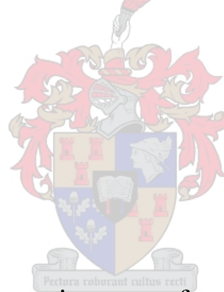
Resistance in South African maize inbred lines to the major ear rot diseases and associated mycotoxin contamination

by

Marili Mouton



Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of AgriSciences at the University of Stellenbosch

Supervisor: Prof. A. Viljoen
Co-supervisors: Ms. L.J. Rose
Prof. B.C. Flett

""Crtkn2014

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Stellenbosch University http://scholar.sun.ac.za

DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 18 January 2014

Copyright © 2014 Stellenbosch University

All rights reserved

Stellenbosch University http://scholar.sun.ac.za

SUMMARY

Maize (*Zea mays* L.) is one of the most important grain crops produced globally and serves as the primary source of carbohydrates and vitamins to millions of people in Africa. Whenever environmental conditions are favourable, fungal species such as *Fusarium verticillioides*, *Fusarium graminearum sensu lato*, *Aspergillus flavus* and *Stenocarpella maydis* frequently infect the ears of maize, reducing yield and grain quality. Of greater economic concern is the contamination of maize kernels with mycotoxins produced by ear rot pathogens due to its association with mycotoxicoses and immune suppression in humans and animals. Outbreaks of ear rot diseases commence in the field, but their associated toxins can be produced along the value chain. Planting resistant cultivars, as part of an integrated management strategy, could provide effective means of controlling preharvest ear rot diseases and mycotoxin accumulation in maize.

Maize cultivars resistant to the major ear rot fungi and their mycotoxins are not yet available in South Africa and therefore should be developed in plant improvement programmes where durable resistance is combined with useful agronomic traits. The first step in introducing resistance into maize cultivars would be to find sources of genetic resistance. Infertility or unwanted traits may be present in wild relatives or other species of maize, and therefore locally adapted breeding material would be the most desirable source.

This research aimed to identify publically available maize genotypes with durable resistance to the major ear rot pathogens and their associated mycotoxins in South Africa. In this study, a collection of inbred lines with diverse genetic backgrounds and valuable agronomic characteristics were evaluated under a range of field conditions. Some inbred lines were resistant to Fusarium ear rot (FER) and fumonisin contamination during artificially inoculated trials over two years. Furthermore, these FER-resistant inbred lines have been tested for resistance to other important maize ear rot diseases including Gibberella ear rot, Diplodia ear rot and Aspergillus ear rot in a multi-location field trial. Inbred lines with low and high levels of resistance to multiple infections were identified, but significant inbred x location interactions were observed. This suggests that potentially resistant lines will require further testing in an extra season to confirm their resistant status. If confirmed, these sources could be used to investigate the underlying mechanisms conferring resistance, or to develop molecular markers to facilitate the transfer of resistance into commercially valuable cultivars.

Stellenbosch University http://scholar.sun.ac.za

OPSOMMING

Mielies (*Zea mays* L.) is een van die belangrikste graangewasse wat wêreldwyd geproduseer word en dien as primêre bron van koolhidrate en vitamienes vir miljoene mense in Afrika. Tydens gunstige omstandighede, word die koppe van mielies dikwels geïnfekteer deur swam spesies soos *Fusarium verticillioides*, *Fusarium graminearum sensu lato*, *Aspergillus flavus* en *Stenocarpella maydis* wat lei tot 'n afname in opbrengs en graan kwaliteit. Kontaminasie van mieliepitte met mikotoksiene wat geproduseer word deur kopvrot patogene, is egter van groter ekonomiese belang aangesien dit verband hou met mikotoksikoses en imuun onderdrukking in mens en dier. Uitbrake van kopvrot siektes begin in die veld, maar die produksie van geassosieerde mikotoksiene kan regdeur die voedselketting geskied. Die aanplanting van weerstandbiedende kultivars, as deel van geïntegreerde siektebestuurmaatreëls, kan effektief wees in die beheer van voor-oes kopvrot siektes en mikotoksien-kontaminasie.

Mielie kultivars wat weerstand bied teenoor die hoof kopvrot swamme en hul mikotoksiene is nog nie in Suid-Afrika beskikbaar nie en moet dus ontwikkel word in plant verbeteringsprogramme waar duursame weerstand met nuttige agronomiese eienskappe gekombineer word. Die eerste stap in die verbetering van weerstand in mielie kultivars sal wees om bronne van genetiese weerstand te vind. Plaaslik-aangepaste teeltmateriaal bied die mees geskikte bron, omdat daar geen komplikasies van onvrugbaarheid of ongewenste kenmerke behoort te wees wat van wilde familielede of ander spesies afkomstig mag wees nie.

Mielie genotipes met stabiele weerstand teen kopvrot patogene en hul geassosieerde mikotoksiene in Suid Afrika is in hierdie studie geïdentifiseer na evaluasie van 'n versameling inteellyne met verskillende genetiese agtergronde en waardevolle agronomiese eienskappe. Na afloop van geïnokuleerde proewe is daar gevind dat sekere inteellyne weerstandbiedend is teenoor Fusarium kopvrot (FKV) en fumonisien-kontaminasie. Die FKV-weestandbiedende inteellyne is toe vir weerstand teen ander belangrike kopvrot siektes, insluitend Gibberella kopvrot, Aspergillus kopvrot en Diplodia kopvrot, getoets. Inteellyne met lae en hoë vlakke van weerstand teen verskeie infeksies is geïdentifiseer, maar 'n beduidende inteellyn x lokaliteit interaksies is waargeneem. Dit dui daarop dat potensiële weerstandbiedende inteellyne verder getoets moet word om hul weerstandstatus te bevestig. Hierdie lyne kan dan gebruik word om die onderliggende meganismes wat weerstand teweeg bring te ondersoek of om molekulêre merkers te ontwikkel wat die oordrag van weerstand in kommersiële kultivars vergemaklik.

Stellenbosch University http://scholar.sun.ac.za

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following people and institutions:

Prof. Altus Viljoen, Lindy Rose and Prof. Bradley Flett. Thank you for your guidance and encouragement throughout this study. I value all your inputs and hope to continue a long working relationship with you in the future!

The financial assistance of **The South African Maize Trust and National Research foundation (NRF)** towards this research is also acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the Maize Trust or NRF.

Medical Research Council – Programme on Mycotoxins and Experimental Carcinogenesis for providing *Aspergillus* isolates MRC 3951, - 3952, - 3954 and - 0200.

The Agricultural Research Council – Grain Crops Institute for providing the trial locations and facilities for inoculum preparation. **Sharon McFarlane** at the South African Sugarcane Research Institute for help with inoculum preparation.

Maureen Fritz at the Agricultural Research Council – Institute for Soil, Climate and Water for providing weather data.

Madré Booyse and Marieta van der Rijst from the Agricultural Research Council – Institute for Fruit and Fruit Technology for help with statistical analyses.

Dr. Marietjie Stander from the Central Analytical Facility at Stellenbosch University, for mycotoxin analyses.

Ilze Beukes, Ilana Visser, Martin Benz, Jaco van Rhyn, Dewald Gouws, Karlien van Zyl, Meagan Vermeulen, Fanyan Mashinini, Yvonne Maila and Kgotso Ramakhale for technical assistance, either in the field or laboratory.

My fellow colleagues from the Department of Plant Pathology at Stellenbosch University and my **family and friends**, for their kindness, support and motivation.

Most of all, I would like to thank my **Heavenly Father** who's perfect love, hope and blessings are the real strength behind all my achievements.

Stellenbosch University http://scholar.sun.ac.za

LIST OF FIGURES

CHAPTER 1

Figure 1 Poor husk coverage **59**

CHAPTER 2

Figure 1 The five localities where maize samples were collected **85**

Figure 2 Artificial silk channel inoculation at Blister (R2) stage **86**

Figure 3 Graphic representation of Fusarium ear rot disease assessment **86**

Figure 4 GGE biplot showing Fusarium ear rot severity of 11 maize inbred lines **87**

Figure 5 GGE biplot showing fungal biomass of 11 maize inbred lines **88**

Figure 6 GGE biplot showing total fumonisin accumulation of 11 maize inbred lines **89**

Figure 7 Weather data recorded from the ARC's weather stations for the 2010/2011 and 2011/2012 seasons at the respective field locations **90**

CHAPTER 3

Figure 1 Gel image representing PCR-based identification of *Stenocarpella maydis* using the primer pair Maydis3 F/Maydis3 R **129**

Figure 2 GGE biplot showing total mycotoxin content (TCT B + ZEA) of nine maize inbred lines **130**

Figure 3 GGE biplot showing total aflatoxin content (AFB_1 + AFB_2) of nine maize inbred lines **131**

Figure 4 Relationship between *Stenocarpella maydis* infection potential and observed fungal biomass concentrations in resistant, intermediate and susceptible maize inbred lines **132**

Figure 5 GGE biplot showing *Stenocarpella maydis* biomass of nine maize inbred lines **133**

Figure 6 Weather data recorded from the ARC's weather stations for the 2012/2013 season at the respective field locations **134**

Stellenbosch University http://scholar.sun.ac.za

LIST OF TABLES

CHAPTER 1

Table 1	Regulatory limits for mycotoxins in feed and food as issued by the United States Food and Drug Administration	**59**

CHAPTER 2

Table 1	Maize inbred lines evaluated for resistance to Fusarium ear rot severity, fungal biomass and fumonisin accumulation	**79**
Table 2	AMMI ANOVA for resistance to Fusarium ear rot severity, fungal biomass and fumonisin accumulation	**80**
Table 3	Evaluation of 11 maize inbred lines for resistance to Fusarium ear rot	**81**
Table 4	Evaluation of 11 maize inbred lines for resistance to fungal biomass	**82**
Table 5	Evaluation of 11 maize inbred lines for resistance to fumonisin accumulation	**83**
Table 6	AMMI stability values (ASV) and ranking orders for Fusarium ear rot severity, fungal biomass and fumonisin contamination	**84**

CHAPTER 3

Table 1	Maize inbred lines evaluated for resistance to Gibberella, Aspergillus and Diplodia ear rot and associated mycotoxin contamination	**118**
Table 2	Visible symptoms of the major ear rot diseases of maize in Sub-Saharan Africa	**119**
Table 3	Genbank accession numbers for gene sequences used to design species-specific primers for the quantification of *Stenocarpella maydis* in maize	**120**
Table 4	Evaluation of nine maize inbred lines for resistance to Gibberella ear rot in a multi-location field trial in South Africa during the 2012/2013 growing season	**122**
Table 5	AMMI ANOVA for resistance to Gibberella ear rot severity, fungal biomass and mycotoxin accumulation in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season	**123**

Stellenbosch University http://scholar.sun.ac.za

Table 6	AMMI stability values (ASV) and ranking orders for trichothecenes B, zearalenone and aflatoxin contamination and *Stenocarpella maydis* biomass of the nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season	**124**
Table 7	Evaluation of nine maize inbred lines for resistance to Aspergillus ear rot in a multi-location field trial in South Africa during the 2012/2013 growing season	**125**
Table 8	AMMI ANOVA for resistance to Aspergillus ear rot severity, fungal biomass and aflatoxin accumulation in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season	**126**
Table 9	Evaluation of nine maize inbred lines for resistance to Diplodia ear rot and fungal biomass in a multi-location field trial in South Africa during the 2012/2013 growing season	**127**
Table 10	AMMI ANOVA for resistance to Diplodia ear rot severity and fungal biomass in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season	**128**
Table 11	Estimates for the relationship between *Stenocarpella maydis* infection potential and fungal biomass concentrations in the maize inbred lines	**129**

Stellenbosch University http://scholar.sun.ac.za

LIST OF ACRONYMS AND ABBREVIATIONS

AER	Aspergillus ear rot
AMMI	Additive main effects and multiplicative interaction
ANOVA	Analysis of variance
ARC-GCI	Agricultural Research Council Grain Crops Institute
ASV	AMMI stability values
BABA	β-amino-butyric acid
CAC	Codex Alimentarius Commission
DER	Diplodia ear rot
DON	Deoxynivalenol
ELISA	Enzyme linked immunosorbent assays
ET	Ethylene
FAR	Food and Agriculture of the United Nations
FER	Fusarium ear rot
GER	Gibberella ear rot
GGE	Genotype main effect and genotype by environment interaction
HPLC	High performance liquid chromatography
HR	Hypersensitive response
HSCAS	Hydrated sodium calcium aluminosilicates
IARC	International Agency for Research on Cancer
JA	Jasmonic acid
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LSD	Least significant difference

Stellenbosch University http://scholar.sun.ac.za

NIV	Nivalenol
PC1	First Principle Component
PC2	Second Principle Component
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PR	Pathogenesis-related
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
SA	Salicylic acid
SAGL	South African Grain Laboratory NPC
SADC	Southern African Developing Community
SAR	Systemic induced resistance
TCT B	B trichothecenes
TLC	Thin-layer chromatography
ZEA	Zearalenone
WHO	World Health Organization

Stellenbosch University http://scholar.sun.ac.za

PREFACE

Maize is the most important staple food for many South Africans. The crop may be affected by ear rot fungi that results in poor grain quality and yield. More importantly, these fungi produce mycotoxins that have been implicated in severe illness and immune suppressions in humans and livestock. Due to major economic losses, food safety concerns and health risks associated with the consumption of contaminated maize products, strategies are required to manage this problem effectively. Such preventative strategies should protect the crop prior to harvest, as damage and production of certain mycotoxins are known to be most extensive in the field.

Chapter 1 provides an overview of maize production in South Africa, important ear rot diseases such as Fusarium ear rot, Gibberella ear rot, Aspergillus ear rot and Diplodia ear rot, and the contamination of grain with mycotoxins. Current disease management tactics are discussed and the role of host resistance emphasised. Finally, the use of conventional plant breeding to improve host resistance in maize to ear rot fungi and their mycotoxin is discussed.

