantly in their fumonisin concentrations ($F (29, 59 = 15.468, p = < 2.2e\text{-}16)$) and yet some crosses had same fumonisin levels. These included groups; a 1 (3.266mg/kg), 2 (3.2595mg/kg) and 3 (3.04525mg/kg); abcd 6 (2.51244mg/kg) and 7 (2.4974mg/kg), abcde 8 (2.0519mg/kg), 9 (1.86496mg/kg), 10 (1.86465mg/kg) and 11 (1.84976mg/kg); abcdef 12 (1.68236mg/kg), 13 (1.48792mg/kg), 14 (1.3286mg/kg), 15 (1.30648mg/kg), 16 (1.2941mg/kg) and 17 (1.27084mg/kg); abcdefg 18 (1.1305mg/kg), 19 (1.21997mg/kg), 20 (1.13758mg/kg) and 21 (0.97411mg/kg); cdefg 23 (0.81196mg/kg), 24 (0.7601mg/kg) and 25 (0.75595mg/kg). However, 26.67% of the crosses had different amounts of fumonisins. These included groups ab 4 (2.75mg/kg), abc (2.737mg/kg), bcdefg (1.601mg/kg), defg (0.751mg/kg), efg (0.627mg/kg), fg (0.514mg/kg), g (0.389mg/kg) and h (0.037mg/kg).

Average temperatures and rainfall during the hybrid’s growth periods were as shown in Figure 4-15 and Figure 4-16.

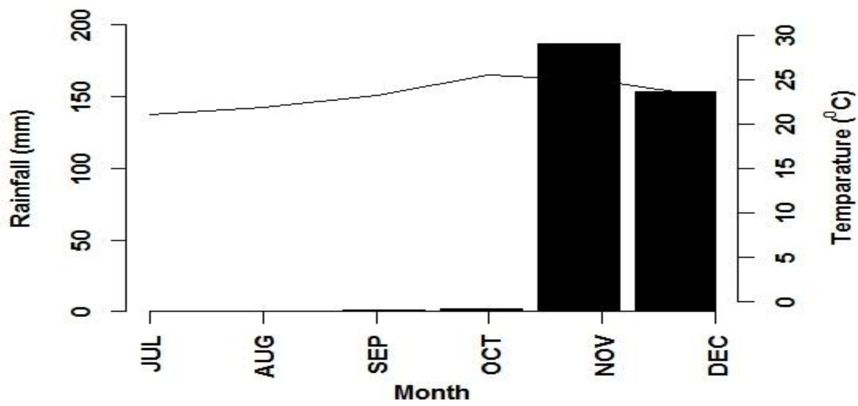

Figure 4-15: Average temperature and rainfall recorded for growing season II from July to December 2014

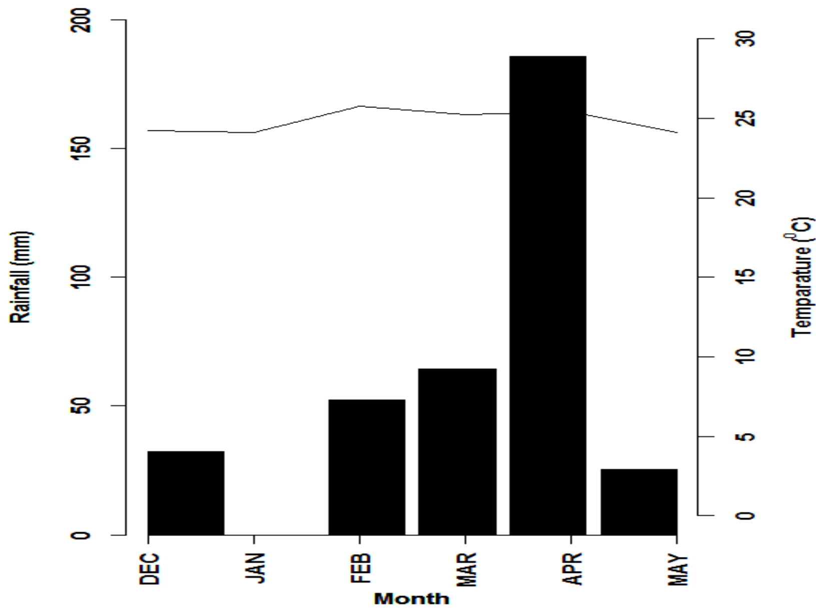

Figure 4-16: Average rainfall and temperature data at Kiboko for the season III crosses from December 2014 to March 2015

CHAPTER FIVE

DISCUSSION

5.1 Mating types in *Aspergillus flavus* populations from Makueni and Nandi

Fungi that produce functional *MAT1-1* and *MAT1-2* genes on the same thallus are self-fertilizing while those with a single MAT gene require compatible *MAT1-1* or *MAT1-2* nuclei from two different individuals. In the Eastern and Rift valley regions, MAT 1 - 2 isolates frequency was high compared to *MAT1-1* isolates and isolates with both *MAT1-1* and *MAT1-2* genes. However, *MAT1-2* frequency in the Nandi region was higher (66.67%) than in Makueni (56.52%). The existence of mating types in all the 44 *A. flavus* isolates is a key signal of possibility for sexual recombination among the isolates in the aflatoxin gene cluster. Wada et al. (2012) supports that MAT genes are important governors of sexual existence all over the fungal kingdom and seem to be a necessity for sexual reproduction.

Laboratory crosses between strains of *A. flavus* of opposite mating type genes have successfully been applied to induce sexual recombination between *MAT1-1* and *MAT1-2* isolates (Horn *et al.*, 2014; Janos *et al.,* 2014 and Horn *et al.,* 2016). In our study, most of the isolates from Makueni were either *MAT1-1* or *MAT1-2* (Table 5-1), indicating that they are likely heterothallic. Four of the isolates sampled in Makueni contained fragments of both MAT genes, indicating the potential for a homothallic existence (Table 5-1). The nearly equal distribution of single mating-types, as well as the presence of isolates exhibiting evidence of two MAT genes, in this region could be an indication that there is active recombination occurring in the fields where these isolates were sampled. Five of the Nandi isolates exhibited evidence for containing both MAT genes. Of the isolates that contained one or the other MAT gene, no *MAT1-1* isolates were identified (Table 5-2). From this we can infer that recombination is not occurring at a rate comparable to Makueni. Mating tests would allow us to confirm or refute the functionality of these loci.

Table 5-1: Mating type distribution for *A. flavus* isolates sampled in Makueni

Strain identity	Aflatoxin level (μg/kg)	Mating type identity using PCR multiplex assay
1VM201365	152965	MAT1-1 & MAT1-2
1VM40018	97184	MAT1-1 & MAT1-2
1VM100095	92249	MAT1-1
1VM100130	88878	MAT1-1
2M2002LG	74367	MAT1-2
2M1983G	67350	MAT1-1
1VM250WY	47653	MAT1-2
2VM964	29484	MAT1-2
1VM132G	23069	MAT1-2
2VM882G	19038	MAT1-2
1VM414	13574	MAT1-2
1VM100957	11971	MAT1-2
1VM201122	11292	MAT1-1
1VM130LG	10199	MAT1-1
1VM100079	9089	MAT1-2
1VM350G	2912	MAT1-1 & MAT1-2
3VM566G	1850	MAT1-2
2M1983DG	1165	MAT1-2
2M1002LG	962	MAT1-2
1VM195	785	MAT1-1
1VM131LG	602	MAT1-2
3VM566	332	MAT1-2
1VM201204	22	MAT1-1 & MAT1-2
1VM300566	0	MAT1-1

Table 5-2: Mating type distribution for *A. flavus* isolates sampled in Nandi

Strain identity	Aflatoxin level (μg/kg)	Mating type identity using PCR multiplex assay
40041BM38Y6	80664	*MAT1-2*
60801BSIYG	23883	*MAT1-2*
40224BSIYG	22203	*MAT1-2*
40296BSY	12803	*MAT1-1* & *MAT1-2*
60756	11292	*MAT1-2*
50400BM2YG	247	*MAT1-2*
40243BMIB	151	*MAT1-1* & *MAT1-2*
40013BM126	145	*MAT1-2*
40267BSIYG	96	*MAT1-2*
60661BMIYG	91	*MAT1-2*
504820BMIYG	77	*MAT1-1* & *MAT1-2*
50536BMIYG	77	*MAT1-2*
60690BMIYG	57	*MAT1-2*
40143BM112G	46	*MAT1-1* & *MAT1-2*
60572 BM2Y	46	*MAT1-2*
60795BM8BJ	22	*MAT1-2*
40025BSIYG	22	*MAT1-2*
50444 BSIYG	0	*MAT1-1* & *MAT1-2*

In Makueni, 17.39% of the isolates were homothallic with MAT genes encoding both MAT 1 - 1 and MAT 1 - 2 genes at a locus while Nandi had 23.81% homothallic isolates

hence are self-fertile and can complete their sexual cycle without the need for a partner. However, heterothallic isolates in Nandi were MAT 1 - 1 (9.52%), MAT 1 - 2 (71.43%) and in Makueni MAT 1 - 1 were 26.09%, MAT 1 - 2 (66.67%). Understanding genetic variation within *A. flavus* through mating type analysis is thus an important step in developing a strong biocontrol strategy. The non-aflatoxigenic strains among the isolates need to be selected carefully and gene flow traced. This is because isolated strains used in the same niche have proved effective according to Mamo et al. (2017) and Ehrlich, (2014).