Host resistance, as part of an integrated management strategy, provides affordable and opportune means for controlling the mycotoxin problem. If resistant cultivars are not available, they should be developed in plant improvement programmes where durable resistance is combined with useful agronomic traits. The first step in establishing such a programme would be to find sources of genetic resistance, preferably in locally-adapted maize genotypes such as inbred lines. **Chapter 2** describes how 11 genetically diverse maize inbred lines were tested for their resistance to Fusarium ear rot (FER) and fumonisin production in a multi-location trial over 2 years in South Africa. This was done to ensure that their resistance remained stable under different environmental conditions. The performance of these inbred lines were evaluated by visually rating ear rot symptoms and quantifying fungal concentration and fumonisin content by means of quantitative real-time PCR and liquid chromatography tandem mass spectrometry, respectively.

Chapter 3 describes how six FER-resistant maize inbred lines were evaluated for their ability to resist Aspergillus ear rot, Gibberella ear rot and Diplodia ear rot and mycotoxin accumulation at three local field sites during the 2012/2013 season. This resistance, if stable, could be used in commercial and public conventional breeding programmes and be extremely advantageous to the local maize industry. The lines could be used to gain insight into host-pathogen interactions and plant resistance responses, and to develop molecular markers that can aid in accelerating the process of conventional plant breeding.

Stellenbosch University http://scholar.sun.ac.za

CONTENTS

Fulfilment I

Declaration II

Summary/Opsomming III

Acknowledgements V

List of Figures VI

List of Tables VII

List of Acronyms and Abbreviations IX

Preface XI

Chapter 1: Resistance in maize to ear rot pathogens and their mycotoxins: A review 16

Introduction 16

Maize production in South Africa 18

Ear rots of maize 19

Ear rot pathogens 19

Fusarium species 19

Aspergillus species 20

Stenocarpella species 20

Lifecycle of ear rot pathogens 21

Mycotoxins and their toxicological effects 22

Aflatoxins 22

Fumonisins 23

Diplonine 24

Trichothecenes 24

Zearalenone 24

Prevention and control of food-borne mycotoxins 25

Regulatory limits 25

Risk assessment of food commodities 25

Detection and quantification of mycotoxins 26

Detection and quantification of mycotoxigenic fungi 26

Current control strategies to reduce mycotoxin contamination 27

Agronomic practices and postharvest storage 27

Physical control 28

Biological control 29

Stellenbosch University http://scholar.sun.ac.za

Chemical control 29
Host-plant resistance 30
Native defence in plant-pathogen interactions 31
Constitutive defence 31
Structural barriers 31
Biochemical barriers 32
Induced defence 32
Localised and systemic induced resistance 32
Improvement of crop performance via host resistance 33
Biotechnological approaches 33
Plant breeding 34
Resistance breeding: the basic principles 35
Sources of resistance 35
Types of genetic resistance 35
Gene effects and heritability 36
Evaluating and selecting for resistance 37
Conclusion 38
References 39
Chapter 2: Multi-site screening for resistance in South African inbred lines to Fusarium ear rot and fumonisin contamination **60**
Abstract 60
Introduction 61
Materials and Methods 62
Plant material 62
Field sites 62
Land preparations 62
Production of fungal inoculum 63
Artificial inoculation of maize ears 63
Disease severity 64
Grain processing 64
Quantification of *Fusarium verticillioides* in maize grain 64
Quantitative detection of *F. verticillioides* in maize using qRT-PCR 65
Fumonisin analysis 65
Liquid chromatographic tandem mass spectrometry (LC-MS/MS) analysis 66
Statistical analysis 66

Stellenbosch University http://scholar.sun.ac.za

Results 67
Disease severity 68
Quantification of *Fusarium verticillioides* 68
Fumonisin analysis 69
Correlation between ear rot severity, fungal biomass and fumonisin contamination 70
Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses 71
Weather data 72
Discussion 73
References 76
Chapter 3: Evaluation of Fusarium ear rot-resistant maize inbred lines as potential sources of resistance to other major ear rot ear rot diseases and associated mycotoxin contamination **91**
Abstract 91
Introduction 92
Materials and Methods 94
Plant material 94
Field sites 94
Production of fungal inoculum 94
Artificial inoculation of maize ears 95
Disease severity 96
Development of species-specific primers for *Stenocarpella maydis* 96
Fungal isolates 96
Genomic DNA extractions, PCR amplification and sequencing 96
Primer design 97
Specificity of the primer sets to *S. maydis* 98
Quantification of *F. graminearum* s.l., *A. flavus* and *S. maydis* in maize grain 98
Genomic DNA extractions 98
Quantitative detection of maize ear rot fungi using qRT-PCT 99
Mycotoxin analysis 100
Statistical analysis 102
Results 102
Gibberella ear rot 102
Disease severity, fungal biomass and mycotoxin production 102

Stellenbosch University http://scholar.sun.ac.za

Correlations between ear rot severity, fungal biomass and mycotoxin contamination 104
Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses 104
Aspergillus ear rot 105
Disease severity, fungal biomass and aflatoxin production 105
Correlations between ear rot severity, fungal biomass and aflatoxin contamination 106
Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses 106
Diplodia ear rot 107
Disease severity and fungal biomass 107
Correlations between ear rot severity and fungal biomass 108
Regression analysis 108
Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses 108
Weather data 109
Discussion 109
References 113

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 1

Resistance in maize to ear rot pathogens and their mycotoxins: A review

INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) describes maize (*Zea mays* L.) as one of the most important staple food crops produced worldwide (FAO, 1992). In Southern Africa, maize is the dominant grain crop with daily consumption levels ranging from 400 g to 500 g per person in certain regions (Shephard, 2008). Maize is also the largest field crop produced locally. The Free State, Mpumalanga and North West provinces are the primary producers, accounting for about 83% of total production (SAGL, 2011). It is estimated that approximately 50% of maize produced in the country is consumed by humans. Maize is the primary source of vitamins and carbohydrates and therefore plays an essential role in the livelihoods of millions of people. The rest of the maize produced is used for the animal feed industry (40%) and seed and industrial uses (10%)(National Department of Agriculture, 2009). Due to its adaptability to different geographical zones, ease of cultivation, high yield per hectare cropland and storage characteristics, maize has now become a commercial crop that many industries depend on for their raw materials (Asiedu, 1989; Iken and Amusa, 2004). However, sustainable maize production is compromised by the infestation of pathogens and pests prior to and after harvest (Lillehoj, 1987; White, 1999).

In the field and under postharvest conditions, fungal infection damages the maize plant by causing leaf and stalk diseases as well as kernel decay. These diseases result in significant yield losses and decrease the quality and nutritive value of the grain (WHO, 2006; White, 1999). The fungal genus *Fusarium* infects the maize, causing seedling diseases as well as root, stalk and ear rot, which can impact negatively on yield and plant growth (Munkvold and Desjardins, 1997). The species most frequently associated with ear rots of maize are *Fusarium verticillioides* (Sacc.) Nirenberg and *Fusarium graminearum sensu lato* (s.l.) (Ncube, 2008; Boutigny *et al*., 2011). *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium subglutinans* (Wollenberg and Reinking) Nelson, Toussoun and Marasas, *Aspergillus flavus* Link ex Fries, *Aspergillus parasiticus* Speare, *Stenocarpella maydis* (Berck) Sutton and *Stenocarpella macrospora* Earle, also remain important causal agents of this disease (Munkvold, 2003b). Several of these species are responsible for producing a wide range of mycotoxigenic secondary metabolites, commonly referred to as mycotoxins.

Stellenbosch University http://scholar.sun.ac.za

Mycotoxin contamination in maize grain is currently of public concern due to its detrimental effect on both human and animal health (Wu, 2006; Murphy *et al.,* 2006; Wu, 2007; Balazs and Schepers, 2007). Although numerous fungal secondary metabolites can be found in maize-based foods and feeds, international research has focused on certain mycotoxins that pose the greatest danger. These include fumonisins produced primarily by *F. verticillioides* and *F. proliferatum*, aflatoxins produced by *A. flavus* and *A. parasiticus*, and deoxynivalenol, nivalenol and zearalenone produced by *Fusarium graminearum* s.l. (Munkvold, 2003a; Boutigny *et al*., 2012). To date, the food-borne mycotoxins likely to be of greatest importance in Africa are the fumonisins and aflatoxins (Gelderblom *et al*., 1988; WHO, 2006), with the former being of current concern because it is a prevalent contaminant in South African maize (Ncube, 2008; Waalwijk *et al*., 2008). Due to their negative impact on society, advisory guidelines for mycotoxin content in maize products intended for commercial use have been implemented in several countries (Haumann, 1995; Van Egmond *et al*., 2007).

In South Africa, regulatory legislation measures to facilitate safe and fair international trade of maize-based products are not in place except for aflatoxins (maximum limit of 10 ppb) which are poorly enforced (Shephard, 2008; Rheeder, 2009). Consequently, a considerable part of the maize crop grown in the country could be receptive to mycotoxigenic fungi when environmental conditions are favourable. Detection of mycotoxins in maize products has resulted in pressure from international markets, regulatory, and trade policies in the form of reduced prices and even export rejection (WHO, 2006; Wu, 2007). The potential threat of ear rot diseases and subsequent mycotoxin production is exacerbated by an increasing human population and limited food supply. According to the Codex Alimentarius Commission (CAC)(2003) there is an urgent need for effective preventative management strategies and good agricultural practices to minimize economic losses and health risks associated with maize mycotoxin threat to food safety and security.

Chemical and cultural practices have been relatively unsuccessful in eliminating infection and high mycotoxin levels in maize (Headrick and Pataky, 1991; Munkvold, 2003a). The most effective strategy would be via host-plant resistance as it provides an affordable and opportune means to control mycotoxin contamination by ear rot pathogens. Maize hybrids and inbred lines have, therefore, been evaluated internationally for resistance to ear rot pathogens, especially *A. flavus* and *F. verticillioides*, but little is known about the resistance status of breeding material and commercial cultivars used in South Africa (Clements *et al*., 2004; Afolabi *et al.,* 2007). In a recent investigation, potential resistance to

Stellenbosch University http://scholar.sun.ac.za

Fusarium ear rot and fumonisin contamination has been identified in a collection of maize inbred lines well adapted to South African growing conditions (Small *et al*., 2012). Additionally, Van Rensburg and Ferreira (1997) and Van Rensburg *et al.* (2003) also reported on local breeding material with high resistance to Diplodia ear rot.

Studies have indicated that plant improvement for resistance to ear rot fungi may be achieved through plant breeding or genetic engineering (Munkvold and Desjardins, 1997; Iken and Amusa; 2004; Acquaah, 2007). Therefore, the objective of this literature review is to provide general background on host resistance in maize towards major ear rot diseases such as Fusarium ear rot, Gibberella ear rot, Aspergillus ear rot and Diplodia ear rot and their associated mycotoxins. The use of conventional plant breeding to improve host resistance in maize towards ear rot diseases and mycotoxin contamination is then discussed.

MAIZE PRODUCTION IN SOUTH AFRICA

Maize is the most important agricultural commodity in South Africa. It is estimated that South Africa has more than 9000 maize producers, who produced an average of 10 million tonnes annually between 1999 and 2011 (SAGL, 2011). These statistics indicate that South Africa is the largest maize producer in the Southern African Developing Community (SADC) region. Maize production systems in South Africa range from smallholder, subsistence farming to large-scale commercial farming. The latter accounts for 85% of maize grown in the country due to the use of improved agronomic practices (Bänziger and De Meyer, 2002).

Maize is cultivated in a challenging environment. Public and socio-economic insecurity as well as a myriad of biotic and abiotic influences make it difficult to farm efficiently and profitably with maize. One of the major socio-economic problems associated with obtaining optimum yields involves the limited access of smallholder and subsistence farmers to additional inputs such as fertilisers, fungicides, education and employment (Bänziger and De Meyer, 2002). These farmers often avoid investing in their agricultural practices because of poverty or the risk of adverse economic responses. Additionally, biotic and abiotic maize production constraints include drought and heat stress, competition by weeds, plant diseases as well as the feeding damage caused by insects and rodents (Heisey and Edmeades, 1999; Pingali and Pandey, 2001). Public ignorance of the existence of mycotoxins exacerbates its potential threat to both humans and animals consuming contaminated maize products.

Stellenbosch University http://scholar.sun.ac.za

EAR ROTS OF MAIZE

Fungal infestation of maize ears and kernels can be divided into distinct diseases that result in discolouration and the significant decrease in kernel density, yield, quality and feed value of the grain (White, 1999; WHO, 2006). These are Fusarium ear rot (FER), Gibberella ear rot (GER), Aspergillus ear rot (AER) and Diplodia ear rot (DER), of which FER and DER are the greatest economical concern to farmers in South Africa (Boutigny *et al*., 2012; Schoeman and Flett, 2012).

Ear rot pathogens

Fusarium species: The genus *Fusarium* is complex and consists of several species which are adapted to a broad range of environments throughout the world (Summerell *et al*., 2001). Many species grow as natural endophytes of maize in vegetative and/or reproductive tissue at some point during its lifecycle, while others are considered pathogens and cause rots, wilts and other diseases in agronomically important crops (Sikora *et al*., 2003). Pathogenic species of maize include *F. verticillioides, F. proliferatum, F. subglutinans* as well as *F. graminearum sensu lato* (Munkvold, 2003b). In most African countries, *F. verticillioides* is the predominant *Fusarium* species occurring on maize (Rheeder *et al.*, 2002; Fandohan *et al.*, 2003; Ncube, 2008) and is responsible for causing FER.

FER thrives in warm, dry weather and is characterized by a cottony, white-pinkish mold that usually occurs at the tip or in scattered areas on the maize ear (Munkvold, 2003b). Diseased kernels may also exhibit a distinctive "starburst symptom" which is observed as white streaks radiating out from the point of silk attachment or from the base of the kernel (Bush *et al*., 2004). *Fusarium verticillioides* grows over a broad range of temperatures and water activities (Reid *et al*., 1999) and infects the plant tissue during all development stages, in some cases without causing any visible symptoms (Munkvold *et al*., 1997; Bottalico, 1998; Munkvold, 2003b). High levels of infection have been reported in the western Free State, North West and Northern Cape provinces in South Africa (Boutigny *et al*., 2012).

GER, primarily caused by *F. graminearum* s.l. (Boutigny *et al.*, 2011), spreads from the tip of the maize ear, covers it extensively with pink or red mycelial growth, and results in yield and grain quality reduction (Logrieco *et al*., 2002). Natural epidemics of this disease are often localized and sporadic and thus difficult to predict (Viger *et al*., 2001). GER predominates in cooler areas such as the eastern Free State, Mpumalanga and KwaZulu-Natal

provinces (Boutigny *et al*., 2012). Low temperatures and high humidity favour infections to typically occur during the three week period after silking (Viger *et al*., 2001).