We may be able to infer a correlation between mating type distribution and aflatoxin outbreaks based on our findings. For example, aflatoxin outbreaks in in Makueni (having a more equal distribution of mating types) have been reported since 1981, while no aflatoxin outbreaks have been reported in Nandi, which has a predominance of *MAT1-2* genes (Kangethe *et al.,* 2017; Sirma *et al.,* 2015; Okoth *et al.,* 2016; Okoth *et al.,* 2017). Sexual recombination between toxigenic and non-aflatoxigenic *A. flavus* strains in the soil is viewed as a major cause of diversity enabling some of the *A. flavus* progenies to inherit the ability to produce aflatoxin (Horn *et al.,* 2009; Olarte *et al.,* 2012; Moore *et al.,* 2013; Olarte *et al.,* 2013; Horn *et al.,* 2014; Chepsergon *et al.,* 2014 and Ehrlich *et al.,* 2014).

Currently, biocontrol is the most favorable technique for lowering pre-harvest aflatoxin contamination. The strategy includes spreading non-aflatoxigenic *A. flavus* strain spores, which result in greatly reduced levels of aflatoxin. Both aflatoxin and non-aflatoxin producing strains occupy the same niches in the soil (Yan-ni *et al.,* 2008). In our study, the 44 *A. flavus* strains characterized are agricultural isolates (Nyongesa *et al.,* 2015 and Okoth *et al.,* 2012). Existence of a nearly equal distribution of MAT genes in Makueni indicate potential for sexual recombination (Table 5-1). Atoxigenic *MAT1-1* IVM300566 (0 µg/kg) as biocontrol could outcross with toxigenic *MAT1-2* in the two regions which could facilitate recombination. Therefore, it is advisable to search for an atoxigenic *MAT1-2* strain as a candidate biocontrol strain. Ehrlich, (2014) suggests that it is important to test frequency of genetic recombination in agricultural environments where non-aflatoxin producing biocontrol have been introduced. Based on our findings, and extrapolation to the field, it is important to assess this distribution for a field prior

to release of fertile biocontrol strains that can recombine with the indigenous population. We must also verify the fecundity of strains that contain both MAT genes to determine if they are functionally bisexual as Horn et al. did with *A. nomius* (2011).

Despite the two regions having high MAT 1 - 2 isolates compared to other MAT genes and Nandi having high frequency of isolates with both MAT 1 - 1 and MAT 1 - 2 genes, aflatoxin outbreaks have always been reported in the Eastern part of Kenya unlike in the Rift Valley region. The trend in mating type genes among the isolates in the Eastern part of Kenya therefore need to be traced because previously, it was thought that reproduction in ascomycetes fungi was strictly asexual. However, molecular genetic analysis has provided evidence of sexual reproduction in ascomycete fungi (Ramirez-prado *et al.,* 2008) and in nature, outcrossing among different fungal populations in the soil enable gene flow to occur. Ehrlich et al. (2014) supports that *A. flavus* has a sexual compatibility and vegetative incompatibility system. Both play a role in directing gene flow in populations and the diversification of *A. flavus* is also suspected to be as a result of its potential to outcross by sexual recombination under special conditions in the soil. This suggests that there could be favourable conditions in the soil promoting growth and virulence of *A. flavus* in Makueni compared to Nandi region hence causing aflatoxicoses outbreaks in the Eastern region. It is thus important to trace gene flow in the isolates from Makueni in their natural populations for safe *A. flavus* bio-control development.

5.2 Germination in maize inbred lines

Seeds with delayed germination rates like R2565Y are likely to reduce the yield of individual plants in a stand, therefore, every breeder would prefer seeds with high germination rates (CML 390, CML 495, CKL050150 and CKL05019). Crop yield and efficiency of resource is determined by successful establishment of the plant in the field and poor germinating lines (R2565Y-2, R119W and CML 264) are likely to affect crop yield especially when grown in areas where the growing season is short since in such areas, the crops must have their developments faster in order to maximize yield. Further still, performance of F_1 greatly depends on the parent lines used in developing crosses (Bushra and Jones, 1983) which makes most breeders select for maize lines with fast, uniform germination and seedling emergence in order to maximize on yield. In addition

to fast stand establishment, the short ASI (-2 to 2 days) recorded by the inbred lines would be an important trait in consideration during breeding. Among the inbred lines, 48.7% of the lines presented negative ASI which is a drought escaping phenomenon signalled by the occurrence of silking before tassels were produced. The short ASI is associated with drought tolerance because the genotypes make use of the limited moisture more efficiently during flowering (Ngugi *et al*., 2013). Selection of maize lines with short ASI is an effective approach for synchronisation for male and female flowering under stress (Bernahu *et al*., 2017). This trait together with other useful secondary traits can be used by breeders for selecting parental lines for breeding purposes.

In Kenya, most farmers prefer seeds that germinate fast evading seed rot germination diseases. In Rift Valley, it was reported in the daily nation (www. nation.co.ke) that maize production dropped from 21 million bags to 16 million bags in 2016 due to maize diseases like head smut and maize lethal necrosis disease. The farmers also pointed out poor germination of the supplied seeds with most seeds rotting as a key cause of yield drop. In May 2017, the farmers also expressed fears of serious yield decline due to head smut which they attributed to poor germination pattern of the seeds (www. nation.co.ke). Breeders therefore need to select for seeds that have good germineability and low rotting during germination.

Leonard, (1998) and Han et al. (2014), support that plant vigour is one of the principal characteristics which support good yields and seed germination rate is an important component of the seed vigour. It is the seed vigour that determines the ability of seeds to germinate and form seedlings quickly, uniformly and robustly across diverse environmental conditions. Elevated seed vigour is therefore key in determining agricultural production because of related prospective for increased growth and productivity. Seed vigour therefore affects selection of seeds by most breeders since a wide better part of the crops that are produced in the world of agriculture are sowed first using seeds in order to establish a new plant in the environment. Therefore, triumphant establishment of the seeds is the first important step for crop production because it determines the success or failure of future harvest. It is thus a primary

objective of every agricultural industry and breeder to improve seed vigour in order to increase the critical and yield defining stage of crop establishment.

5.3 Flowering in maize inbred lines

Flowering stage in maize is the key period in maize development determining grain yield characterized by silking and pollen shed. Silking data was recorded at first silk emergence, days to 25% and 50% silk emergence. Among all the inbred lines, South African line VLO6688 silked earliest (51st day after planting). Lines of South African origin that silked latest included R119W and R2565Y (59th day after planting). Among the Kenyan maize lines, CKLO5022 silked earliest (61st day) followed by CML 264, CML 247 and CML 442 which silked on the 63rd day after planting. Important distinctions occurred in time of silking among Kenyan and South African inbred lines and in all the silking stages, South African lines silked before Kenyan lines. Additionally, in all the stages, VLO6688 took the least number of days to silk.

In our breeding program, outcrossing unrelated inbred lines was highly preferred for this is known to increase hybrid vigour since the crossed genes are unrelated. Therefore, outcrossing Kenyan lines with South African lines was considered best. Line CKL05022 was the Kenyan inbred line that silked earliest on the 61st day after planting. However, CKL05022 silked after all the South African inbred lines had silked. Therefore, in order to maximize outcrossing if the Kenyan inbred line was the female plant, then CKL05022 would have to be planted earlier depending on which male South African line was targeted for crossing and the number of days to its pollen shed.

Pollen shedding data was thus very useful in planting the lines targeted for crossing because in maize, pollen shedding occurs before silking. In our study, pollen shedding in the inbred lines commenced at 51st day and stretched to the 68th day. Line I137tmw was the first to shed pollen (51st day) followed by US2540W (53rd). However, La Posta and CML 247 were the last (68th and 67th respectively) in pollen shed. Since silking stages of the inbred lines had substantial variations in silking at first silking ($p < 2.2e\text{-}16$, F (42.669)) and lowest at 25% silking ($p < 2.2e\text{-}16$, F (29.629)), proper synchronization through delaying the sowing dates of males was important for successful hybrid development.

Time of pollen shedding in the inbred lines of South African origin differed with those of the Kenyan origin. The South African inbred lines shed their first, 25% and 50% pollen earlier compared to the Kenyan inbred lines except CML 390 and R119W which shed pollen late compared to any other South African line. This meant that in outcrossings where CML 390 and R119W were the male plants, then the two lines had to be planted more than once in the crossing block depending on the silking dates of the targeted Kenyan female lines. Moreover, since South African lines shed their pollen earlier than the Kenyan lines, then South African male inbred lines had to be planted late depending on the silking dates of the targeted Kenyan female line. However, those lines that took same number of days to 25%, 50% and 100% pollen shed would be planted close to each other depending on the female plant in the breeding program because the lines shed pollen almost at the same time. The same pattern would also apply in those inbred lines that silked within the same time. This is important in order to maximize on the quality of pollen tapped, reduce the distance travelled by the breeder during the process of pollination and maximize the number of pollinations carried out per day.

According to Westphal, (2013), for successful development of hybrids, a vigilant examination procedure of the inbred lines throughout their growth and development cycle enables the researchers to trail each parent lines strength and weaknesses. Therefore, it is necessary for the researcher to track dates of the inbred lines when the lines reached first silking, first pollen shed, 50% silking and 50% pollen shed, disease presence and susceptibility among other factors. The production research data is thus essential to the breeder for the breeder is guided on how the lines perform in different environments.