Aspergillus species: Members of the genus *Aspergillus* are distributed worldwide, but are most abundant between 26° and 35° latitudes north and south of the equator and more common in tropical and subtropical regions (Klich *et al*., 1994). This genus contains economically important species such as *Aspergillus flavus* and closely related species *A. parasiticus*. Both species are primarily fungal saprophytes surviving on decaying plant matter but may be regarded as opportunistic pathogens in humans and animals (Klich *et al*., 1994). *Aspergillus flavus* is more common in maize and cottonseed due to its adaptation to upper-ground niches whereas *A. parasiticus*, adapted to soil environments, is predominantly isolated from groundnuts (Yu *et al*., 2005).

Aspergillus flavus and *A. parasiticus* are responsible for causing AER, one of the most familiar ear rot diseases in maize fields in west-central Africa, south-eastern United States and Texas (Henry *et al*., 2009). *Aspergillus flavus* appears as an olive-green and *A. parasiticus* as a green-gray powdery mold and are commonly observed at the tip of the ear during hot and humid seasons (Fennel *et al*., 1975). Similar to *F. verticillioides*, *A. flavus* can contaminate grain without causing any visible signs of infection (Henry *et al*., 2009). Extensive surveys by the South African Maize Board since 1986 have consistently indicated very low levels of aflatoxin contamination in both commercial and home-grown maize in South Africa (Janse van Rensburg, 2012). Although local maize is virtually free of aflatoxins, improper harvest and storage practices may cause *A. flavus* and *A. parasiticus* and subsequent aflatoxin accumulation (Shephard, 2005).

Stenocarpella species: Stenocarpella maydis and the closely related *S. macrospora* are frequently reported in the literature as prevalent spoilage field fungi of maize (Odriozola *et al.* 2005). Although extensively published as species of *Diplodia* in previous years, *S. maydis* and *S. macrospora* belong to the order Diaporthales rather than the Botryosphaeriales as revealed by their distinct conidiogenesis (Sutton, 1980).

Stenocarpella maydis and *S. macrospora* causes DER of maize where infection typically starts as a dense white to greyish mold growing from the base of the ear upward. In addition, *S. maydis* produces fruiting bodies (pycnidia) which appear as raised black bumps that may be scattered on the husks, ears and sides of rotten kernels (Lamprecht *et al*., 2011). Latent infection of *S. maydis* shows no apparent symptoms, but when the ears are broken in half and

the kernels removed, pycnidia can be found on the kernels whose embryos are discoloured (Nowell, 1997).

The climatic conditions favouring *S. maydis* and *S. macrospora* differ dramatically. *Stenocarpella maydis* is monocyclic in nature and widely distributed over the entire maize-producing area in South Africa (Van Rensburg and Flett, 2010; Lamprecht *et al*., 2011). It also prefers late season rains in warm climates (Flett and McLaren, 1994; Flett and McLaren, 2001). Warm and humid weather favour infection by *S. macrospora* which is a polycyclic disease known to cause leaf lesions. DER caused by *S. macrospora* is frequently observed in the south-eastern regions of KwaZulu-Natal (Kloppers and Tweer, 2009).

Life cycle of ear rot pathogens

The cycle of infection and disease development in maize-pathosystems is complex and yet to be clarified (White, 1999). *Fusarium* species such as *F. verticillioides* and *F. graminearum* s.l, overwinter as thickened mycelia or fruiting structures on maize residues on the soil surface, or in the soil following mechanical incorporation. Soil-borne mycelia are believed to be the source of abundant conidia in maize fields during the growing season (Munkvold and Desjardins, 1997; Cotton and Munkvold, 1998). These spores are easily carried to the maize ear by wind, water and insects and serve as inoculum for infections (Munkvold, 2003b; Oren *et al*., 2003). Several infection pathways for *F. verticillioides* have been identified such as systemic movement from seed, root or stalk to the kernels, contamination of insect and bird damage as well as silk infection (Reid *et al*., 1996; Munkvold *et al*., 1997). Ear-invading lepidopteron pests such as stalk borers (*Busseola fusca* Fuller), ear borers (*Ostrinia nubilalis* Hübner), sap beetles (*Carpophilus* spp.; *Glischrohilus quadrisignatus* Hood) and thrips (*Frankliniella williamsi* Say) play a key role in the distribution of *F. verticillioides* and in FER disease development. They can act as wounding agents causing injury to the plant tissue that predisposes the kernels to fungal penetration, or they can act as vectors spreading the fungus from the origin of inoculum to new plantings (Fig. 1C) (Flett and Van Rensburg, 1992; Munkvold and Desjardins, 1997; Dowd, 1998). The passive movement of spores along the surface of silks, perhaps via capillarity action, is considered to be the more important infection pathway for *F. verticillioides* into the ear (Manns and Adams, 1923; Koehler, 1942; Headrick and Pataky, 1991; Munkvold and Carlton, 1997). Duncan and Howard (2010) have recently suggested the phenotype of the stylar canal as a probable mechanism by which *F. verticillioides* enter developing kernels.

Stellenbosch University http://scholar.sun.ac.za

Sources of inoculum for *A. flavus* and *A. parasiticus* are conidia, mycelia and sporogenic scerotina (Calvo *et al*., 1999) that are produced by these fungi to prolong their survival in soil and plant debris (Yu *et al*., 2005). The large number of microconidia that are produced in the field stubble are easily wind dispersed and vectored in hot and humid weather. Spores may land on the silk tissue where they germinate and enter the ears prior to pollination. According to Payne (1999), green silks are relatively resistant to infection whereas senescent silks can be colonised by *A. flavus* and *A. parasiticus*.

The life cycle of *Stenocarpella* species commence with a saprophytic phase when the fungi overwinter on unburied maize stubble as viable pycnidia or mycelia (Flett *et al.,* 1992). Under warm moist conditions spores are extruded from the pycnidia and spread by wind or rain splashed onto several parts of the maize plant. *Stenocarpella maydis* and *S. macrospora* infect behind the leaf sheath at the stele and progress up the shank causing the ear to rot (Bensch *et al*., 1992).

Mycotoxins and their toxicological effects

In addition to causing ear rots, certain *Fusarium, Aspergillus* and *Stenocarpella* species also have the ability to produce a variety of mycotoxins with proven detrimental effects on both humans and animals (Pereira *et al*., 2011). Mycotoxins are secondary metabolites that are produced by many fungi in food crops and result in mycotoxicoses when ingested by humans and animals. The role of these substances in nature remains an open issue. Compelling evidence suggests that *Fusarium graminearum* s.l. may use its trichothecenes for pathogenicity (Desjardins and Hohn, 1997; Harris *et al*. 1999) and that *F. verticillioides* may produce fumonisins to obtain a competitive advantage during stressful environmental conditions such as water- and nutrient deficiency (Picot *et al*., 2010).

Many mycotoxins are economically important, but the five most often encountered on maize are aflatoxins, fumonisins, deoxynivalenol, nivalenol and zearalenone. Moreover, the neurotoxin diplonine that was recently isolated and characterized locally is also attracting public attention (Snyman *et al*., 2011).

Aflatoxins: Aflatoxins are a family of structurally related polyketides with the four major aflatoxins being B_1, B_2, G_1 and G_2, primarily produced by strains of *A. flavus* and *A. parasiticus* (Pitt *et al*., 1993; Pitt *et al*., 1994). The "B" and "G" designations refer to the blue and green fluorescent colours displayed under long wave ultraviolet light in physicochemical assays, while the subscript numbers 1 and 2 indicate chromatographic mobility. Acute

Stellenbosch University http://scholar.sun.ac.za

exposure to aflatoxins can lead to growth impairment, childhood stunting as well as jaundice and in severe cases, death (Gong *et al*., 2002). To date, the largest outbreak of aflatoxicosis occurred in Kenya in 2004 where 215 recognized deaths were reported (Lewis *et al*., 2005). The International Agency for Research on Cancer (IARC) has evaluated aflatoxin B_1 and mixtures of aflatoxins as Group 1 carcinogens producing liver cancer in humans (IARC, 2002). Aflatoxins in animal feed have also led to liver necrosis, oxidative stress and haemorrhage in broiler chickens, pigs and cattle (Eraslan *et al*., 2005; Osweiler, 2005).

Fumonisins: While aflatoxins are the most damaging mycotoxin reported in the international scientific literature, fumonisins are more prevalent in South African maize (Waalwijk *et al*., 2008; Ncube, 2008) and the discovery of their oesophageal cancer-promoting activity has resulted in extensive research efforts (Gelderblom *et al.,* 1988). Fumonisins are produced by several *Fusarium* species, of which *F. verticillioides* is the most prolific and common fumonisin producer belonging to *Gibberella fujikuroi* mating population A (Marasas, 2001). While members of the D mating population, such as *F. proliferatum*, also produce large quantities of fumonisins, they are found on maize in relatively low frequencies (Shephard *et al.,* 1996). Fumonisins are comprised of chemically related structures that consist of a minimum of 28 analogues (Nelson *et al*., 1993). The most commonly found in naturally contaminated maize-based foodstuff and feeds worldwide (Shephard *et al*., 1996; Rheeder *et al*., 2002) are the fumonisins B_1, B_2 and B_3 (Marasas, 2001). Research has shown that fumonisin B_1 disrupts the sphingolipid metabolism in many types of cells and also changes poly-unsaturated fatty acid pools, followed by cellular deregulation and finally cell death (Wang *et al*., 1991; Gelderblom *et al*., 2001). Therefore, circumstantial evidence linking the consumption of fumonisin contaminated maize to incidences of human oesophageal cancer in regions of South Africa and central China is plausible (Gelderblom *et al*., 1988; Chu and Li, 1994; Marasas *et al*., 2004). Fumonisin is also considered a potential risk factor causing neural tube defects in newborns internationally where maize is a staple food, and reduced immunity which increases the possible onset of AIDS in HIV-compromised individuals in Sub-Saharan Africa (Stack, 1998; Missmer *et al*., 2006; Williams *et al*. 2011). The acute effects of FB_1 in animal feed has resulted in hepatosis and leukoencephalomalacia in equines (Kriek *et al*., 1981; Kellerman *et al*., 1990), pulmonary oedema syndrome in pigs, nephrosis in sheep (Harrison *et al*., 1990) and liver cancer in experimental mice and rats (Gelderblom *et al*., 1994; Marasas, 2001). Based on these findings, the IARC has classified fumonisins as possible carcinogens (group 2B) to humans (IARC, 2002).

Stellenbosch University http://scholar.sun.ac.za

Diplonine: Recent investigation by Snyman *et al.* (2011) indicated the ability of *S. maydis* to produce diplonine, a neurotoxin that causes symptoms similar to those caused by diplodiosis. In South Africa, diplodiosis is rated the sixth most important mycotoxicosis of sheep and cattle (Snyman *et al.,* 2011), resulting in nervous system defects and neonatal losses, as well as acute toxicity in ducklings and chickens (Kellerman *et al*., 1985; Rabie *et al*., 1987; Kellerman *et al*., 1991). Diplonine is yet to be linked to detrimental effects in humans (Barros *et al*., 2008; Snyman *et al*., 2011). Further research is currently underway to isolate and characterise the possible mycotoxins produced by *S. maydis*. Publically available standards of such mycotoxins will enable accurate risk assessment in grazing fields, and could facilitate studies into pathogenesis that could lead to a cure or preventative treatment for diplodiosis in livestock (Snyman *et al*., 2011).

Trichothecenes: Deoxynivalenol (DON) and its acetylated forms 3-acetyl-deoxynivalenol (3-A-DON) and 15-acetyl-deoxynivelanol (15-A-DON) as well as nivalenol (NIV) are classified type B trichothecenes, a large group of sesquiterpenoid molecules. These mycotoxins are produced by *F. graminearum* s.l. (Boutigny *et al.,* 2011). Type B trichothecenes are extremely potent inhibitors of eukaryotic proteins, and RNA and DNA synthesis and interact with the cell wall membrane (Pestka, 2007). DON and NIV are two of the most common mycotoxins present in grains such as maize, wheat, oats, barley and rice and when ingested in high doses can cause nausea, vomiting, and diarrhoea in farm animals. At lower doses, pigs exhibit feed refusal and decreased weigh gain (Rotter *et al*., 1996). DON and NIV are often found in association with zearalenone (ZEA), because all are produced by *F. graminearum* s.l. (Boutigny *et al*., 2012).

Zearalenone: ZEA and its derivative zearalenol are nonsteroidal molecules with estrogenic properties. While biologically potent, they are hardly toxic (Kuiper-Goodman *et al*., 1987). Ingestion of ZEA as low as 1 ppm can cause infertility, abortion or other reproductive disorders in mammals and farm animals (Nelson *et al*., 1993). Since ZEA and zearalenol's ability to block estrogen receptors was discovered in the early 1970's, pharmaceutical companies have manipulated it to serve as growth-promoting hormones and postmenopausal treatment (Utian *et al*., 1973; Hidy *et al*., 1977).

Stellenbosch University http://scholar.sun.ac.za

PREVENTION AND CONTROL OF FOOD-BORNE MYCOTOXINS

Regulatory limits

On the grounds that there are possible health risks associated with exposure to mycotoxins, maximum tolerable limits allowed for human and animal consumption have been established by international authorities in more than 100 countries (Haumann, 1995; Van Egmond *et al*., 2007) (Table 1). Under South African national policy (Act No. 54 of 1972, as amended by Government Notice No. R. 1145 of 8 October 2004), only limits for aflatoxins in all foodstuffs but specifically peanuts and dairy milk (maximum limit of 10 ppb), and patulin in apple-based fruit juices (maximum limit of 50 ppb) has been legalised (Rheeder *et al*., 2009). In local commercial systems, however, maize grain are graded according to their aflatoxin, fumonisin, DON and ZEA content as published in the Agricultural Product Standards Act (Act No. 119 of 1990, as amended by the Government Notice No. 32190 of 8 May 2009) (SAGL, 2011).

A survey conducted by Ncube (2008) in the 2006 and 2007 planting seasons illustrated that the majority of moldy grain harvested by subsistence farmers in South Africa are used in brewing traditional beer and as stock feed. Worryingly, most African countries lack the basic infrastructure, formal food markets systems and capacity to detect and control mycotoxins in food and feed prior to consumption, with the result that mycotoxin-contaminated commodities present greater health risks in these regions. It is thus crucial to investigate and develop quick and reliable detection and *in vivo* screening tests for either mycotoxigenic fungi or their mycotoxins that can be made publicly available at low cost.

Risk assessment of food commodities

Mycotoxins are ubiquitous and exposure occurs worldwide, with a large part of the food and feed chain contaminated to some extent (Magan and Olsen, 2004). Screening for mycotoxins in commodities can aid in the prevention and diagnosis of mycotoxicoses. Potential health risks are usually determined at the silos during grading where disease severity is visually assessed (SAGL, 2011). This is not a true indicator of the presence of mycotoxin-producing fungi due to the ability of different organisms to induce similar kernel symptoms. The identification and quantitative measurement of mycotoxigenic ear rot fungi and their mycotoxins *in situ* generally requires accurate sampling, sample preparation and analytical techniques that are easy, rapid, selective and sensitive (Small, 2010). According to Miraglia

Stellenbosch University http://scholar.sun.ac.za

et al. (2005), the sampling technique is the most important step in risk assessment since only a representative sample will yield reliable and useful results.

Detection and quantification of mycotoxins: Many standard methods have been developed and employed for the screening and routine analysis of mycotoxins which generally require mycotoxin extraction from the matrix with an adequate solvent, a clean-up step to eliminate interference from the extract and finally, detection of the mycotoxin by suitable analytical instruments (Pascale and Visconti, 2008). Analytical methods, such as thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) are commonly used in practice but are incapable of dealing with multi-analytes in complicated food matrices (Sulyok *et al*., 2005). Modern technology such as liquid chromatography with tandem mass spectrometry (LC-MS/MS) offers higher selectivity as its simple dilute and shoot approach enables the detection of several masked and diverse mycotoxins without dedicated sample clean-up (Berthiller *et al*., 2005; Sulyok *et al.,* 2010). Other advanced technologies for mycotoxin analysis include lateral flow devices, infrared spectroscopy, fluorescence polarization immunoassays, biosensors, molecularly imprinted polymers and chip technology to name a few (Sulyok *et al.,* 2010). These techniques are, however, very expensive and require specialist expertise.

Enzyme linked immunosorbent assays (ELISA) for detection of mycotoxins have become very popular due to their affordability and easy application (Goryacheva *et al*., 2007). Commercially available ELISA kits are based on a competitive assay design that uses a monoclonal antibody to target a specific molecule. These tests can be rapid, highly specific and portable (Stanker *et al*., 2008). The disadvantage of these kits lies in the fact that they are made for single use only and become cost-prohibitive in bulk screening. Additionally, ELISA tests have a limited detection range due to the narrow-based sensitivity of the antibodies (Stanker *et al*., 2008).

Detection and quantification of mycotoxigenic fungi: Quantification of mycotoxigenic fungi in maize has involved plate counts and infection rates (percentage of seed that show fungal growth after surface disinfection) as an indication of fungal biomass (Schwadorf and Müller, 1989; Saxena *et al.*, 2001). These conventional methods are time-consuming and in the case of infection rate, not applicable to milled samples. Furthermore, microscopic identification of fungi *in planta* requires technical skill.

Ergosterol assays has proven to be useful in the early detection of fungi in grain crops such as rice (Gourama and Bullerman, 1994), wheat (Saxena *et al.*, 2001) and maize (De Castro *et al.*, 2002). Ergosterol is a core sterol component specific to fungal cell membranes that may be influenced by elevated oxygen levels and water activity. Ergosterol assays are therefore not trustworthy and cannot be used to measure specific fungal mass in a sample.

The limitations of conventional methods have prompted scientists to develop nucleic acid based methods for the rapid identification and quantification of mycotoxigenic fungi. Although conventional polymerase chain reaction (PCR) is accurate and sensitive, Barros *et al*. (2008), Mideros *et al.* (2009) and Boutigny *et al.* (2012) found real-time PCR (qRT-PCR) to be more sensitive and specific, and therefore an effective tool for the quantification of ear rot fungi in dry milled maize. Presently, qRT-PCR is frequently used in laboratory assays as it is reproducible, time-effective and more reliable than culturing and visual ratings. This data may also be used to determine the distribution of certain ear rot pathogens (Boutigny *et al.* (2012).

Current control strategies to reduce mycotoxin contamination

Technology is not yet available to completely eliminate mycotoxins from maize products or other food commodities (Munkvold and Desjardins, 1997). However, the implementation of sound control strategies during production followed by good storage and manufacturing practices could ensure reduced pathogen and mycotoxin concentrations in foods and feeds. Such practices include preharvest management, postharvest handling, storage, transportation and processing phases (Wagacha and Muthomi, 2008). According to the Codex Alimentarius Commission (CAC)(2003) the following agronomic and storage practices will help reduce fungal infection and consequent mycotoxin formation in cereal crops.

Agronomic practices and postharvest storage: In the field, the incidence and severity of mycotoxin accumulation in maize depends on the coincidence of host susceptibility, inoculum pressure and environmental conditions conducive to both the epidemiology of mycotoxin-producing pathogens and mycotoxin production (Munkvold, 2003a). Therefore, preharvest measures recommended to decrease unacceptable mycotoxin levels, include i) seed bed preparation and crop-rotation with non-host crops to reduce inoculum build-up (Flett, 1993; Munkvold, 2003a), ii) planting locally adapted maize cultivars with enhanced resistance to ear diseases and mycotoxin accumulation (Munkvold, 2003a) iii) enriching the soil with adequate nitrogen and other essential growth nutrients to maintain plant health and

Stellenbosch University http://scholar.sun.ac.za

reduce susceptibility (Blandino *et al*., 2008), iv) adhering to earlier planting dates in temperate areas, and recommended row widths and plant densities to reduce water stress (Mukanga *et al.,* 2011), v) irrigation during critical periods reduces mycotoxin levels in maize by avoiding elevated temperature and drought stress known to enhance fumonisin and aflatoxin production (Jones *et al*., 1980; Cole *et al*., 1985), vi) preharvest insecticides should be applied to control infection routes created by certain insects (Munkvold, 2003a) and vii) preharvest herbicide treatments are important to reduce plant stress as a result of competition with weeds for nutrients, space and water (Fandohan *et al*., 2003).

In South Africa, maize is left to dry naturally in the field to a moisture content of 15%. It should be harvested as soon as practically possible, since delayed harvest under conditions favourable for ear rot and/or mycotoxin development results in elevated mycotoxin levels (Chulze *et al*., 1996; Bush *et al*., 2004). In subsistence farming systems, broken and rotten grain should be sorted and separated from the bulk before storing it in clean bins equipped with ventilation systems to allow dry and cool conditions (Afolabi *et al*., 2006). In commercial farming systems, the grain is graded according to national grading standards which take broken and rotten kernels into account (SAGL, 2011). Under commercial storage conditions, grain must be kept at a suitable temperature (preferably 1 to 4°C) and moisture content (< 15%). Storage facilities, whether subsistence or commercial, must also protect the grain from the damage caused by unpredicted weather (rain, wind and hail) and bird, rodents and insects. Implementation of basic sanitation measures and appropriate control actions of pests will minimize infection of maize in storage (Wagacha and Muthomi, 2008; Mukanga *et al.,* 2011).

Developing countries rarely have the technical infrastructure and financial resources to optimise traditional management practises for mycotoxin control (Small, 2010). In light of this, research is aimed at integrating different management programmes for alternative control of maize ear rots and mycotoxin development.

Physical control: Physical treatments include heating, washing, polishing, mechanical sorting and separation, UV radiation and ultrasound treatment to name a few (Fandohan *et al*., 2005). In subsistence farming systems, washing and crushing in combination with de-hulling maize grain have been shown to effectively prevent further accumulation of fumonisins and aflatoxins after harvest (Siwela *et al*., 2005; Fandohan *et al*., 2008). Physical control strategies have met limited success because their application is labour and time-intensive and

most mycotoxins are chemically stable and thus not destroyed by long term storage and boiling (IARC, 1993; Howard *et al*., 1998).

Biological control: Biological methods have shown great promise in reducing mycotoxin levels in the field, because it is safe to the environment and most specific to the particular pest (Meissle *et al*., 2009). Interactions between different fungal species can significantly affect the conditions of disease development and subsequent mycotoxin production. With respect to *Fusarium* species, Marin *et al*. (1998) found *F. verticillioides* to competitively exclude *A. flavus* at a water activity above 0.96 under both field and laboratory conditions. In field studies, Reid *et al*., (1999) suggested *F. verticillioides* to dominate *F. graminearum* whereas *in vitro* studies contradicted this finding (Velluti *et al*. 2000; Velluti *et al*., 2001).

Many bacterial species have shown potential to limit fungal growth effectively under laboratory conditions. Bressen and Figuieredo (2005) reported the antagonistic abilities of *Streptomyces* to manage *S. maydis* in maize seeds and seedlings whereas *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains completely inhibit *A. flavus* and its aflatoxins (Palumbo *et al.,* 2006). The use of endophytic bacteria to reduce fumonisin-producing *Fusarium* species has also been demonstrated by Bacon *et al*. (2001). Although bacteria appear to be effective *in vitro*, their use in the field remains a challenge due to their inability to be effective under field conditions (Dorner, 2004).

The biological control strategy with the greatest potential to reduce fumonisin and aflatoxin contamination, involves the application of atoxigenic strains to maize fields (Desjardins and Plattner, 2000). Atoxigenic strains naturally outcompete and displace the toxigenic isolates in the same niche (Phillips *et al*., 2005; Pitt and Hocking, 2006; Dorner, 2008). Based on this technology, commercial biopesticides such as Alfaguard™ and Aflasafe™ have been developed and are readily available to reduce aflatoxin contamination in American and African maize, respectively (Abbas *et al*., 2006).

Chemical control: There are no effective fungicides registered in South Africa to control the major ear rot diseases of maize (Janse van Rensburg, 2012). A possible reason for this could be that the maize ear is difficult to reach when using a spray programme. Even if there were fungicides available, it might not be a sustainable solution in the long-term in most African countries since it is not economically feasible at small-scale farm level (Wagacha and Muthomi, 2008). While some approaches are directed at managing mycotoxin accumulation

with biopesticides together with the use of fungicides, others are focused on decontaminating maize after harvest.

To date, innovative attempts to detoxify mycotoxins in maize products by chemical means have met with limited success. Ammoniation and oxidising agents are well recognized internationally in the maize industry because they successfully detoxify aflatoxins in milled maize (Park *et al*., 1992). Their efficacy to reduce sufficient quantities of FB_1, however, is inconsistent (Moustafa *et al*., 2001). In contrast, non-enzymatic browning (Lu *et al*., 1997) and nixtamalization (Park *et al*., 1996) were found to reduce FB_1 concentrations in maize.

Another manner to reduce the uptake of mycotoxins from contaminated animal feed is with the use of mycotoxin binders. These adsorbent materials are intended to absorb mycotoxins in the gastrointestinal tract, thus preventing their uptake in the blood and distribution to target organs. Many absorbent materials such as activated carbon, hydrated sodium calcium aluminosilicates (HSCAS) and polymers have been studied (Mayura *et al*., 1998; Solfrizzo *et al*., 1998) and some have been promoted as animal feed additives in intensive pig and chicken farming systems (Pasha *et al*., 2007; Jacela *et al*., 2010). However, most of them appear to be highly specific. HSCAS, for example, are very effective in animals as they alleviate the toxic effects of aflatoxins, but have little or no beneficial effect against fumonisins, trichothecenes or zearalenone (Lindemann *et al*., 1993). Additionally, the uptake and utilization of essential nutrients from the feed may also be inhibited (Mayura *et al*., 1998; Solfrizzo *et al*., 1998). Clinical trials regarding the short-term safety evaluation of processed calcium montmorillonite clay (NovaSil) in reducing human exposure to aflatoxin are currently underway (Wang *et al*., 2005; Phillips *et al*., 2008).

Host-plant resistance: Breeding maize varieties to improve plant resistance is the most efficient strategy thus far in controlling the mycotoxin menace. This technology is convenient for farmers and producers as it provides affordable and environmentally sound control over ear diseases and mycotoxins in grain without the reliance on pesticides. Enhanced plant resistance has widely been used since the 20th century and sources of resistance to ear rot pathogens have been found internationally (Biffen, 1905; Dorrance *et al*., 1998; Keen, 2000; Clements *et al*., 2004; Presello *et al*., 2004; Kleinschmidt *et al*., 2005; Afolabi *et al*., 2007; Mesterházy *et al*., 2012). DER-resistance in breeding material (Wiser *et al*., 1960; Van Rensburg and Ferreira, 1997; Van Rensburg *et al*., 2003) and hybrids (Rheeder *et al*., 1990; Flett and McLaren, 1994) tested under local growing conditions have also been reported. In a recent investigation by Small *et al*. (2012) locally-adapted maize inbred lines with high

Stellenbosch University http://scholar.sun.ac.za

resistance to *F. verticillioides* and fumonisin contamination have been identified. These lines were selected by maize breeders for their good combinability and agronomic traits and are used extensively in the Agricultural Research Council Grain Crops Institute's breeding programmes (Potchefstroom, South Africa).

NATIVE DEFENCE IN PLANT-PATHOGEN INTERACTIONS

Green plants are an attractive source of nutrients for organisms that are not able to produce their own food. In order to discourage consumption by potentially harmful micro-organisms and animals, plants have developed defence strategies; some that are preformed and prevent invasion of the attackers (constitutive) and others that act in response to pathogen detection by means of active host-defence (inducible) (Niks *et al.*, 2011).

Constitutive defence

Plants have various defence mechanisms in the form of structural and biochemical barriers as well as other protein-based defences to combat potential pathogens and insects that target and damage their cells (Guest and Brown, 1997). These barriers represent the first line of defence against attackers.

Structural barriers: The physical shape and structure of plants play a key role in limiting pathogens to successfully attach, invade and cause infection in host tissue. Some plants shield their leaves with a thick, waxy cuticle layer while others have evolved thorns and spines to deter insect predators. Many of the defence mechanisms in maize plants are poorly understood. However, the type of cultivar and certain features of the maize ear and kernel morphology have been indicated in the resistance of major ear rot pathogens.