Production of the hybrids in this study was majorly targeted at crossing South African lines and the Kenyan inbred lines, the production research data was therefore of great importance in determining performance of the lines. For example, if crossing was targeted between CKLO5022 (male parent) and CB 222 (female parent) and line CKLO5022 was 5 to 4 days later in pollen shed, CKLO5022 would be planted 5 to 4 days earlier than CB 222 in order to obtain good nick of the lines for pollen and silk. In this case, the production data would help the breeder understand importance of a

comprehensive system of the production research as opposed to the research that merely centres on capacity to make possible crosses. Since the production research examines all the factors including quality, purity and germination, best parent lines for development of crosses suitable to respond competently in the given environment are selected. Financial losses are thus minimized, and high yields achieved. However, great financial losses can be incurred by the breeder if the lines used in the production process are not considered. The breeder will have no knowledge of whether the inbred lines are adapted to the area where the lines are to be grown and the hybrids produced. For example, when a breeder intends to produce a hybrid in an area where the male plant is not adapted, there will be no pollen hence no seed, resulting in great financial loss.

Successful breeding program therefore depends on the timing of outcrossing between two targeted inbred lines determined by the silking and pollen shed days. Ngugi *et al.* (2013) associates poor kernel set with poor timing of pollen shed with emergence of silk which results into low yield. Therefore, when maize flowers under drought, there is delay in silking and the period between male and female flowering increases giving rise to anthesis silking interval (ASI). Studies by Banziger et al. (1999) related high grain yield under a range of stress intensities with a short ASI and earlier flowering dates, increased plant and ear heights, increased number of ears per plant and delayed leaf senescence.

Bernahu et al. (2017) supports that wider ASI reduces high yield on maize because ASI has a high negative correlation with grain yield under stress conditions since drought stress before or at flowering delays silk elongation though has no or little effect on pollen shed. Thus, indirect selection to minimize ASI has been an effective approach for synchronisation of male and female flowering under stress. Kiboko and Katumani are hot areas known to experience drought stress hence selection of parent lines with short ASI is important for effective synchronisation of male and female flowering. Apart from selecting for lines with low mycotoxin accumulation levels, breeders would select for inbred lines with uniformity in pollination and early silking lines which are important in gene mapping for early maturity. Early silkers with uniformity in pollination under a range of stress intensities would ensure short ASI during breeding leading to high yield. According to Okoth, (2016) most local varieties are resistant to

aflatoxin and fumonisin accumulation but produce low yields and this does not offer solution to food security in Kenya.

Successful hybrid seed production was achieved through synchronization between anthesis and silking of parental inbred lines. This is because in maize plant pollen shedding occurs earlier than silking. Therefore, in the cross-breeding programme, synchronization was achieved by delaying the sowing dates of male lines depending on the recorded silking and pollen shed data of the trials.

5.4 Blister development in the inbred lines

Blistering in the inbred lines started at the 65th day up to the 109th day and line VLO6688 blistered earliest (65th day). Variations in time taken to blister development by different lines at different stages with some lines blistering within the same period were important for farm management practices aiming at maximizing yield. This was because control of pests and diseases was highly maximized at this stage in preparation for harvesting. For example, insect infestation of the field was controlled by spraying using appropriate insecticides, invasion of the field by wild pigs through employing watchmen to guard the field at night among others. Additionally, blistering data at different stages was of great importance because harvesting time could be estimated, and proper preparations made. Collected data on blistering may also be of great usefulness to any breeder who would in future prefer to use the inbred lines.

5.5 Ear rots, aflatoxin and fumonisin levels in maize inbred lines

Among the 23 lines in this study, no inbred lines appeared to resist fungal infection by both *A. flavus* and *F. verticilloides.* However, lines VL06688 and I137tnw were susceptible to both fungi. It is important that there are no lines that appear to be resistant to both fungi though line VL06688 had high kernel infection by both *A. flavus* and *F. verticilloides*. Lanubile et al. (2017) supports that resistance or susceptibility of the lines to ear rots could be due to genetic composition of the lines. For example, FER resistance has proved to be a quantitative trait controlled by polygenes. Therefore, lines VL06688 and I137tnw could be used to determine whether the same genes confer susceptibility to both pathogens. Information obtained from mapping of possible susceptibility genes

in lines VL06688 and I137tnw could be useful in breeding for resistance to ear rot by *A. flavus* and *F. verticilloides.* This can be likely through excluding any inbred line with the same genes for susceptibility to both fungi in the two susceptible lines from the breeding program. This is because the most efficient way to improve resistance in hybrids is to assess and choose among inbred lines before using resources to produce hybrids.

In the Katumani experiment, there was significant positive correlation between *Fusarium* ear rot and fumonisin levels in the inbred lines ($r = 0.334$, $p < 0.05$) which supports the findings of Balconi et al. (2014). However, the positive correlation coefficient between FER and total fumonisin level in the inbred lines was low. The low correlation is supported by several evaluated maize inbred lines that accumulated highest fumonisin levels not being the highest in FER. Positive correlation between the two variables suggests that factors affecting kernels infection might operate dependently to those affecting fumonisin productions. Line I137tnw was the fourth highest in the level of accumulated total fumonisin being the highest in FER. Similarly, R119W was the third highest in accumulated fumonisin level and was the second highest in FER. However, CML 442 was the highest inbred line in total fumonisin level and was the most resistant line among the other inbred lines in disease severity. The varieties P502 and R2565Y were among the susceptible lines to fumonisin accumulation and the lines were neither susceptible nor resistant to FER. The low correlation ($r = 0.334$) suggests that fumonisin levels accumulated by each line cannot be directly determined by disease severity alone, instead a chemical test must be performed to quantify the toxin level in each line.

Though there were some lines that appeared to be resistant/susceptible to both *A. flavus* and *F. verticilloides* infection, no significant correlation occurred between AER and FER among the lines ($r = -0.130$, $p > 0.05$). This suggests that different factors control AER and FER resistance. Therefore, those factors that affect kernel infection in *Aspergillus* spores infected lines are independent from those affecting kernel infections in *Fusarium* spores infected lines creating no relationship between the two variables.

The lines P502, I137tnw, R119W, R2565Y, US2540W, R0549W, V0617Y-2, VL06688, MIRTC5 and CKL05003 showed high concentrations of aflatoxin and CML

495, CB 222, CML 390, CML 444, CB 248, CML 247 and La Posta had low concentrations of aflatoxin. However, lines R2565Y, V0617Y-2, CML 442 and R119W accumulated high levels of fumonisin while CML 390, CML 247 and CKL05003 had low fumonisin levels. The results suggest that R2565Y, V0617Y-2 and R119W could be susceptible to both aflatoxin and fumonisin contamination whereas P502, I137tnw, R119W, US2540W, R0549W, VL06688, MIRTC5 and CKL05003 were vulnerable only to aflatoxin. Lines CML 495, CB 222, CML 444, CB 248 and La Posta were invulnerable only to aflatoxin while inbred line CKL05003 was only resistant to fumonisin and CML 445 susceptible only to fumonisin. Among the lines, CML 495 (1.091µg/kg) and CB 222 (1.1µg/kg) accumulated least aflatoxin levels compared to any other evaluated inbred line whereas CML 247 and CML 390 accumulated least fumonisin level (0.115mg/kg and 0.076mg/kg respectively). Remarkably, CML 390 and CML 247 appeared to be resistant to accumulation of both aflatoxin and fumonisin.

Lines CB 222, CML 495, CML 390, CML 444, CML 247 accumulated least aflatoxin levels below 10 µg/kg. In a different study (Okoth *et al*., 2017), CB 222, CML 444, CML 495, CML 247 and CML 390 were reported to be resistant to *A. flavus* infection and subsequent accumulation of aflatoxin. A dissimilar study reported resistance to aflatoxin accumulation in CML 247 (Williams *et al*., 2017). Further still, low aflatoxin accumulation and low AER has been documented in CML 247 (Okoth *et al*., 2017). In this study, germplasms CML 390, CML 247 and CKL05003 accumulated fumonisin levels below 2mg/kg. It is also encouraging to note that CML 390 and CML 247 accumulated lowest fumonisin and aflatoxin levels. In a study conducted to determine resistance to infection by *F. verticillioides* and subsequent accumulation of fumonisins, line CML 390 was found to be resistant to *Fusarium* ear rot (FER) and fumonisin accumulation (Rose *et al*., 2016). Therefore, CML 390 and CML 247 germplasms may be useful sources of resistance in maize breeding programs targeted at lowering both aflatoxin and fumonisin contamination. As expected, some maize inbred lines showed resistance only to aflatoxin instead of fumonisin contamination, and vice versa (Table 3.1). Lines CML 264, CML 495, and La Posta accumulated aflatoxin levels below 10µg/kg and fumonisin above 2mg/kg. Likewise, to CKL05003, which accumulated fumonisin levels below 2 mg/kg and aflatoxin < 10µg/kg. However, these lines may have different levels of resistance when evaluated in different environments. They

could be suitable for mapping populations directed at identifying quantitative trait loci (QTL) involved in resistance to infection by *A. flavus* or *F. verticilloides* and contamination by aflatoxins, and fumonisins respectively. Though a positive correlation ($r = 0.431$, $P > 0.05$) occurred between aflatoxin and fumonisin levels in the inbred lines, the correlation coefficient was low. Selection of inbred lines with low aflatoxin levels would not probably select for lines with low fumonisin levels in most cases. For example, CML 495 accumulated very low aflatoxin levels (1.09µg/kg) but recorded fumonisins above 2mg/kg (3.49mg/kg). A previous study found a positive correlation between aflatoxin and fumonisin levels in the inbred maize lines (Ngugi *et al*., 2013) and further assessment of a collection of germplasms for resistance is recommended. The QTL analysis would therefore determine if there are common loci for the aflatoxins, and fumonisins resistant phenotypes.