With respect to FER, maize traits that may act as barriers for penetration include closed ear tips (Fig. 1A) and tightness of the husk (Warfield and Davis, 1996); droopy ears (Munkvold, 2003a); stems that do not lodge easily (Alakonya *et al.*, 2008); delayed silk senescence after pollination (Headrick and Pataky, 1991); pericarp thickness (Sampietro *et al.*, 2009); the structure of the stylar canal (Duncan and Howard, 2010); the waxy and black layers of the kernel (Headrick and Pataky, 1991; Guo *et al.*, 1995) as well as decreased tendency of the kernel to split (Odvody *et al.*, 1990). Additionally, Brown *et al.* (1993) demonstrated the viability of the embryo to mediate resistance to AER and aflatoxin accumulation whereas Munkvold (2003a) suggested pendant ears (Fig. 1B) and ears with

Stellenbosch University http://scholar.sun.ac.za

loose husks to be more susceptible to GER development after heavy rainfall. Physical barriers documented for DER-resistance include husk protection (Koehler, 1953), pericarp thickness and droopy ears (Koehler, 1959).

Biochemical barriers: Biochemical barriers are molecules secreted in cells or onto the plant surface preventing tissue infection by pathogens. In specific cases these secretions may be essential for infection by certain pathogens. An example of such a situation would be the eggs of the potato cyst nematode, *Globodera rostochiensis,* which cannot hatch in the absence of specific exudates (Guest and Brown, 1997). The bitter-tasting properties of tannins and alkaloids repel herbivores, whereas phytotoxic mixtures of phenolic compounds, defensins and antioxidant enzymes contribute to the anti-microbial activity in resistant maize genotypes (Brown *et al*., 1999; Chen *et al.,* 2001; Bily *et al*., 2003). Assabgui *et al*. (1993) reported on the correlation of kernel (E)-ferulic acid content in maize with resistance to GER infection. Furthermore, a study conducted by Chen *et al*. (1999) revealed a 14 kDa trypsin inhibitor protein that arrested the production of aflatoxin by *A. flavus*. Toxic chemicals and defence-related proteins are not actively produced by the plant until pathogens are detected because of the high energy cost to the plant and nutrient requirements associated with their production and maintenance (Freeman and Beattie, 2008).

Induced defence

Biotrophic pathogens must be able to interact with host cells to manipulate them to their advantage. Once the pathogen overcomes the initial line of defence, the pathogen must then contend with a secondary line of defence that is actively deployed by the host to stop the pathogen's spread within the tissue.

Localized and systemic induced resistance: Induced resistance requires recognition of specific elicitor molecules, which can either be of foreign origin such as microbial proteins, or derived from the plant itself after it has been damaged (Van Wees *et al.*, 2008). Elicitors are perceived by the plant as danger signals, thereby activating an array of signalling networks and production of anti-microbial compounds. In the course of coevolution, certain pathogens have developed counter measures to suppress plant defence responses. Among these, secretions of effectors or virulence factors interfere with signal transduction pathways required to trigger plant immunity (Van Wees *et al.*, 2008). In turn, certain plants have gained the means to detect effectors by the production of pathogenesis-related (PR) proteins.

Stellenbosch University http://scholar.sun.ac.za

Effector recognition often leads to localized plant cell death, the hypersensitive response (HR), which ultimately results in long-lasting and broad-spectrum resistance throughout the host plant (Van Wees *et al.*, 2008). This systemic induced resistance (SAR) uses the plant's own resources as defence mechanisms in case of further pathogen attack. Plants are able to induce systemic resistance upon colonisation of roots by beneficial soil-borne micro-organisms such as rhizobacteria and mycorrhizal fungi (Van Loon *et al.*, 2006; Van Wees *et al.*, 2008).

Besides these biological elicitors, SAR can be triggered with natural chemical activators such as plant hormones. Salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and β-amino-butyric acid (BABA) are known to play central roles in the regulation of plant defence (Navarro *et al.*, 2006; Bari and Jones, 2009; Vlot *et al.*, 2009). Previous investigation by Cohen (2002) has shown that BABA effectively reduced *F. verticillioides* infection in maize seedlings whereas field and glasshouse studies by Small *et al.* (2012) contradicted this finding. Synthetic substances based on the structural analogues of these plant hormones are gaining favour in the agricultural community as they are effective against certain crop diseases and less detrimental to humans and wildlife than the use of fungicides or antibiotics (Walters *et al*., 2005; Freeman and Beattie, 2008; Walters *et al*., 2009).

IMPROVEMENT OF CROP PERFORMANCE VIA HOST RESISTANCE

Resistance to maize ear rots and mycotoxins can be enhanced either by conventional (plant breeding) and unconventional (molecular biology and biotechnology) breeding techniques. To understand the basis of resistance in plants, an intensive knowledge of the pathogen, the host and environmental factors that affect disease development and the interactions between these factors is required. The ultimate goal of plant improvement is to develop crop selections that have durable resistance to diseases and the resultant mycotoxins whilst having good recombining potential and desirable agronomic traits (Robertson *et al*., 2005). The development of new cultivars consists of several phases namely i) creating genetic diversity via plant breeding or biotechnology, ii) evaluating and identifying elite crop material and iii) seed certification, release and distribution of commercial cultivars (Acquaah, 2007).

Biotechnological approaches

The use of biotechnology provides alternative means of creating resistant germplasm when resistance is not readily available or significant in crop plants against biotic stresses. Genetic

Stellenbosch University http://scholar.sun.ac.za

modification of maize to reduce mycotoxins is aimed at either i) preventing fungal infection (Munkvold, 2003a), ii) interfering with mycotoxin formation (Woloshuk and Fakhoury, 2000), or to iii) neutralize mycotoxin effects (Kimura *et al*., 1998; Duvick, 2001; Kant *et al*., 2012). Transgenic maize possessing insecticidal crystal proteins from *Bacillus thuringiensis* has shown the greatest potential thus far in conferring resistance to certain mycotoxins. Reduction of stem borer feeding damage has led to reduced fumonisin, aflatoxin, DON and ZEA levels in most locations it has been studied (Munkvold *et al*., 1999; Betz *et al*., 2000; Bakan *et al*., 2002). Other transgenic modification approaches include induced mutations and gene silencing via irradiation with fast neutrons and RNAi technology, respectively. Contrary to the negative public opinion and intellectual property rights surrounding genetically modified cultivars, traditional breeding of plants is widely accepted. Therefore, only the use of classical plant breeding as a method to increase host resistance in maize towards the major ear rot diseases and mycotoxin contamination will be further emphasised in this review.

Plant breeding

Plant breeding has been practiced for thousands of years with the general aim of adding important traits to crops from which society can benefit. Classical plant breeding originated from identifying and selecting varieties with desirable properties followed by crosses to produce hybrid plants (Gepts, 2002). This procedure mainly relies on the i) generation of genetic diversity, ii) the selection and testing of segregating populations and the iii) development of cultivars. Today, selection still remains the primary strategy to establish genetic variation although the modern plant breeder also uses many *in vitro* techniques such as protoplast fusion, embryo rescue and mutagenesis (Baenziger and Otayk, 2007).

Breeding for increased quality, yield and tolerance against environmental stress is conceptually different from breeding for resistance to biotic stresses (pathogen attack). According to Cooper *et al*. (2009), “plant breeders consistently work within a field of trait genetic complexity where some traits show a relative simple underlying genetic basis” and others such as pathogen resistance, may have a complex design. For example, research conducted in California suggests that tight husk covering may reduce FER severity in areas where mechanical damage by insects is a problem, but it may also favour *F. verticillioides* infection because of slow ear drying (Munkvold, 2003a).

The success of breeding maize for reduced ear rot development and mycotoxin levels has mainly been attributed to subjecting plant material to high inoculum pressure and then

Stellenbosch University http://scholar.sun.ac.za

selecting physiological traits that suppress fungal activity directly or indirectly (Munkvold, 2003a).

Resistance breeding: the basic principles

Different responses are observed when a plant is exposed to high disease pressure. In a breeding context, these responses are categorised as "resistant" or "susceptible". While resistant genotypes contain the lowest disease levels, susceptible genotypes can either show moderate signs of infection (intermediate) or be very prone to disease development depending on what the genotype is compared to (Niks *et al*., 2011).

Sources of resistance: The first requirement of breeding for resistance against a certain pathogen is to find possible germplasm donors from which the resistance (R) genes can be introduced. Such sources may originate from exotic or indigenous cultivars, wild types of the same species or even closely related species or genera (Acquaah, 2007) and the technical ease of introducing resistance into a crop decreases, respectively. For example, unwanted traits may derive from interspecific hybrids and wild relatives whereas the use of related species and genera presents the complication of infertility (Acquaah, 2007). Inbred lines that are locally adapted and available remain the most useful sources of resistance in a breeding programme (Niks *et al*., 2011). Significant differences among maize inbred lines and hybrids for resistance to ear rot diseases has been thoroughly investigated in the past decade and genotypes with great potential to resist certain ear rot fungi have been identified, locally and internationally (Brown *et al*., 2001; Rossouw *et al*., 2002; Van Rensburg *et al*., 2003; Williams *et al*., 2003; Hefny *et al*., 2012; Kelly *et al*., 2012; Small *et al*., 2012; Tembo *et al*., 2012). Limited progress, however, has been made to successfully exploit the genetic resistance within the breeding material due to its polygenic nature and poor agronomic performance (Munkvold, 2003a).

Types of genetic resistance: Disease resistance in plants is divided into two distinct classes: i) specific resistance governed by one or a few genes, called qualitative resistance and ii) general resistance governed by many genes with additive effects on the phenotypic expression, called quantitative resistance (Van der Plank, 1968). In elite maize varieties, most disease resistance displays a quantitatively inherited pattern which is often influenced by environmental conditions, making proper selection difficult (Ali and Yan, 2012). Qualitative resistance is easier to work with in crop genomics as it has high heritability, but breeders

prefer to incorporate quantitative resistance in their breeding programme due to its broad specificity and high durability (Lindthout, 2002).

Gene effects and heritability: It is not crucial for plant breeders to understand exactly how resistance is inherited but it is useful to know whether the R genes are dominant or recessive and whether it is located on the chromosome or in the cytoplasm. With the onset of molecular markers technologies, estimating the number of R genes, their chromosomal location and effects involved in quantitative resistance, has become feasible (Lindthout, 2002).

A plant's R genes interact with other genes in their surroundings, with these interactions influencing the expression of resistance (Russell, 1978). Gene action can be partitioned into additive, dominant and epistatic effects. R genes are known to be additive in their effects when each of the R genes involved enhances the expression of resistance by equal increments. Alternatively, the expression of the R gene may depend on the presence of two or more "modifier genes" (epistatis) and in some cases one of these genes may mask the effect of the R gene. Epistatis can be compared with dominance, which is the interaction between pairs of alleles at the same locus. A single resistance mechanism may also be conditioned by duplicate genes, with the presence of any one of these genes affording non-additive effects (Russell, 1978). Studies have indicated that the resistance found in maize to important ear rot diseases are mostly conditioned by additive gene effects whereby resistance to AER and GER are also conditioned by epistasis and non-additive gene action, respectively (Mukanga *et al*., 2010). Some knowledge of gene interactions can be useful in any programme when breeding for resistance. For instance, combining several R genes with additive effect in a single variety is likely to increase expression of resistance (Russell, 1978).

Progress on enhancing quantitative resistance in field maize varieties against fungal pathogens has been slow due in part to the complexity of this trait. This has prompted scientists to explore quantitative trait loci (QTL) responsible for resistance to ear rot diseases and mycotoxins. It was concluded that these traits are distinct with relatively low heritability and high environmental influence (Pérez-Brito *et al.*, 2001; Ali *et al*., 2005; Brooks *et al*., 2005; Ding *et al*., 2008). In contrast, Robertson-Hoyt *et al*. (2006) found a strong genotypic correlation between FER and fumonisin contamination with both of these traits having moderate to high entry mean heritability. Similar conclusions were drawn by Martin *et al*. (2011; 2012) from the findings of GER and DON resistance in four maize populations. Generally, pedigree and marker-assisted selection is suggested to facilitate the process of resistance breeding for GER and reduced DON contamination due to the consistency of the

Stellenbosch University http://scholar.sun.ac.za

QTLs across the mapping populations. Additionally, these QTL studies yielded strong evidence of a fixed QTL for GER, FER and reduced mycotoxin concentrations. Robertson-Hoyt *et al*. (2007) and Henry *et al*. (2009) also indicated a significant relationship between FER and AER; and fumonisin and aflatoxin accumulation, respectively, in a set of hybrids. This implies that a common resistance mechanism may function for ear rot diseases, and breeding for enhanced host resistance against one form of ear rot may lead to reduced disease and mycotoxin levels of another and vice versa.

Evaluating and selecting for resistance: Maize can be tested in the field, the greenhouse or in the laboratory. In the latter, parameters such as light, humidity and temperature can be controlled. However, selecting for disease resistance in field crops under laboratory conditions are not representative for commercial cultivation and therefore the results are irrelevant. (Niks *et al*., 2011).

The main objective in plant selection is to easily discriminate between resistant and susceptible genotypes. It is thus important that the entire plant population be homogeneously exposed to the same level of disease pressure (Russell, 1978). Since the severity of natural infections is not consistent from one year to the next, maize breeders have learnt that genotypic differentiation can only be achieved by the use of appropriate artificial inoculation methods (Barug *et al.,* 2003). Many artificial inoculation methods and their variants have been developed to screen for ear rot pathogens and related mycotoxins with the oldest being Young's toothpick method (1943). Currently, literature suggests that the silk channel method is the most effective in screening for resistance against FER, GER and AER. This method involves the injection of a spore suspension down the silk channel of maize ears using syringes or cattle vaccinators (Bush *et al*., 2004; Afolabi *et al*., 2007). The timing and amount of inoculum usually varies for the respective ear rot diseases. For FER, a spore suspension of 1×10^6 conidia ml^{-1} at the R2 stage is used (Presello *et al*., 2008) while earlier inoculations (7 days after mid-silking) with 1×10^5 conidia ml^{-1} for GER (Löffler *et al.* 2010) and 1×10^8 conidia ml^{-1} for AER are generally recommended (LaPrade and Manwiller, 1977). It should be noted that another inoculation technique described by Flett and McLaren (1994) is preferred when screening for improved DER resistance in maize. Additionally, many authors stress the importance of testing resistant material at multiple locations and seasons to account for significant genotype x environmental effects (Afolabi *et al*., 2007; Eller *et al*., 2008; Small *et al*., 2012).

Stellenbosch University http://scholar.sun.ac.za

Selecting for improved phenotypic resistance in maize against ear rot diseases and mycotoxins should be effective due to the modest to high heritability of these traits (Robertson-Hoyt *et al*., 2007). Of these traits, mycotoxin content has proven to be more reliable than visual ratings of disease symptoms. Toxin analysis, however, can be time-consuming and cost-prohibitive whereas visual ratings can be relatively simple and extended to thousands of genotypes (Eller *et al*., 2008). Accordingly, the debate continues on whether or not to include toxin evaluation in a breeding programme.

CONCLUSION

Ear rot diseases and mycotoxin contamination in maize present daunting challenges for the maize producers in subsistence and commercial farming systems. Enhanced host resistance, as part of an integrated disease management strategy, could be a viable solution to the mycotoxin problem. The first requirement of any resistance breeding program is to obtain usable sources of resistance. Ideally, these sources would be in the form of locally adapted maize varieties such as inbred lines. Currently, there are no known maize cultivars available with immunity to any of the ear rot pathogens and their related mycotoxins in South Africa. It is, therefore, crucial to continue the search for durable resistance sources to ultimately introduce into high-yielding and agronomically superior female lines.

Breeding for improved ear rot and mycotoxin resistance has been slow due to its genetic complexity likely to be influenced by external factors. However, the many QTL mapping studies for disease resistance in maize have provided an abundance of DNA marker trait associations (Mesterházy *et al*., 2012). The use of molecular marker technologies to assist classical plant breeding could accelerate the development of crops resistant to fungal and mycotoxin contamination.

Stellenbosch University http://scholar.sun.ac.za

REFERENCES

Abbas, H.K., Zablotowicz, R.M., Bruns, H.A. and Abel, C.A. 2006. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. Biocontrol Science and Technology 16: 437-449.

Acquaah, G. 2007. Principles of plant genetics and breeding. Blackwell, Oxford, 569 pp.

Afolabi, C.G., Ojiambo, P.S., Ekpo, E.J.A., Menkir, A. and Bandyopadhyay, R. 2007. Evaluation of maize inbred lines for resistance to Fusarium ear rot and fumonisin accumulation in grain in tropical Africa. Plant Disease 91: 279-286.

Alakonya, A.E., Monda, E.O. and Ajanga, S. 2008. Effect of delayed harvesting on maize ear rot in Western Kenya. American-Eurasian Journal of Agriculture and Environmental Science 4: 372-380.

Ali, F. and Yan, J., 2012. Disease resistance in maize and the role of molecular breeding in defending against global threat. Journal of Integrative Plant Biology 54: 134-151.

Ali, M.L., Taylor, J.H., Jie, L., Sun, G., William, M., Kasha, K.J., Reid, L.M. and Pauls, K.P. 2005. Molecular mapping of QTLs for resistance to Gibberella ear rot, in corn, caused by Fusarium graminearum. Genome 48: 521-533.

Asiedu, J.J. 1989. Processing tropical crops: A technological approach. Macmillan Press, London, 266 pp.

Assabgui, R.A., Reid, L.M., Hamilton, R.I and Arnason, J.T. 1993. Correlation of kernel (E)-ferulic acid content of maize with resistance to *Fusarium graminearum*. Phytopathology 83: 949-953.

Bacon, C.W., Yates, I.E., Hinton, D.M. and Meredith, F. 2001. Biological control of Fusarium moniliforme in maize. Environmental Health Perspectives 109: 325-332.

Baenziger, P.S. and Otayk, S. 2007. Plant Breeding in the 21st Century. Proceedings of the 8th African Crop Science Society Meeting, El-Minia, Egypt, October 27-31.

Bänziger, M. and De Meyer, J. 2002. Collaborative maize variety development for stress-prone environments in Southern Africa. Pages 269-296 in: Farmers, scientists and plant breeding: Integrating knowledge and practice. D.A. Cleveland and D. Soleri, eds. CABI Publishing, Wallingford, United Kingdom.

Bakan, B., Melcion, D., Richard-Molard, D. and Cahagnier, B. 2002. Fungal growth and *Fusarium* mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. Journal of Agricultural and Food Chemistry 50: 728-31.

Balazs, E. and Schepers, J.S. 2007. The mycotoxin threat to world safety. International Journal of Food Microbiology 119: 1-2.

Bari, R. and Jones, J.D.G. 2009. Role of plant hormones in plant defence responses. Plant Molecular Biology 69: 473-488.

Barros, E., Crampton, M., Marais, G. and Lezar, S. 2008. A DNA-based method to quantify *Stenocarpella maydis* in maize. Maydica 53: 125-129.

Barug, D., van Egmond, H.P., Lopez-Garcia, R., van Osenbruggen, T. and Visconti, A. 2003. Meeting the Mycotoxins Menace. Wageningen Academic, The Netherlands. 320 pp.

Bensch, M.J., Van Staden, J. and Rijkenberg, J.H.F. 1992. Time and site of inoculation of maize for optimum infection of ears by *Stenocarpella maydis*. Journal of Phytopathology 136: 265-269.

Berthiller, F., Dall' Asta, C., Schuhmacher, R., Lemmens, M., Adam, G. and Krska, R. 2005. Masked mycotoxins: determination of deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. Journal of Agricultural and Food Chemistry 53: 3421-3425.

Betz, F.S., Hammond, B.G. and Fuchs, R.L. 2000. Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regulatory Toxicology and Pharmacology 32: 156-173.

Biffen, R.H .1905. Mendel's laws of inheritance and wheat breeding. Journal of Agricultural Science 1: 4-48.

Bily, A.C., Reid, L.M., Taylor, J.H., Johnston, D., Malouin, C., Burt, A.J., Bakan, B., Regnault-Roger, C., Pauls, K.P., Arnason, J.T. and Philogene, B.J.R. 2003. Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: Resistance factors to *Fusarium graminearum*. Phytopathology 93: 712-719.

Blandino, M., Reyneri, A. and Vanara, F. 2008. Influence of nitrogen fertilization on mycotoxin contamination of maize kernels. Crop Protection 27: 222-230.

Bottalico, A. 1998. Fusarium diseases of cereals: species complex and related mycotoxin profiles in Europe. Journal of Plant Pathology 80: 85-103.

Bressan, W. and Figueiredo, J.E.F. 2005. Biological control of *Stenocarpella maydis* in maize seed with antagonistic *Streptomyces* sp. isolates. Journal of Phytopathology 153: 623-626.

Brooks, T.D., Williams, W.P., Windham, G.L., Willcox, M.C. and Abbas. H.K. 2005. Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. Crop Science 45: 171-174.

Stellenbosch University http://scholar.sun.ac.za

Brown, R.L., Chen, Z.-Y., Cleveland, T.E. and Russin, J.S. 1999. Advances in the development of host resistance in corn to aflatoxin contamination by *Aspergillus flavus*. Phytopathology 89: 113-117.

Brown, R.L., Chen, Z.-Y., Menkir, A., Cleveland, T.E., Cardwell, K., Kling, J. and White, D.G. 2001. Resistance to aflatoxin accumulation in kernels of maize inbreds selected for ear rot resistance in West and Central Africa. Journal of Food Protection 64: 396-400.

Brown, R.L., Cotty, P.J., Cleveland, T.E. and Widstrom, N.W. 1993. Living maize embryo influences accumulation of aflatoxin in maize kernels. Journal of Food Protection 56: 967-971.

Boutigny, A.-L., Beukes, I., Small, I., Zühlke, S., Spiteller, M., Van Rensburg, B.J., Flett, B.C., and Viljoen, A. 2012. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. Plant Pathology 61: 522-531.

Boutigny, A.-L., Ward, T.J., Van Coller, G.J., Flett, B.C., Lamprecht, S.C., O'Donnell, K. and Viljoen, A. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48: 914-920.

Bush, B.J., Carson, M.L., Cubeta, M.A., Hagler, W.M. and Payne, G.A. 2004. Infection and fumonisin production by *Fusariumverticillioides* in developing maize kernels. Phytopathology 94: 88-93.

CAC. 2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and trichothecenes CAC/RCP 51-2003. Codex Alimentarius Commission. Online publication: www.codexalimentarius.net/web/standard_list.do?lang=en (29 September 2013).

Calvo, A.M., Hinze, L.L., Gardner, H.W. and Keller, N.P. 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Applied Environmental Microbiology 65: 3668-3673.

Chen, Z.-Y., Brown, R.L., Cleveland, T.E., Damann, K.E. and Russin, J.S. 2001. Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. Journal of Food Protection 64: 1785-1792.

Chen, Z.-Y., Brown, R.L., Russin, J.S., Lax, A.R. and Cleveland, T.E. 1999. A maize trypsin inhibitor with antifungal activity inhibits *Aspergillus flavus* α-amylase. Phytopathology 89: 902-907.

Stellenbosch University http://scholar.sun.ac.za

Chu, F.S. and Li, G.Y. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in mouldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. Applied and Environmental Microbiology 60: 847-852.

Chulze, S.N., Ramirez, M.L., Farnochi, M.C., Pascale, M., Visconti, A. and March, G. 1996. *Fusarium* and fumonisin occurrence in Argentina corn at different ear maturity stages. Journal of Agricultural and Food Chemistry 44: 2797-2801.

Clements, M.J., Maragos, C.A., Pataky, J.K. and White, D.G. 2004. Sources of resistance to fumonisin accumulation in grain and Fusarium ear and kernel rot of corn. Phytopathology 94: 251-260.

Cohen, Y.R. 2002. Beta-aminobutyric acid-induced resistance against plant pathogens. Plant Disease 86: 448-457.

Cole, R.J., Sanders, T.H., Hill, R.A. and Blankenship, P.D. 1985. Mean geocarposphere temperatures that induce preharvest aflatoxin contamination of peanuts under drought stress. Mycopathologia 91: 41-46.

Cooper, M., Van Eeuwijk, F.A., Hammer, G.L., Podlich, D.W. and Messina, C. 2009. Modeling QTL for complex traits: Detection and context for plant breeding. Current Opinion in Plant Biology 12: 231-240.

Cotton, T.K. and G.P. Munkvold. 1998. Survival and seed transmission of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium subglutinans* in maize stalk residue. Phytopathology 88: 550-555.

De Castro, M.F.P.M., Bragagnolo, N. and de Toledo Valentini, S.R. 2002. The relationship between fungi growth and aflatoxin production with ergosterol content of corn grains. Brazilian Journal of Microbiology 33: 22-26.

Desjardins, A.E. and Hohn, T.M. 1997. Mycotoxins in plant pathogenesis. Molecular Plant-Microbe Interactions 10: 147-152.

Desjardins, A.E. and Plattner, R.D. 2000. Fumonisin B (1)-nonproducing strains of Fusarium verticillioides cause maize (Zea mays) ear infection and ear rot. Journal of Agricultural and Food Chemistry 48: 5773-5780.

Ding, J., Wang, X., Chander, S., Yan, J. and Li, J. 2008. QTL mapping of resistance to Fusarium ear rot using a RIL population in maize. Molecular Breeding 22: 395-403.

Dorner, J.W. 2004. Biological control of aflatoxin contamination of crops. Journal of Toxicology: Toxin Reviews 23: 425-450.

Dorner, J.W. 2008.Management and prevention of mycotoxins in peanuts. Food Additives and Contaminants 25: 203-208.

Dorrance, A.E., Hinkelmann, K.H. and Warren, H.L. 1998. Diallel analysis of Diplodia ear rot resistance in maize. Plant Disease 82:699-703.

Dowd, P.F. 1998. Involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions. Pages 307-350 in: Mycotoxins in agriculture and food safety. K.K. Sinha and D. Bhatnagar, eds. Marcel Dekker Publishers, New York, USA.

Duncan, K.E. and Howard, R.J. 2010. Biology of maize kernel infection by *Fusarium verticillioides*. The American Phytopathological Society 23: 6-16.

Duvick, J. 2001. Prospects for reducing fumonisin contamination of maize through genetic modification. Environmental Health Perspectives 109: 337-342.

Eller, M., Holland, J. and Payne, G. 2008. Breeding for improved resistance to fumonisin contamination in maize. Toxin Review 27: 371-389.

Eraslan, G., Akdogan, M., YaSouth African, E., Sahindokuyucu, F., Essiz, D., and Altintas, L. 2005. The effects of aflatoxins on oxidative stress in broiler chickens. Turkish Journal of Veterinary and Animal Sciences 29: 701-707.

FAO. 1992. Maize in human nutrition. (FAO Food and Nutrition Series, No. 25) FAO, Rome, Italy.

Fandohan, P., Hell, K. and Marasas W.F.O. 2008. Food processing to reduce mycotoxins in Africa. Pages 302-309 in: Mycotoxins: Detection methods, management, public health and agricultural trade. J.F. Leslie, R. Bandyopadhyay and A. Visconti, eds. CABI Publishing, Wallingford, UK.

Fandohan, P., Gnonlonfin, B., Hell, K., Marasas, W.F.O. and Wingfield, M.J. 2005. Natural occurrence of Fusarium and subsequent fumonisin contamination in preharvest and stored maize in Benin, West Africa. International Journal of Food Microbiology 99: 173-183.

Fandohan, P., Hell, K., Marasas, W.F.O. and Wingfield, M.J. 2003. Infection of maize with *Fusarium* species and contamination with fumonisin in Africa. African Journal of Biotechnology 12: 570-579.

Fennel, D.I., Lillehoj, E.B. and Kwolek, W.F. 1975. *Aspergillus flavus* and other fungi associated with insect damaged field corn. Cereal chemistry 52: 314-321.

Flett, B.C. 1993. The potential of crop rotation in the control of maize ear and stalk rots caused by *Stenocarpella maydis.* Phytophylactica 25: 184.

Flett, B.C. and McLaren., N.W. 1994. Optimum disease potential for evaluating to *Stenocarpella maydis* ear rot in corn hybrids. Plant Disease 78: 597-589.