As expected, some lines showed resistance only to aflatoxin rather than to fumonisin contamination and vice versa. These lines may manifest divergent levels of resistance when investigated in other environments or may be important for breeding for resistance to aflatoxin or fumonisin. The lines may also be important in mapping populations based on distinguishing quantitative trait locus (QTL) involved in resistance to aflatoxin and fumonisin. Of interest among the lines investigated in the study were CML 390 and CML 247 which accumulated total aflatoxin levels below 20µg/kg and fumonisin levels less than 2mg/kg. These two lines with stability in resistance to aflatoxin and fumonisin may be useful sources of resistance in maize breeding programs targeted at lowering both aflatoxin and fumonisin contamination.

Abbas et al. (2009) supports that susceptibility of different inbred lines to aflatoxin and fumonisin accumulation is related to ear rots following *A. flavus* and *F. verticilloides* infections and the ear rots associated with insect damage. Genetic make-up of a seed can also make a plant susceptible or resistant to aflatoxin and fumonisin accumulations. This is because the genes responsible for controlling the plants reactions during stressful conditions may also be responsible for the correlation between aflatoxin and fumonisin levels in corn.

Association between aflatoxin and fumonisin levels in the evaluated inbred lines showed significant positive correlation between accumulated aflatoxin and fumonisin

levels in the inbred lines ($r = 0.431$, $p > 0.05$). Though there was significant positive correlation between accumulated aflatoxin and fumonisin levels in the inbred lines, the correlation coefficient was low. It could be difficult to produce high r values due to the interaction of several biological factors of the plant and fungus together with the environment. For example, the rainfall patterns in Kiboko and Katumani during the 2013 and 2014 planting seasons (Figure 4-11 and 4-12) were different during the months of January, February, March, April and May. This directs that the interaction between environmental and biological factors of the maize plant and fungi could have influenced accumulated aflatoxin and fumonisin levels in the maize lines.

Nevertheless, the positive correlation between aflatoxin and fumonisin accumulation levels in the inbred lines show direct relationship between accumulated aflatoxin and fumonisin levels in the inbred lines. Since the r value is low ($r = 0.431$), selection of the lines with low aflatoxin levels would not probably select for lines with low fumonisin levels in most cases. Further studies on quantitative trait locus (QTL) are therefore recommended. This would help breeders select for inbred lines with genes that are resistant to aflatoxin and fumonisin contamination since genetic resistance is the best defense against mycotoxin contamination and at present there are no commercial hybrids that are completely resistant to mycotoxin contamination. Quantitative trait loci analysis would also determine if there are common QTL regions for the two mycotoxin resistant phenotypes, as supported by Warburton, (2009) that resistance to grain aflatoxin accumulation is a polygenic trait with multiple QTL of different importance within a given line.

Guo et al. (2017) findings also supported positive correlation between aflatoxin and fumonisin levels accumulated in the inbred lines. In his study, he suggested further studies to find characters that add to resistance to aflatoxin and fumonisin for actual improvement of breeding programs. Guo et al. (2017), further suggests that it is important to assess the collection of germplasm sources for resistance in order to improve the growth of commercial hybrids with resistance to aflatoxin and fumonisin.

No significant relationship occurred between AER and aflatoxin levels in Katumani and Kiboko ($r = 0.127$). In Katumani ($r = 0.044$) and Kiboko ($r = 0.118$) research site, significant correlations between aflatoxin accumulation levels and AER severity in the

inbred lines did not occur. However, the variation was least in Katumani (r = 0.044) and highest in Katumani + Kiboko (r = 0.127). Absence of positive correlation between aflatoxin concentration and AER suggests that factors affecting kernels infection might operate independently from those affecting aflatoxin productions. This in turn might affect the relationship between accumulated aflatoxin levels and ear rot severity in the inbred lines. Additionally, several evaluated inbred lines with very low disease severity accumulated high aflatoxin levels and lines with high disease severity accumulated low aflatoxin levels. These included CB 248, CML 247, VLO6688, CKLO5003, CKLO5022 and VLO617Y. This further contributed to the lack of correlation between total aflatoxin levels and ear rot severity in the inbred lines.

It would be expected that those lines with low aflatoxin accumulation levels produce low AER severity and susceptible aflatoxin lines produce high AER severity. Some of the inbred lines did not follow the trend. However, CML 390 and CKLO5003 were some of the lines that followed this phenomenon though CML 390 (1.367%) had low AER severity compared to other lines that had accumulated lower aflatoxin levels like CB 222 (6.28%) and CML 495 (3.1%). Among the South African lines, CB 248 was the inbred line that accumulated low aflatoxin level after CML 390 and was among the lines susceptible to AER. Line VL06688 recorded the highest (66%) AER severity though the line was not the most susceptible line to total aflatoxin accumulation. Therefore, the data demonstrates that the grains must always be tested chemically to determine the level of accumulated aflatoxin by the grains and the amount of aflatoxin should not be determined by the level of ear rot severity. The data also suggests that different genes/factors control toxin resistance and fungal rot resistance. The results are like those of Brien et al. (2009). Further studies on quantitative trait locus analysis can be carried out on these inbred lines to determine the location of ear rot and toxin resistance genes.

5.6 Aflatoxin and fumonisin in maize hybrids

5.6.1 Resistance of the hybrids to aflatoxin

Season I *Aspergillus* block treated crosses varied in their aflatoxin levels (*F* (21, 43 = 25.183, p = < 2.2e-16)) with aflatoxin levels ranging between 15.88 - 163.36µg/kg. Among the crosses, only three crosses accumulated aflatoxin levels below the

regulatory action level of 20µg/kg. These included CKLO5022 (F) x CB 248 (M) (15.877µg/kg), CB 248 (F) x CKLO5022 (M) (17.545µg/kg) and CML 247 (F) x CB 222 (M) (18.503µg/kg). The two crosses with the least aflatoxin levels were reciprocal crosses. This indicates that the genes present in CKLO5022 and CB 248 might be important in breeding for resistance to aflatoxin. Line CKLO5022 is of Kenyan origin while CB 248 is an inbred line developed in South Africa. The two lines are thus genetically unrelated possibly combining characteristics for low aflatoxin accumulation levels. Further studies involving QTL analysis are therefore necessary in order to locate the genes responsible for low aflatoxin levels in CKLO5022 (F) x CB 248 (M) and CB 248 (F) x CKLO5022 (M). The inbred lines CKLO5022 (4.5µg/kg), CB 248 (2.475µg/kg), CML 247 (2.8µg/kg) and CB 222 (1.1µg/kg) though were not the lines with least aflatoxin levels among the trials, their parental combinations produced hybrids with aflatoxin levels below 20µg/kg. This indicates that during selection for parental lines for breeding for resistance to aflatoxin, one must not only choose for inbred lines with least aflatoxin levels but should explore a wide range of parental combinations.

In season II and III crosses, significant differences in the accumulated aflatoxin levels occurred with all the hybrids accumulating aflatoxin levels above 20µg/kg. The amount of total aflatoxin in the hybrids ranged between 22.455 - 95.295µg/kg. La Posta (F) x R119W (M) accumulated the highest levels of aflatoxin in season II and III though aflatoxin levels in the same cross in season III (99.263µg/kg) was higher than in season II (95.295µg/kg). In season II crosses CKLO5022 (F) x CML 444 (M) recorded the lowest aflatoxin level (22.455µg/kg) while CML 444 (F) x CKLO5022 (M) had the lowest aflatoxin level in season III (22.18µg/kg).

A strong relationship occurred between season II and III crosses ($r = 0.526$, $p < 0.05$). The strong correlation co-efficient ($r = 0.5262$, $p < 0.05$) in accumulated aflatoxin levels within the crosses in the two phases is supported by the fact that La Posta (F) x R119 W (M) accumulated the highest aflatoxin level in season II (95.295µg/kg) and season III (99.263µg/kg). Between the two phases, it would be expected that those crosses that accumulated low aflatoxin levels in season II maintained the low levels in season III and those with high aflatoxin levels in season II accumulated high aflatoxin levels in season III. The norm was observed in only 42% of the hybrids and did not occur in the

other crosses. For example, Local hybrid I was the second highest (92.63µg/kg) in season II and third highest in season III (83.068µg/kg). Hybrid R119W (F) x CML 390 (M) was the second highest in accumulated aflatoxin levels in season III (91.703 µg/kg) and was third highest in season II (79.14µg/kg). Additionally, hybrid R119W (F) x CB 222 (M) was the fifth highest (65.125µg/kg) in season II and seventh highest (60.23µg/kg) in season III. However, not all the crosses showed close variations in season II and season II.