Flett, B.C., McLaren, N.W. and Wehner, F.C. 2001. Incidence of *Stenocarpella maydis* ear rot of corn under crop rotation systems. Plant Disease 85: 92-94.

Stellenbosch University http://scholar.sun.ac.za

Flett, B.C. and Van Rensburg, J.B.J. 1992. Effect of *Busseola fusca* on the incidence of maize ear rot caused by *Fusarium moniliforme* and *Stenocarpella maydis.* South African Journal of Plant and Soil 9: 177-179.

Flett, B.C., Wehner, F.C. and Smith, M.F. 1992. Relationship between maize stubble placement in soil and survival of *Stenocarpella maydis*. Journal of Phytopathology 134: 33-38.

Freeman, B.C. and Beattie, G.A. 2008. An Overview of Plant Defences against Pathogens and Herbivores. The Plant Health Instructor. DOI: 10. 1094/PHI-I-2008-0226-01.

Gelderblom, W.C.A., Abel, S., Smuts, C.M., Marnewick, J., Marasas, W.F.O., Lemmer, E.R. and Ramljak, D.R. 2001. Fumonisin – induced hepatocarcinogenesis: Mechanisms related to cancer initiation and promotion. Environmental Health Perspectives 109: 291-300.

Gelderblom, W.C.A., Cawood, M.E., Snyman, S.D. and Marasas, W.F.O. 1994. Fumonisin B1 dosimetry in relation to cancer initiation in rat-liver. Carcinogenesis 15: 209-214.

Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R. and Krick, N.P.J. 1988. Fumonisins – Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Applied and Environmental Microbiology 54: 1806-1811.

Gepts, P. 2002. A comparison between crop domestication, classical plant breeding, and genetic engineering. Crop Science 42: 1780-1790.

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

Goryacheva Y., De Saeger S., Eremin, S.A. and Van Peteghem, C. 2007. Immunochemical methods for rapid mycotoxin detection: evolution from single to multiple analyte screening: a review. Food Additives and Contaminants 24: 1169-1183.

Gourama, H. and Bullerman, L.B. 1994. Relationship between aflatoxin production and mold growth as measured by ergosterol and plate count. Lebensmittel-W. und Technologie 28: 185-189.

Guest, D. and Brown, J.F. 1997. Plant defences against pathogens. Pages 263-286 in: Plant pathogens and plant diseases. J.F. Brown and H.J. Ogle, eds. Rockvale Publications, Armidale, Australia.

Guo, B.Z., Russin, J.S., Cleveland, T.E., Brown, R.L. and Widstrom, N.W. 1995. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. Journal of Food Protection 58: 296-300.

Stellenbosch University http://scholar.sun.ac.za

Harris, L.J., Desjardins, A.E., Plattner, R.D., Nicholson, P., Butler, G., Young, J.C., Weston, G., Proctor, R.H. and Hohn., T. H. 1999. Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. Plant Disease 83: 954-960.

Harrison, L. R., Colvin, B.M., Green, J.T., Newman, L.E. and Cole, J.R. 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B1, a toxic metabolite of *Fusarium moniliforme*. Journal of Veterinary Diagnostic Investigation 2: 217-221.

Haumann, F. 1995. Eradicating mycotoxins in food and feeds. Inform 6: 248-256.

Headrick, J.M. and Pataky, J.K. 1991. Resistance to kernel infection by *Fusarium moniliforme* in sweet corn inbred lines and the effect of infection on emergence. Plant Disease 73: 887-892.

Hefny, M., Attaa, S. Bayoumi, T. Ammar, S. and El-Bramawy, M. 2012. Breeding maize for resistance to ear rot caused by Fusarium moniliforme. Pakistan Journal of Biological Sciences 15: 78-84.

Heisey, P.W. and Edmeades, G.O. 1999. Maize production in drought-stressed environments: Technical options and research resource allocation. Pages 1-36 in: Anonymous Part 1. of CIMMYT 1997/98 world maize facts and trends; maize production in drought-stressed environments: Technical options and research resource allocation. CIMMYT, Mexico.

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

Hidy, P.H.R.S., Baldwin, R.L., Greasham, C.L., Keith, T.J, and McMullen, J.R. 1977. Zearalenone and some derivatives: production and biological activities. Advances in Applied Microbiology 22: 59-82.

Howard, P.C., Churchwell, M.I., Cough, L.H., Marques, M.M. and Doerge, D.R. 1998. Formation of N-(carboxymethyl) fumonisin B1, following the reaction of fumonisin B1 with reducing sugars. Journal of Agricultural Food and Chemistry 46: 3546-3557.

IARC. 1993. IARC monographs on the evaluation of carcinogenic risks to humans Vol. 56. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. International Agency for Research on Cancer, Lyon, France. 599 pp.

IARC. 2002. IARC monographs on the evaluation of carcinogenic risks to humans Vol. 82. Some traditional herbal medicines, mycotoxins, naphthalene and styrene. International Agency for Research on Cancer, Lyon, France. 590 pp.

Stellenbosch University http://scholar.sun.ac.za

Iken, J.E. and Amusa, N.A. 2004. Maize research and production in Nigeria. African Journal of Biotechnology 3: 302-307.

Jacela, J.Y., DeRouchey, J.M. and Tokach, M.D. 2010. Feed additives for swine: Fact sheets – flavors and mold inhibitors, mycotoxin binders, and antioxidants. Journal of Swine Health and Production 18: 27-32.

Janse van Rensburg, B. 2012. Modelling the incidence of *Fusarium* and *Aspergillus* toxin producing species in maize and sorghum in South Africa. PhD thesis, University of the Free State, Bloemfontein, South Africa.

Jones, R.K., Duncan, H.E., Payne, G.A. and Leonard, K.J. 1980. Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. Plant Disease 64: 859-863.

Kant, P., Gulati, A., Harris, L., Gleddie, S., Singhand J. and Pauls, K.P. 2012. Transgenic corn plants with modified ribosomal protein L3 show decreased ear rot disease after inoculation with *Fusarium graminearum.* Australian Journal of Crop Science 6: 1598-1605.

Keen, N.T. 2000. A century of plant pathology: A retrospective view on understanding host-parasite interactions. Annual Review of Phytopathology 38: 31-41.

Kellerman, T.S., Marasas, W.F.O., Thiel, P.G., Gelderblom, W.C., Cawood, M. and Coetzer, J.A. 1990. Leukoencephalomalacia in 2 horses induced by oral dosing of fumonisin B1. The Onderstepoort Journal of Veterinary Research 57: 269-275.

Kellerman, T.S., Prozesky, L., Schultz, R.A., Rabie, C.J., Van Ark, H., Maartens, B.P. and Lubben, A. 1991. Perinatal mortality in lambs of ewes exposed to cultures of Diplodia maydis (= Stenocarpella maydis) during gestation. The Onderstepoort Journal of Veterinary Research 58: 297-308.

Kellerman, T.S., Rabie, C.J., Van der Westhuizen, G.C., Kriek, N.P. and Prozesky, L. 1985. Induction of diplodiosis, a neuromycotoxicosis, in domestic ruminants with cultures of indigenous and exotic isolates of Diplodia maydis. The Onderstepoort Journal of Veterinary Research 52: 35-42.

Kelly, R.Y., Williams, W.P., Mylroie J.E., Boykin, D.L., Harper, J.W., Windham, G.L., Ankala, A. and Shan, X. 2012. Identification of maize genes associated with host plant resistance or susceptibility to *Aspergillus flavus* infection and aflatoxin accumulation. PLoS One 7:e36892.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H. Yoneyama, K. and Yamaguchi, I. 1998. Trichothecene 3-Oacetyltransferase protects both the producing

organism and transformed yeast from related mycotoxins. Journal of Biological Chemistry 273: 1654-1661.

Kleinschmidt, C.E., Clements, M.J., Maragos, C.M., Pataky, J.K. and White, D.G. 2005.Evaluation of food-grade dent corn hybrids for severity of Fusarium ear rot and fumonisin accumulation in grain. Plant Disease 89: 291-297.

Kloppers, R. and Tweer, S. 2009. Diplodia leaf streak fact sheet. PANNAR Seed (Pty) Ltd, Greytown, South Africa. Online publication: www.plantwise.org/FullTextPDF /2011/20117800334.pdf. (20 December 2013).

Klich, M.A., Tiffany, L.H. and Knaphus, G. 1994. Ecology of the aspergilli of soils and litter. Pages 329-353 in: Aspergillus Biology and Industrial Applications. M.A. Klich and J.W. Bennett, eds. Butterworth-Heineman Publishers, Boston, USA.

Koehler, B. 1942. Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. Journal of Agricultural Research 64: 421-442.

Koehler, B. 1953. Ratings of some yellow corn inbreds for ear rot resistance. Plant Disease 37: 440-444.

Koehler, B. 1959. Corn ear rots in Illinois. Agricultural Experimental Station Bulletin 639.

Kriek, N.P.J., Kellerman, T.S. and Marasas, W.F.O. 1981. A comparative study of the toxicity of *Fusarium verticillioides* (= *Fusarium moniliforme*) to horses, primates, pigs, sheep, and rats. The Onderstepoort Journal of Veterinary Research 48: 129-133.

Kuiper-Goodman, T., Scott, P.M. and Watanabe, H. 1987. Risk assessment of the mycotoxin zearalenone. Regulatory Toxicology and Pharmacology 7: 253-306.

Lamprecht, S.C., Crous, P.W., Groenewald, J.Z., Tewoldemedhin, Y.T. and Marasas, W.F.O. 2011. *Diaporthaceae* associated with root and crown rot of maize. International Mycological Association 2: 13-24.

LaPrade, J.G and Manwiller, A. 1977. Relation of insect damage, vector, and hybrid reaction to aflatoxin B1 recovery from field corn. Phytopathology 67: 544-547.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahive, A., Misore, A., DeCock, K., Rubin, C. and the Kenya Aflatoxicosis Investigation Group. 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environmental Health Löffler Perspectives 113: 1763-1767.

Lillehoj, E.B. 1987. The aflatoxin in maize problem: the historical perspective. In: Aflatoxin in maize: a proceedings of the workshop. M.S. Zuber, E.B. Lillejoh, and B.L. Renfro, eds. CIMMYT, Mexico, D.F.

Stellenbosch University http://scholar.sun.ac.za

Lindemann, M.D, Blodgett, D.J., Kornegay, E.T and Schurig, G.G. 1993. Potential ameliorators of aflatoxicosis in weanling/growing in swine Journal of Animal Science 71: 171-178.

Lindthout, P. 2002. The perspectives of polygenic resistance in breeding for durable disease resistance. Euphytica 124: 217-226.

Löffler, M., Kessel, B., Ouzunova, M. and Miedaner. T. 2010. Population parameters for resistance to *Fusarium graminearum* and *Fusarium verticillioides* ear rot among large sets of early, mid-late and late maturing European maize (*Zeamays* L.) inbred lines. Theoretical and Applied Genetics 120: 1053-1062.

Logrieco, A., Mule`, G., Moretti, A. and Bottalico, A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. European Journal of Plant Pathology 108: 597-609.

Lu, Z., Dantzer, R.W., Hopmans, E.C., Prisk, V., Cunnick, J.E., Murphy, P.A. and Hendrich, S. 1997. Reaction with fructose detoxifies fumonisin B1 while stimulating liver associated natural killer cell activity in rats. Journal of Agricultural Food and Chemistry 45: 803-809.

Magan, N. and Olsen, M. 2004. Mycotoxins in food. Detection and control. Woodhead Publishing; Abington, MA, USA. 227 pp.

Manns, T.E. and Adams, J.F. 1923. Parasitic fungi internal of seed corn. Journal of Agricultural Research 23: 495-524.

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: A historical perspective. Environmental Health Perspectives 109: 239-243.

Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martinez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A. Voss, K.A. Wang, E. and Merrill Jr., A.H. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. Journal of Nutrition 134: 711-716.

Marin, S., Sanchis, V., Ramos, A.J., Vinas, I. and Magan, N. 1998. Environmental factors, *in vitro* interactions, and niche overlap between *Fusarium moniliforme*, *F. proliferatum*, and *F. graminearum*, *Aspergillus* and *Penicillium* species from maize grain. Mycological Research 102: 831-837.

Stellenbosch University http://scholar.sun.ac.za

Martin, M., Miedaner, T., Dhillon, B.S., Ufermann, U., Kessel, B., Ouzunova, M., Schipprack, W. and Melchinger, A.E. 2011. Colocolization of QTL for Gibberella ear rot resistance and low mycotoxin contamination in early European maize. Crop Science 51: 1935-1945.

Martin, M., Miedaner, T., Schwegler, D.D., Kessel, B., Ouzunova, M., Dhillon, B.S., Schipprack, W., Utz, H.F. and Melchinger, A.E. 2012. Comparative quantitative trait loci mapping for Gibberella ear rot resistance and reduced deoxynivalenol contamination across connected maize populations. Crop Science 52: 32-43.

Mayura, K., Abdel-Wahhab, M.A., McKenzie, K.S., Sarr, A.B., Edwards, J.F., Naguib, K., and Phillips, T.D. 1998. Prevention of maternal and developmental toxicity in rats via dietary inclusion of aflatoxin sorbents: hidden risks. Toxicology Science 41: 175-182.

Meissle, M., Mouron, P., Musa, T., Bigler, F., Pons, X., Vasileiadis, V. P. Otto, S., Antichi, D., Kiss, J., Pa´ linka´ s, Z. Dorner, Z., van der Weide, R., Groten, J., Czembor, E., Adamczyk, J., Thibord, J.-B., Melander, B., Cordsen Nielsen, G., Poulsen, R. T., Zimmermann, O., Verschwele, A. and Oldenburg, E. 2009. Pests, pesticide use and alternative options in European maize production: current status and future prospects. Journal of Applied Entomology 134: 357-375.

Mesterházy, A., Lemmens, M. and Reid, L.M. 2012. Breeding for resistance to ear rots caused by *Fusarium* spp. in maize – a review. Plant Breeding 131: 1-19.

Mideros, S.X., Windham, G.L., Williams W.P. and Nelson, R.J. 2009. Aspergillus flavus biomass in maize estimated by quantitative Real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. Plant Disease 93: 1163-1170.