Some crosses accumulated high aflatoxin levels in season II and low levels in season III or vice versa weakening the correlation co-efficient ($r = 0.526$, $p < 0.05$). These included Local hybrid 2 which accumulated 60.108µg/kg in season II and 40.077µg/kg in season III, La Posta (F) x CKLO5022 (M) had 32.355µg/kg in season II and 69.044µg/kg in season III, CML 444 (F) x CB 222 (M) had 29.23µg/kg in season II and 64.373µg/kg in season III. Of the crosses, 30% differed in aflatoxin levels concentrations between season II and season III with a range of 5-10µg/kg. These included R119W (F) x CB 248 (M) which accumulated 45.858µg/kg in season II and 45.119µg/kg in season III , CB 222 (F) x La Posta (M) accumulated 29.767µg/kg in season II and 26.018µg/kg in season III, CML 444 (F) x CB 248 (M) 35.482µg/kg in season II and 33.985µg/kg in season III , La Posta (F) x CML 444 (M) 38.555µg/kg in season II and 34.397µg/kg in season III, CML 444 (F) x CML 390 (M) 27.53 µg/kg in season II and 22.637µg/kg in season III, La Posta (F) x CML 444 (M) had 38.555µg/kg in season II and 34.397µg/kg in season III and R119W (F) x CB 222 (M) with 65.233µg/kg in season II and 60.233µg/kg in season III. However, 42.5% of the crosses varied within the range of above 10µg/kg and above weakening the correlation co-efficient ($r = 0.526$, $p < 0.05$). All the hybrids in season II and season III crosses accumulated aflatoxin levels above the maximum recommended levels by the FDA of 20µg/kg and are thus not suitable for commercial hybrids' development.

5.6.2 Resistance of the hybrids to fumonisin

Hybrids CML 444 (F) x CKLO5015 (M) (0.50227mg/kg) accumulated the least fumonisin level followed by CML 444 (F) x CML 495 (M), CML 495 (F) x CML 444 (M) and P502 (F) x CML 444 (M) with fumonisin levels ranging between 0.8211 to 0.904mg/kg). Interestingly, hybrids CML 444 (F) x CML 495 (M) and CML 495 (F) x

CML 444 (M) are reciprocal crosses and accumulated low fumonisins. Inbred line CML 444 is a parent line in all the crosses that accumulated low fumonisin levels either as a male or female plant though CML 444 was a fumonisin susceptible line with fumonisin levels of 5.715mg/kg. Additionally, the hybrids were developed from maize lines of Kenyan and South African origin. This indicates that heterosis in maize breeding program is best achieved by crossing completely unrelated lines. Genotype by environment interactions was likely to be very important in determining fumonisin levels in the season I *Fusarium* crosses. These crosses with low fumonisin level in season I need to be further analyzed in order to determine if resistance to fumonisin accumulation is determined by polygenes as proposed by Guo *et al*., 2017.

Two reciprocal crosses MIRTC5 (F) x R119W (M) (2.793mg/kg) and R119W (F) x MIRTC5 (M) accumulated fumonisin above the recommended levels by the FDA, that is, 2mg/kg in season I. The two lines, MIRTC5 and R119W did not appear in the crosses that had low fumonisin levels demonstrating that the genes in these two reciprocal crosses are responsible for susceptibility to fumonisin accumulation. Consequently, the susceptibility genes need to be mapped and any maize lines containing the genes excluded in the maize breeding program for the development of *Fusarium* resistant hybrids. Alternatively, a population derived from crossing either MIRTC5 or R119W with maize inbred lines that showed resistance to fumonisin accumulation like CML 390 (1.08mg/kg), CML 247 (1.43mg/kg) and CKLO5003 (1.78mg/kg) can be developed in order to determine whether the same genes confer resistance mechanisms to fumonisin as proposed by Brien *et al*., 2009.

In season II crosses, hybrid MIRTC5 (F) x CML 444 (M) accumulated the highest amount of fumonisin (2.714mg/kg) compared to any other cross and 80% of the crosses accumulated fumonisin levels below 2 mg/kg. However, among the season III crosses, 73.33% of the crosses accumulated fumonisin levels below 2mg/kg with fumonisin levels ranging between 0.037-1.865mg/kg. Hybrid, CKLO5015 (F) x CML 444 (M) accumulated the least amount of fumonisin (0.03735mg/kg). Other crosses that recorded very low fumonisin levels included CML 444 (F) x P502 (M) (0.3894mg/kg), CKLO5015 (F) x R119W (M) (0.51436mg/kg), CML 444 (F) x R119W (M) (0.62668mg/kg). However, CML 495 (F) x R119W (M) accumulated (3.0453mg/kg),

MIRTC5 (F) x CML 444 (M) and MIRTC5 (F) x CKLO5015 (M) accumulated the highest total fumonisin levels (3.267mg/kg and 3.259mg/kg respectively). Most of the crosses in which line CML 444 was either a male or female plant, the hybrid accumulated low fumonisin levels except in a cross where CML 444 was crossed with MIRTC5 or MIRTC5 crossed with CKLO5015.

The trend of a cross made of CML 444 either as a male or female plant accumulating low fumonisin levels has been maintained all the way from season I to season III crosses. The same trend has been observed with CKLO5015. Further studies on genes present on CML 444 and CKLO5015 is recommended such that if CML 444 and CKLO5015 have a mechanism that limits or controls the fumonisin production pathway, this character should be explicated and introduced into other lines as suggested by Brien et al. (2007). Meanwhile, the genes in line MIRTC5 need to be mapped so that the genes are omitted and any other lines containing the same genes excluded during maize breeding program. However, crosses that accumulated fumonisin levels below 2mg/kg are important for selection for further studies involving quantitative trait locus analysis since the crosses are potential commercial hybrids.

Season II and III showed a very strong positive correlation ($r = 0.908$, $p < 0.05$) between fumonisin levels. This indicated that most of the crosses showed close variations in the amounts of accumulated fumonisins in the two seasons. Therefore, the crosses that accumulated high fumonisin levels in season II probably had high levels in season III. The same trend followed by the crosses that accumulated low fumonisin levels in season II accumulating low fumonisins in season III and vice versa.

Generally, genes that command plant stress response may subscribe to the relationship between aflatoxin and fumonisin levels in the inbred lines. Apart from maize genotypes, the greatest powerful threat influences with respect to FER and fumonisin buildup are temperature, drought stress and insect damage among others. Genotype by environment relations is probably very significant in causing mycotoxin contamination. Though many plant breeders depend on natural infection to generate satisfactory level of disease severity for the choice of disease resistant genotypes, artificial infection has been established to be appropriate approach for analyzing host genome types towards resistance to mycotoxin production (Guo *et al*., 2017). In this study, artificial infection

was desired and the same *A. flavus* and *F. verticilloides* inoculum strains were used to infect inbred lines and crosses avoiding a possible difference in complex fumonisin and aflatoxin producer fungus community.

CONCLUSION AND RECOMMENDATIONS

This study underscores the importance of investigating the mating-type distribution in the field, prior to biocontrol selection and release, to minimize the potential for sexual recombination while promoting efficacy of the biocontrol strain. Our findings indicate that *A. flavus* strains in Kenya have the potential to harbour both MAT genes, although we are uncertain of their functionality. Also, we show that not all fields will have the same distribution of mating types. The Makueni field population may have higher genetic and chemotype diversity, and potential for sexual recombination, due to the observed distribution of mating types. Selection of a naturally infertile atoxigenic strain as biocontrol would be better here. For Nandi, the distribution is less diverse, and if the *MAT1-1+MAT1-2* isolates are incapable of self-fertilizing or outcrossing, then use of *MAT1-2* biocontrol strain would be effective. Another characteristic that would be helpful would be to ensure the biocontrol strain lacks aflatoxin cluster genes since it might be more difficult to inherit the entire cluster during recombination.

Early germination and short ASI are important in mapping for early maturity. The inbred lines had a short ASI of between -2 to 2 days except La Posta (3 days) which is an indication of presence of a drought tolerant trait. Apart for selecting for early maturity, most breeders would prefer lines that accumulate aflatoxins and fumonisins below the positive controls. Lines CML 390 and CML 247 would be preferable because despite having short ASI, and good germination rates, they accumulated least aflatoxin and fumonisin levels compared to the other inbred lines. The two lines could be useful sources for breeding for resistance to aflatoxins and fumonisins. Aflatoxin (CB222, CML 495 and CML444) and fumonisin (CKL05003) resistant lines could be investigated further for inclusion into maize breeding program for resistance to aflatoxins, and fumonisins respectively. In the study, not all inbred lines that accumulated low aflatoxin levels recorded low fumonisins and vice versa. Lines CML 495, La Posta, CML 444, CB 248 and CB 222 had aflatoxin levels below 10µg/kg and fumonisins above 2mg/kg while CKL05003 showed resistance to fumonisin but was susceptible to aflatoxin accumulation. Further investigation on the genes controlling resistance to aflatoxins and fumonisins accumulation in maize is recommended.

All the 44 *Aspergillus flavus* isolates including strains 201365, 100130 and 100095 used for spore inoculation of maize lines and crosses had mating type genes indicating the potential for sexual reproduction leading to gene flow. Therefore, a need for gene flow studies on the atoxigenic strains among the isolates cannot be ignored for this is important in developing safe biocontrol strategies for aflatoxin and fumonisin accumulation in maize. Among the maize lines, VL06688 was a fast germinator and an early silker which are good traits suitable for agronomic studies such as shortening anthensis silking interval (ASI) to ensure successful synchronization and high yield. However, VLO6688 was susceptible to aflatoxin, fumonisin and kernel infections by *A. flavus* and *F. verticilloides*. Though, early germination and silking are important in mapping for early maturity, breeders would prefer lines that mature fast and are resistant to mycotoxin accumulation hence VLO6688 is likely to be rejected in the maize breeding programs. CML 390 and CML 247 showed resistance to both aflatoxin and fumonisin accumulation in Katumani and Kiboko and the lines can be used for gene mapping for resistance to aflatoxin and fumonisin. Maize lines that had aflatoxin levels below 20µg/kg included CML 495, CB 222, CML 444, CB 248, and fumonisin levels below 2mg/kg were CML 247 and CKL05003. *Fusarium* spores infected crosses that had resistance included CML 444(F) x CKLO5015 (M), CKLO5015 (F) x CML 444 (M), CML 444 (F) x CML 495 (M), CML 495 (F) x CML 444 (M), P502 (F) x CML 444 (M), CKLO5015 (F) x R119W (M) and CML 444 (F) xR1119W (M). The inbred lines and crosses can be recommended for maize breeding programs for resistance to aflatoxin and fumonisin. Positive correlations occurred between aflatoxin and fumonisin levels in the maize lines and crosses indicating that those inbred lines and crosses with high aflatoxin levels are likely to have high fumonisin levels and vice versa.

REFERENCES

Abbas, H. K., Wilkinson, J. R., Zablotowicz, R. M., Accinelli, C., Abel, C. A., Bruns, H. A., Weaver, M. A. (2009). Ecology of *Aspergillus flavus*, regulation of aflatoxin production and management strategies to reduce aflatoxin contamination of corn. *Toxins review 4: 142-153.*

Abdin, M., Malik, M., Saleem, J. (2010). Advances in molecular detection of *Aspergillus*: an update. *Arch Microbiology* 192:409-425

ACDI/VOCA. 2015a. *Kenya Maize Development Program (KMDP).* ACDI/VOCA, Washington, DC, USA.

Alakonya, A. E., Monda, E. O., Ajanga, S. (2008). Effect of delayed harvesting on maize ear rot in Western Kenya. *American Eurasian Journal of Agriculture and environmental science.* 3: 372-380.

Atehnkeng, J., Ojiambo, P.S., Donner, M., Ikotun, T., Sikora, R.A., Cotty, P.J., Bandyopadhyay, R. 2008. 'Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria'. *International Journal of Food Microbiology* 1: 74–84.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Schurz, R. H., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L. F., Misore, A., DeCock, K., Rubiin, C., Slutsker, L., Aflatoxin Investigation Group. (2005). Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environmental Health Perspect*.113: 1779-1783.

Balconi, C., Bevardo, N., Locatelli, S., Lanzanova, C., Torri, A., Redaelli R. (2014). Evaluation of ear rot (*F. verticilloides*) resistance and fumonisin accumulation in Italian maize inbred line. *Phytopathologia Mediterranean*. 53: 14-26

Bankole, S.A., Adebanjo, A. (2003). 'Mycotoxins in food in West Africa: Current situation and possibilities of controlling it'. African Journal of Biotechnology 2: 254–263.

Bernahu, T. E., Beyeni, Y., Biswanata, D., Mugo, S., Olsen, M., Oikeh, S., Juma, C., Labuschagne, M., Prasanna, B. M. (2017). Combining ability and test cross

performance of drought tolerant maize inbred lines under stress and non-stress environments in Kenya. *Plant breeding* 136: 197-205.

Bii, F., Wanyoike, W., Nyande, A. B., Gituru, R. W., Bii C. (2012). Fumonisin contamination of maize (*Zea mays*) in aflatoxin hot zones in Eastern Province of Kenya. *African Journal of Health Sciences* 20: 28-36

Brien, H.W., Williams, W.P., Windham, G. L., Hawkins, L.K. (2009). Evaluation of maize inbred lines for resistance to Aspergillus and Fusarium ear rot and mycotoxin accumulation. *Agronomy*. 101: 1219-1226

Bhat, R. V, Shetty, P. H., Amruth, R. P., Sudersham, R. V. (1997) A food-borne disease outbreak due to consumption of moldy sorghum and maize containing fumonisin mycotoxins. *Journal of Clinical Toxicology* 35: 249–255

Bushra, M., Jones D. A. (1983). The genetics of maize (*Zea mays L.)* growing at low temperatures. *Euphytica*. 2: 535-542.

Castelino, J.M., Routledge, M.N., Wilson, S., Dunne, D.W., Mwatha, J.K., Gachuhi, K., Wild, C.P., Gong, Y.Y. (2015. ‘Aflatoxin exposure is inversely associated with IGF1 and IGFBP3 levels in vitro and in Kenyan schoolchildren’. *Molecular Nutrition and Food Research* 3: 574–581.

Center for Disease Control and Prevention (CDC). (2004a). ‘Outbreak of aflatoxin poisoning –eastern and central provinces, Kenya, January–July 2004’. *Morbidity and Mortality Weekly Report* 34: 790.

Charles, W. S. (2013). Breaking the mold. *Environmental Health Perspectives*. 9: 270-275.

Cotty, P. J., Misaghi, I. J. (1984). Zinniol production by Alternaria species. *Phytopathology* 74: 785-788

Dhillon, B. S., Prassana, B. M. (2001). Maize. In V. L. Chopra (ed.) Breeding field crops; Theory and practice. Oxford and IBH, New Delhi.

Donald, G. W. (1999). Compendium of corn diseases. 3rd edition. The American Phytopathological Society.

Ehrlich, K.C., Cotty, P. J. (2004). ‘An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene’. *AppliedMicrobiology and Biotechnology* 65: 473–478.

Ehrlich, K.C. (2014). ‘Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: Advantages and limitations. *Frontiers in Microbiology* 5: 50.

Ehrlich, K.C., Moore, G.G., Mellon, J.E., Bhatnagar, D. (2014). ‘Challenges facing the biological control strategy for eliminating aflatoxin contamination’. *World Mycotoxin Journal* 2: 225–233.

Ezekiel, C.N., Warth, B., Ogara, I.M., Bia, W.A., Ezekiel, V.C., Atehnkeng, J., Sulyok, M., Turner, P.C., Tayo, G.O., Krska, R., Bandyopadhyay, R. (2014). ‘Mycotoxin exposure in rural residents in northern Nigeria: A pilot study using multi-urinary biomarkers. *Environment International* 66: 138–145.

Fandohan P., Hell, K., Marasas, W. F., Wingfield, M.J., (2013). Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African journal of biotechnology.* 12: 570-579.

Fandohan, P., Gnonloufin, B., Hell, K., Marasas, W.F., Wingfield, M. J. (2005). Natural occurrence of Fusarium and subsequent fumonisin contamination in pre-harvest and stored maize in Benin, West Africa. 2: 173-183.

Felicia, W., Clarre, N., Marites, T., Yan L. (2011). The health economics of aflatoxin. Global burden of disease. *Aflatoxin control* 4: 1-20

Felipe de Mendiburu (2017). agricolae:Statistical Procedures for Agricultural Research. R package version 1.2-8.http://CRAN. R-project.org/package=agricolae

Food and Drug Administration. (2001). Guidance for Industry: Fumonisin levels in human foods and Animal Feeds; Final Guidance. *Centre for food safety and Applied Nutrition.* 202: 260-0631.

Goggi, A. S., Pollak, L., Golden, J., Devries, M., McAndrews, G., Montgomery, K. (2007). Impact of early seed quality selection on maize inbreds and hybrids. *Maydica*. 52: 223-233.

Gong, Y.Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P.C., Hall, A.J., Wild, C.P. (2002). 'Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study'. *British Medical Journal* 325 (7354): 20–21

Grubisha, L., Cotty, P. (2010). Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus *Aspergillus flavus*. *Molecular Ecology* 19:269-280.

Guo, B., Xiangyun, J., Xinzhi, N., Jake, C., Hong, L., Hammed, K., Robert, D., Brian, T. (2017). Evaluation of maize inbred lines for resistance to pre-harvest aflatoxin and fumonisin accumulation in the field. *Crop Journal.*259-264.

Han, Z., Ku, L., Zhang, Z., Zhang, J., Guo, S., Liu, H., Zhao, R., Ren, Z., Zhang, L., Su, H., Dong, L., Chen, Y. (2014). Quantitative trait loci for seed vigour- related traits identified in maize seeds germinated under artificial aging conditions. *PLos ONE* 9: 3.

Horn, B., Moore, G., Carbone, I. (2009). Sexual reproduction in *Aspergillus flavus*. *Mycologia* 3:423-429—19.

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P., Denning, D. W. (2007). *Aspergillus flavus:*human pathogen, allergen and mycotoxin producer. *Microbiology.* 153: 1677-1692

Hell, K., Fandohan, P., Bandyopadhyay, R., Cardwell, K., Kiewnick, S., Sikora, R., Cotty, P. (2008). 'Pre- and post-harvest management of aflatoxin in maize'. *In* Leslie, J.F., Bandyopadhyay, R. and Visconti, A. (eds) *Mycotoxins:* Detection Methods, Management, Public Health and Agricultural Trade. CABI Publishing, Wallingford, UK.

Hosen, M., Farhana, M., Shalim, M., Ratan, K., Kumar D. (2014). Maintenance and characterization of new exoticinbred line of maize. *Agriculture and veterinary science.* 7: 62-66

Howard, D. H. (2003). Pathogenic Fungi in Humans and Animals (2nd Ed.) Marcel Dekker. ISBN 0-8247-0683-8.

Khodarahmpour, Z. (2012). Evaluation of maize (*Zea mays*) hybrids, seed germination and seedling characters in water stress conditions. *African Journal of Agricultural Research.*7: 6049-6053.

Krishnan, S., Elias, K., Pranatharthi H. (2008). *Aspergillus flavus;* an emerging non-fumigatus *Aspergillus* species of significance. Blackwell verlerg GmbH. doi:10.1111/j.1439-0507.2008.01642.x.

Jolly, P., Jiang, Y., Ellis, W., Awuah, R., Nnedu, O., Phillips, T., Jolly, C. (2006). 'Determinants of aflatoxin levels in Ghanaians: Socio demographic factors, knowledge of 84 aflatoxin and food handling and consumption practices. *International Journal of Hygiene and Environmental Health* 4: 345–358.

Kang, M.S., Nkurunziza, P., Muwanika, R., Qian, G., Tang, L., Song, X., Wang, J.S. (2015). 'Longitudinal evaluation of aflatoxin exposure in two cohorts in south-western Uganda'. *Food Additives & Contaminants Part* 8: 1322–1330.

Kibe, N. (2015). Thesis. Occurrence of mycotoxigenic fungi in maize from food commodity markets in Kenya. Ghent university.

Kenya Business Daily Newspaper, Thursday 28th April 2016 p. 1

Lanubile, V. M., Borelli, V. M., Lorenzo, S., Antonio, F. L., Adriano M. (2017). Molecular basis of resistance to *Fusarium* ear rot in maize. *Plant science.*

Leonard, D. (1998). Traditional field crops. Peace corps volunteers.

Leroy, J.L., Wang, J.S., Jones, K. (2015). 'Serum aflatoxin B 1-lysine adduct level in adult women from Eastern Province in Kenya depends on household socio-economic status: A cross sectional study'. *Social Science & Medicine* 146: 104–110.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A., Misore, A., Decock, K., Rubin, C., The Kenya aflatoxicosis investigation group. (2005). Aflatoxinn contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central *Kenya. Environmental Health Pespectives*. 12:1763-1767

MacRobert, J.F., Setimela, P.S., Gethi, J., Worku, M. (2014). Maize. Hybrid Seed Production Manual. Mexico, D.F.: CIMMYT. ISBN: 978-607-8263-264

Mandal, B. C. (2014). Maize Breeding and Seed Production Manual. Food and Agriculture Organization of the United Nations. Office of the Food and Agriculture Organization in DPR Korea.

Mamo, F. T., Jonathan, N. S., Wang, Y., Yang, L. (2017). Possibility of recombination among isolates of *A. flavus* in the aflatoxin gene cluster. *Journal of applied and environmental microbiology.*

Mwakhisi, J., Patel, K., Buziba, W., Ritich J. (2005). Oesophageal cancer in North Rift Valley of Western Kenya. *African Health Sciences.* 2: 157-163.

Mwalwayo, D., Thole, B. (2016). Prevalence of aflatoxin and fumonisins (B_1 +B_2) in maize consumed in rural Malawi. 3: 173-179.

Mwihia, T., Straetmans, M., Ibrahims, A., Njau, J., Muhenje, O., Guranja, A., Gikundi, S., Mutonga, D., Tetteh, C., Likimani, S., Breiman, R., Njenga, K., Lewis L. (2008). Aflatoxins level in locally grown maize from Makueni district, Kenya. *East African Medical Journal* 7: 311-317

Natamba, B.K., Wang, J.S., Young, S.L., Ghosh, S., Griffiths, J.K. (2016). 'HIV-infected pregnant and lactating women have higher serum aflatoxin levels than HIV-uninfected women and aflatoxin levels are higher during early postpartum than during pregnancy among HIV-infected women'. *The FASEB Journal* 30(1): 668–5.

National Farmers Information Service. (2012). Ministry of Agriculture-Kenya

Ngindu, A., Kenya, P.R., Ochieng, D.M., Omondi, T.M., Ngare, W., Gatei D. (1982). Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. *Lancet* 1, 1346–1348.

Ngugi, K., Jerono, C., Muchira, C., Chemining'wa, G. (2013). Anthesis to silking interval usefulness in developing drought tolerant maize. *Journal of renewable agriculture*.1: 84-90.

Nyongesa, B.W., Okoth, S., Vincent, A. (2015). Identification key for *Aspergillus* species isolated from maize and soil of Nandi county, Kenya. *Advances in Microbiology*, 5: 205-229

Obura, A. (2013). Aflatoxins: Finding solutions for improved food safety. Aflatoxicosis: Evidence from Kenya. Agriculture for Nutrition and health.

Okoth, S., Lindy, J.R., Ouko, A., Beukes, I., Sila, H., Mouton, M., Flett, B.C., Makumbi, D., Viljoen A. (2017). Field evaluation of resistance to aflatoxin accumulation in maize inbred lines in Kenya and South Africa. *Journal of Crop Improvement,* 31: 862–878

Okoth, S. (2016). Improving the evidence base on aflatoxin contamination and exposure in Africa. Strengthening the Agriculture-Nutrition Nexus. Review. CTA Working paper 16/13.

Okoth, S.A., Nyongesa, B., Joutsjoki, V., Korhonen, H., Ayugi, V., Kang'ethe, E.K. (2016). Sclerotia Formation and Toxin Production in Large Sclerotial *Aspergillus flavus* Isolates from Kenya. *Advances in Microbiology*, **6**, 47-56. http://dx.doi.org/10.4236/aim.2016.61005

Okoth, S., Nyongesa, B., Ayugi, V., Kangethe, E., Korhonen, H., Vesa, J. (2012). Toxigenic potential of *Aspergillus* species occurring in maize kernels from two agro- ecological zones. *Toxins*. 4: 491-1007.

Okoth, S., Kola M. (2012). Market samples as a source of chronic aflatoxin exposure in Kenya. *African Journal of Health Science.* 20:56-61.

Onyemelukwe, G.C., Ogoina, D., Ibiam, G. E., Ogbadu, G.H. (2012). 'Aflatoxins in body fluids and food of Nigerian children with protein-energy malnutrition'. *African Journal of Food, Agriculture, Nutrition and Development* 12: 6553–6566.

Pascale, M., Visconti, A. (2008). 'Overview of detection methods for mycotoxins. *In* Leslie, J.F., Visconti, A. (eds.) *Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade*. Cromwell Press, Trowbridge, Wiltshire, UK.

Paulina, G. (2015). Determination of *Gibberella* ear rot resistance in new maize hybrids. Thesis-Institute for Biotechnology in Plant production, IFA-Tulln

Pierce, P. (2016). Identifying and quantifying ear rots. *Crop protection*. Ohio State University. www.striptillfarmer.com

Probst C., Schulthess, F., Cotty, P.J. (2010). Impact of *Aspergillus* Section Flavi community structure on the development of lethal levels of aflatoxins in Kenyan maize (*Zea mays*). *Applied Mycology* 2: 600-610

Rose, L., Mouton, M., Beukes, I. (2016). Multi-environment evaluation of maize inbred lines for resistance to Fusarium ear rot and fumonisin. *Plant Disease,* 100:10

R Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rodrígues, P., Soares, C., Kozakiewicz, Z., Paterson, R., Lima, N., Venancio, A. (2007). Identification and characterization of *Aspergillus flavus* and aflatoxins. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology, ed. Mendez-Vilas*. 34: 527-534.

Ramirez-Prado, J., Moore, G., Horn, B., Carbone, I. (2008). Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genetics and Biology* 49:1292-1299--32.

Schmidt, C. W. (2013). Breaking the mold: New strategies of fighting aflatoxins. *Environmental health perspectives*. 121 (9).

Suleiman, R. A., Rosentrater, K. A. (2015). Current maize production, post-harvest losses and the risk of mycotoxins contamination in Tanzania. Agriculture. 1-128.

Shimizu, K., Julie, K. H., Tzu-Pi, H., Nancy, P. K. (2003). The significance of *Aspergillu*s and *Penicillium* in mammalian disease is heightened by their production of potent mycotoxins. *Genetics society of America*.165: 1095-1104

Wada, R., Maruyama, J., Yamaguchi, H., Yamamoto, N., Wagu, Y., Paoletti, M., Archier, D., Dyer P., Kitamoto, K. (2012). Presence and functionality of mating type genes in the supposedly asexual filamentous fungus *Aspergillus oryzae*. *Applied and environmental microbiology.*

Warburton, M.L., Brooks, T.D., Krakowsk,y M.D., Shem, X., Windham, G.L., Williams W.P. (2009). Identification and mapping of new sources of resistance to aflatoxin accumulation in maize. *Crop Science*. 49: 1403-1408.

Westgate, M. E., Lizaso, J., Batchelor W. (2003). Quantitative relationships between pollen density and grain yield in maize crop. *Crop Science* 43:934-942.

Westphal, D. (2013). Lessons from Africa. The importance of production research. Partners for advancing African research (R). https//contextnet.com/lessons-from-africa-the-importance-of-production-research.

Williams, W.P. (2006). Breeding for resistance to aflatoxin accumulation in maize. *Mycotoxins*. 22: 27-32.

APPENDICES

Appendix 1: Aflatoxin levels (µg/kg) in season II maize hybrids in Katumani Research station

Season II maize hybrids	Aflatoxin concentration (µg/kg)
LAPOSTA(F) x R119W(M)	1.97[a]
Local maize hybrid- I	1.96[a]
R119W(F) x CML 390(M)	1.89[ab]
R119W(F) x LAPOSTA(M)	1.82[abc]
R119W(F) x CB 222(M)	1.80[abcd]
CB 248(F) x R119W(M)	1.77[abcde]
Local maize hybrid-2	1.76[abcdef]
CML 390(F) x LAPOSTA(M)	1.68[abcdefg]
R119W(F) x CB 248(M)	1.65[abcdefg]
CB 222 (F) x R119W(M)	1.64[abcdefg]
LAPOSTA(F) x CB 248(M)	1.61[abcdefg]
CB 222(F) x CML 390(M)	1.59[abcdefg]
CML 444(F) x R119W(M)	1.59[abcdefg]
LAPOSTA(F) x CML 444(M)	1.57[bcdefg]
CML 390(F) x CML 444(M)	1.57[bcdefg]
CML 444(F) x LAPOSTA(M)	1.55[bcdefg]
CB 222(F) x CML 444(M)	1.55[bcdefg]
CKL05022(F) x R119W(M)	1.54[cdefg]
CML 444(F) x CB 248(M)	1.53[cdefg]
CB 222(F) x CKL05022(M)	1.51[cdefg]
CKL05022(F) x LAPOSTA(M)	1.51[cdefg]
CB 248(F) x CB 222(M)	1.49[cdefg]
CKL05022(F) x CML 390(M)	1.49[cdefg]
LAPOSTA(F) x CKL05022(M)	1.49[cdefg]

Season II maize hybrids	Aflatoxin concentration (µg/kg)
CB 248(F) x LAPOSTA(M)	1.49^{cdefg}
CML 390(F) x CB 222(M)	1.48^{cdefg}
CB 248C(F) x KL05022(M)	1.48^{cdefg}
CB 222(F) x LAPOSTA(M)	1.46^{defg}
CB 248(F) x CML 444(M)	1.46^{defg}
LAPOSTA(F) x CB 222(M)	1.46^{defg}
CML 444(F) x CB 222(M)	1.46^{defg}
CML 390(F) x CB 248(M)	1.45^{defg}
CML 444(F) x CML 390(M)	1.43^{efg}
CML 390(F) x CKL05022(M)	1.43^{efg}
CML 444(F) x CKL05022(M)	1.43^{efg}
CKL05022(F) x B 248(M)	1.41^{fg}
CB 222(F) x B 248(M)	1.39^{g}
CB 248(F) x CML 390(M)	1.39^{g}
CKL05022(F) x CB 222(M)	1.38^{g}
CKL05022(F) x CML 444(M)	1.37^{g}

Appendix 2: Aflatoxin levels (µg/kg) in season III maize hybrids in Katumani Research station

Season III maize hybrids	Aflatoxin concentration (µg/kg)
LAPOSTA(F) x R119W(M)	1.994644^{a}
R119W(F) x CML 390(M)	1.940559^{ab}
Local maize hybrid-1	1.917701^{abc}
LAPOSTA(F) x CKL05022(M)	1.83518^{abcd}
CML 444(F) x CB 222(M)	1.805927^{abcde}
LAPOSTA(F) x CB 248(M)	1.783172^{abcdef}
R119W(F) x CB 222(M)	1.777507^{abcdef}
CB 248(F) x CML 390(M)	$1.75251^{abcdefg}$
CKL05022(F) x LAPOSTA(M)	$1.751186^{abcdefg}$
CKL05022(F) x CML 390(M)	$1.743063^{abcdefg}$
CB 248(F) x R119W(M)	$1.740547^{abcdefg}$
CKL05022(F) x R119W(M)	$1.718988^{abcdefgh}$
CB 248(F) x CML 444(M)	$1.693625^{abcdefgh}$
CB 248(F) x CB 222(M)	$1.679917^{abcdefgh}$
CML 390(F) x CB 222(M)	$1.677516^{abcdefgh}$
CML 390(F) x CML 444(M)	$1.675318^{abcdefgh}$
CML 444(F) x LAPOSTA(M)	$1.67097^{abcdefghi}$
CB 222(F) x CML 390(M)	$1.666488^{abcdefghi}$
R119W(F) x CB 248(M)	$1.653759^{abcdefghi}$
CML 444(F) x R119W(M)	$1.648993^{abcdefghi}$
CB 248C(F) x KL05022(M)	$1.62129^{bcdefghij}$
Local maize hybrid-2	$1.59632^{cdefghij}$
R119W(F) x LAPOSTA(M)	$1.586821^{defghij}$
LAPOSTA(F) x CB 222(M)	$1.576469^{defghij}$
CB 222 (F) x R119W(M)	$1.568316^{defghij}$
CB 248(F) x LAPOSTA(M)	$1.564109^{defghij}$

Season III maize hybrids	Aflatoxin concentration (µg/kg)
CML 390(F) x CKL05022(M)	1.555656[defghij]
CML 390(F) x CB 248(M)	1.554217[defghij]
CKL05022(F) x B 248(M)	1.542912[defghij]
CML 390(F) x LAPOSTA(M)	1.540073[defghij]
LAPOSTA(F) x CML 444(M)	1.533446[defghij]
CML 444(F) x CB 248(M)	1.530635[defghij]
CB 222(F) x B 248(M)	1.482446[efghij]
CKL05022(F) x CB 222(M)	1.4711[fghij]
CKL05022(F) x CML 444(M)	1.452972[ghij]
CB 222(F) x CML 444(M)	1.447771[ghij]
CB 222(F) x CKL05022(M)	1.415665[hij]
CB 222(F) x LAPOSTA(M)	1.409835[hij]
CML 444(F) x CML 390(M)	1.350081[ij]
CML 444(F) x CKL05022(M)	1.320632[j]

Appendix 3: Fumonisin levels (mg/kg) in Season II and III maize hybrids

Season II maize hybrids	Fumonisin level	Season III maize hybrids	Fumonisin level
MIRTC5(F) x CML444(M)	0.43[a]	MIRTC5(F) x CML444(M)	0.51[a]
MIRTC5(F) x R119W(M)	0.38[ab]	MIRTC5(F) x CKL05015(M)	0.48[a]
CML495(F) x R119W(M)	0.36[ab]	CML495(F) x R119W(M)	0.48[a]
CML495(F) x CML444(M)	0.32[abc]	MIRTC5(F) x R119W(M)	0.43[ab]
MIRTC5(F) x CKL05015(M)	0.31[abcd]	P502(F) x CML495(M)	0.40[abc]
R119W(F) x CML495(M)	0.30[abcd]	CML495(F) x CML444(M)	0.38[abcd]
P502(F) x CML495(M)	0.29[abcd]	R119W(F) x CML495(M)	0.38[abcd]
MIRTC5(F) x P502(M)	0.23[abcd]	CML495(F) x P502(M)	0.31[abcde]
COMMERCIAL	0.22[abcde]	COMMERCIAL	0.27[abcde]
CML495(F) x P502(M)	0.18[abcde]	MIRTC5(F) x P502(M)	0.26[abcde]
CML495(F) x CKL05015(M)	0.12[abcde]	CML495(F) x CKL05015(M)	0.26[abcde]
CML495(F) x MIRTC5(M)	0.11[abcde]	R119W(F) x MIRTC5(M)	0.22[abcdef]
R119W(F) x MIRTC5(M)	0.09[abcde]	CKL05015(F) x MIRTC5(M)	0.16[abcdef]
CKL05015(F) x P502(M)	0.07[abcde]	R119W(F) x P502(M)	0.12[abcdef]
CKL05015(F) x MIRTC5(M)	0.07[abcde]	R119W(F) x CKL05015(M)	0.11[abcdef]
CML444(F) x CML495(M)	0.06[abcde]	CML495(F) x MIRTC5(M)	0.09[abcdef]
CML444(F) x MIRTC5(M)	0.03[abcde]	CML444(F) x CML495(M)	0.09[abcdef]
R119W(F) x CKL05015(M)	0.02[abcde]	CKL05015(F) x P502(M)	0.04[abcdefg]
R119W(F) x P502(M)	-0.05[abcdef]	CML444(F) x MIRTC5(M)	0.04[abcdefg]
P502(F) x CKL05015(M)	-0.06[abcdef]	P502(F) x CML444(M)	0.01[abcdefg]
P502(F) x CML444(M)	-0.07[abcdefg]	P502(F) x MIRTC5(M)	-0.01[abcdefg]
P502(F) x MIRTC5(M)	-0.09[bcdefg]	P502(F) x CKL05015(M)	-0.05[bcdefg]
CKL05015(F) x CML495(M)	-0.09[bcdefg]	CKL05015(F) x CML495(M)	-0.10[cdefg]
P502(F) x R119W(M)	-0.11[bcdefg]	P502(F) x R119W(M)	-0.12[cdefg]
CML444(F) x CKL05015(M)	-0.11[bcdefg]	CML444(F) x CKL05015(M)	-0.12[cdefg]

Season II maize hybrids	Fumonisin level	Season III maize hybrids	Fumonisin level
CML444(F) x R119W(M)	-0.18[cdefg]	MIRTC5(F) x CML495(M)	-0.13[defg]
MIRTC5(F) x CML495(M)	-0.20[defg]	CML444(F) x R119W(M)	-0.20[efg]
CKL05015(F) x R119W(M)	-0.28[efg]	CKL05015(F) x R119W(M)	-0.28[fg]
CML444(F) x P502(M)	-0.50[fg]	CML444(F) x P502(M)	-0.47[g]
CKL05015(F) x CML444(M)	-0.65[g]	CKL05015(F) x CML444(M)	-1.42[h]