Miraglia, M., De Santis, B., Minardi, V., Debegnach, F. and Brera, C. 2005. The role of sampling in mycotoxin contamination: a holistic view. Food additives and Contaminants 22: 31-36.

Missmer, S.A., Suarez, L., Felkner, M., Wang, E., Merrill, A.H., Rothman, K.J. and Hendricks, K.A. 2006. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. Environmental Health Perspectives 114: 237-241.

Moustafa, A.M., Losso, J.N., King, J.M. and Park, D.L. 2001. Ammoniation of aflatoxin contaminated raw peanuts at ambient temperature and pressure. Proceedings of the institute of food technologists annual meeting, New Orleans, Louisiana, June 23-27.

Mukanga, M., Derera, J. and Tongoona, P. 2010. Gene action and reciprocal effects for ear rot resistance in crosses derived from five tropical maize populations. Euphytica 174: 293-301.

Stellenbosch University http://scholar.sun.ac.za

Mukanga, M., Derera, J., Tongoona, P. and Laing, M.D. 2011. Farmer's perceptions and management of maize ear rots and their implications for breeding for resistance. African Journal of Agricultural Research 6: 4544-4554.

Munkvold, G.P. 2003a. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology 41: 99-116.

Munkvold, G.P. 2003b. Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. European Journal of Plant Pathology 109: 705-713.

Munkvold, G.P. and Carlton, W.M. 1997. Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds. Plant Disease 81: 211-216.

Munkvold, G.P. and Desjardins, A.E. 1997. Fumonisins in maize: Can we reduce their occurrence? Plant Disease 81: 556-565.

Munkvold, G.P., Hellmich, R.L. and Rice, L.G. 1999. Comparison of fumonisin concentrations in kernels of transgenic Bt maize hybrids and nontransgenic hybrids. Plant Disease 83: 130-138.

Munkvold, G.P., McGee, D.C. and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. Phytopathology 87: 209-217.

Murphy, P.A., Hendrich, S., Landgren, C. and Bryant, C.M. 2006. Food mycotoxins: An update. Journal of Food Science 71: 51-65.

NGFA. National Grain and Feed Association. 2011. FDA mycotoxin regulatory guidance: A guide for grain elevators, feed manufacturers, grain processors and exporters. 5pp.

National Department of Agriculture – Directorate of Agricultural Statistics. 2009. (Abstr.) Pretoria, South Africa, 107 pp.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O. and Jones, J.D.G. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436-439.

Ncube, E. 2008. Mycotoxin levels in subsistence farming systems in South Africa. MSc thesis, University of Stellenbosch, Stellenbosch, South Africa.

Nelson, P.E., Desjardins, A.E. and Plattner, R.D. 1993. Fumonisins, mycotoxins produced by *Fusarium* species – Biology, chemistry, and significance. Annual Review of Phytopathology 31: 233-252.

Niks R.E., Parlevliet J.E., Lindhout P. and Bai Y. 2011. Breeding crops with resistance to diseases and pests. Wageningen Academic Publishers, Wageningen, The Netherlands. 430 pp.

Stellenbosch University http://scholar.sun.ac.za

Nowell, D.C. 1997. Studies on ear rot and grey leaf spot of maize in South Africa. PhD thesis. University of Natal, Pietermaritzburg, South Africa.

Odriozola, E., Odeón, Canton, G., Clemente, G. and Escande, A. 2005. *Diplodia maydis*: a cause of death of cattle in Argentina. New Zealand Veterinary Journal 53: 160-161.

Odvody, G.N., Remmers, J.C. and Spencer, N.M. 1990.Association of kernel splitting with kernel and ear rots of corn in a common hybrid grown in the coastal bend of Texas. Phytopathology 80: 1045.

Oren, L., Ezrati, S., Cohen, D. and Sharon, A. 2003. Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. Applied and Environmental Microbiology 69: 1695-1701.

Osweiler, G. 2005. Aflatoxins and animal health. Iowa State University, Ames, Iowa. 210 pp.

Palumbo, J.D., Baker, J.L. and Mahoney, N.E. 2006. Isolation of bacterial antagonists of *Aspergillus flavus* from almonds. Microbial Ecology 52: 45-52.

Park, D. L., Lopez-Garcia, R., Trujillo-Preciado, S. and Price, R.L. 1996. Reduction of risks associated with fumonisin contamination in corn. Advances in Experimental Medicine and Biology 392: 335-44.

Park, D.L., Rua, S.M., Jr., Mirocha, C.J., Abd-Alla, E.S. and Weng, C. Y. 1992. Mutagenic potential of fumonisin contaminated corn following ammonia decontamination procedure. Mycopathologia 117: 105-108.

Pascale, M. and Visconti, A. 2008. Overview of detection methods for mycotoxins. Pages 171-183 in: Mycotoxins. Detection methods, management, public health and agricultural trade. J.F. Leslie, R. Bandyopadhyay and A. Visconti, eds. Cromwell Press, Trowbridge, UK.

Pasha, T.N., Farooq, M.U., Khattak, F.M., Jabbar, M.A. and Khan, A.D., 2007. Effectiveness of sodium bentonite and 2 commercial products as aflatoxin absorbents in diets for broiler chickens. Animal Feed Science and Technology 132: 103-110.

Payne, G.A. 1999. Ear and kernel rots. Pages 44-45 in: Compendium of corn diseases. D.G., White, ed. Third Edition. APS Press, The American Phytopathological Society, Minnesota, USA.

Pereira, P., Nesci, A., Castillo, C. and Etcheverry, M. 2011. Field studies on the relationship between Fusarium verticillioides and maize (Zeamays L.): Effect of biocontrol agents on fungal infection and toxin content of grains at harvest. International Journal of Agronomy 127: 223-238.

Pèrez-Brito, D., Jeffers, D., Gonzalez-de-Leon, D., Khairallah, M., Cortes-Cruz, M., Velazquez-Cardelas, G., Azpiroz-Rivero, S. and Srinivasan, G. 2001. QTL mapping of *Fusarium moniliforme* ear rot resistance in highland maize, Mexico. Agrocencia 35: 181-196.

Pestka, J.J. 2007. Deoxynivelanol: toxicity, mechanisms and animal health risks. Animal Feed Science and Technology 137: 283-298.

Phillips, T.D., Afriyie-Gyawu, E., Wang, J.S., Ofori-Adjei, D., Ankrah, N., Jolly, P. and Williams, J.H. 2005. Sustainable enterosorbent strategies for the protection of African populations from aflatoxins. Proceedings of the conference on reducing impact of mycotoxins in tropical agriculture with emphasis on health and trade in Africa, Accra, Ghana, September 13-16.

Phillips, T.D., Afriyie-Gyawu, E., Williams, J., Huebner, H., Ankrah, N.-A., Ofori-Adjei, D., Jolly, P., Johnson, N., Taylor, J., Marroquin-Cardona, A., Xu, L., Tang, L. and Wang, J.-S., 2008. Reducing human exposure to aflatoxin through the use of clay: A review. Food Additives and Contaminants 25: 134-145.

Picot, A., Barreau, C., Pinson-Gadais, L., Caron, D., Lannou, C. and Richard-Forget, F., 2010. Factors of the *Fusarium verticillioides*-maize environment modulating fumonisin production. Critical Reviews in Microbiology 36: 221-231.

Pingali, P.L. and Pandey, S. 2001. Part 1. Meeting world maize needs: Technological opportunities and priorities for the public sector. Pages 1-24 in: CIMMYT 1999-2000 World maize facts and trends. P.L. Pingali, ed. Meeting world maize needs: Technological opportunities and priorities for the public sector. CIMMYT, Mexico.

Pitt, J.I. and Hocking, A.D. 2006. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. Mycopathologia 162: 233-243.

Pitt, J.I., Hocking, A.D., Bhudhasamai, K., Miscamble, B.F., Wheeler, K.A. and Tanboon-Ek, P. 1993. The normal mycoflora of commodities from Thailand: Nuts and oilseeds. International Journal of Food Microbiology 20: 211-226.

Pitt, J.I., Hocking, A.D., Bhudhasamai, K., Miscamble, B.F., Wheeler, K.A. and Tanboon-Ek, P. 1994. The normal mycoflora of commodities from Thailand. 2. Beans, rice, small grains and other commodities. International Journal of Food Microbiology23: 35-53.

Presello, D. A., Botta, G., Iglesias, J. and Eyherabide, G. H. 2008: Effect of disease severity on yield and grain fumonisin concentration of maize hybrids inoculated with *Fusarium verticillioides*. Crop Protection 27: 572-576.

Stellenbosch University http://scholar.sun.ac.za

Presello, D. A., Reid, L.M. and Mather, D.E. 2004. Resistance of Argentine maize germplasm to Gibberella and Fusarium ear rot. Maydica 49: 73-81.

Rabie, C.J., Du Preez, J.J. and Hayes, J.P. 1987. Toxicity of *Stenocarpella maydis* to broilers, ducklings and laying chicken hens. Poultry Science 66: 1123-1128.

Reid, L.M., Hamilton, R.I. and Mather, D.E. 1996. Screening maize for resistance to Gibberella ear rot. Agriculture and Agri-Food Canada; Technical Bulletin 196-5E.

Reid, L.M., Nicol, R.W., Ouellet, T., Savard, M., Miller, J.D., Young, J.C., Stewart, D.W. and Schaafsma, A.W. 1999. Interaction of *Fusarium graminearum* and *F-moniliforme* in maize ears: Disease progress, fungal biomass, and mycotoxin accumulation. Phytopathology 89: 1028-1037.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S. and Van Schalkwyk, D.J. 1990. Reaction of South African maize cultivars to ear inoculation with *Fusarium moniliforme*, *F. graminearum* and *Diplodia maydis*. Phytophylactica 22: 213-218.

Rheeder, J.P., Marasas, W.F.O. and Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* species. Applied Environmental Microbiology 68: 2101-2105.

Rheeder, J.P., Shepard, G.S, Vismer, H.F. and Gelderblom, W.C.A. 2009. Guidelines on mycotoxin control in South African foodstuffs: from the application of the Hazard analysis and crotical control point (HACCP) system to new national mycotoxin regulations. MRC policy brief. Online publication: www.mrc.ac/policybriefs/mycotoxinguidelines. (20 December 2013).

Robertson, L.A., Payne, G.A. and Holland, J.B. 2005. Marker-assisted breeding for host resistance to mycotoxin contamination. Pages 423-435 in: Aflatoxin and Food Safety. H.K. Abbas, ed. Marcel Dekker, New York, USA.

Robertson-Hoyt, L.A., Betran, J., Payne, G.A., White, D.G., Isakeit, T., Maragos, C.M., Molnar, T.L. and Holland, J.B. 2007. Relationships among resistances to Fusarium and Aspergillus ear rots and contamination by fumonisin and aflatoxin in maize. Phytopathology 97: 311-317.

Robertson-Hoyt, L.A., Jines, M.P., Balint-Kurti, P.J., Kleinschmidt, C.E. and White, D.G. 2006. QTL mapping for Fusarium ear rot and fumonisin contamination resistance in 2 maize populations. Crop Science 46: 1734-1743.

Rossouw, J. D., van Rensburg, J. B. J., and van Deventer, C. S. 2002. Breeding for resistance to ear rot of maize caused by Stenocarpella maydis (Berk.) Sutton. Evaluation of selection criteria. South African Journal of Plant Soil 19: 182-187.

Stellenbosch University http://scholar.sun.ac.za

Rotter, B.A., Prelusky, D.B. and Pestka, J.J. 1996.Toxicology of deoxynivalenol (vomitoxin). Journal of Toxicology and Environmental Health 48: 1-34.

Russell, G.E. 1978. Plant breeding for pest and disease resistance. Butterworth and Co, Durban, South Africa. 485 pp.

SAGL. South African Crain Laboratory. South African maize crops: Quality report 2010/2011 season. 2011. Online publication: www.sagl.co.za/Portals/0/Full%20Maize%20report%202.pdf (21 December 2013).

Sampietro, D.A., Vattuone, M.A., Presello, D.A., Fauguel, C.M., and Catalan, C.A.N. 2009. The pericarp and its surface wax layer in maize kernels as resistance factors to fumonisin accumulation by *Fusarium verticillioides*. Crop Protection 28: 196-200.

Saxena, J., Munimbazi, C. and Bullerman, L.B. 2001. Relationship of mould count, ergosterol and ochratoxin A production. International Journal of Food Microbiology 71: 29-34.

Schoeman, A. and Flett, B.C. 2012. Diplodia and stalk rot of maize in the spotlight. SA Grain. Online publication: www.grainsa.co.za/diplodia-ear-and-stalk-rot-of-maize-in-the-spotlight (20 December 2013).

Schwadorf, K. and Müller, H.M. 1989. Determination of ergosterol in cereals, mixed feed components, and mixed feeds by liquid chromatography. Association of Official Analytical Chemists 72: 457-462.

Shephard, G.S. 2005. Aflatoxin and food safety: recent African perspectives. Pages 15-17 in: Aflatoxin and food safety. H.K. Abbas, ed. CRC Press, Taylor and Francis group, Boca Raton, USA.

Shephard, G.S. 2008. Impact of mycotoxins on human health in developing countries. Food Additives and Contaminants 25: 146-151.

Shephard, G.S., Thiel, P.G., Stockenstrom, S. and Sydenham, E.W. 1996. Worldwide survey of fumonisin contamination of corn and corn-based products. Journal of AOAC International 79: 671-687.

Sikora, R.A., Niere, B. and Kimenju, J. 2003. Endophytic microbial biodiversity and plant nematode management in African agriculture. Pages 179-191 in: Biological control in IPM systems in Africa. P. Neuenschwander, C. Borgemeister and J. Langewald, eds. CAB International, Wallingford, England.

Siwela, A.H., Siwela, M., Matindi, G., Dube, S. and Nziramasanga, N. 2005. Decontamination of aflatoxin-contaminated maize by dehulling. Journal of the Science of Food and Agriculture: 85: 2535-2538.

Stellenbosch University http://scholar.sun.ac.za

Small, I.M. 2010. Resistance in maize to *Fusarium verticillioides* and fumonisin. MSc thesis, University of Stellenbosch, Stellenbosch, South Africa.

Small, I.M., Flett, B.C., Marasas, W.F.O., McLeod, A., Stander, M.A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticillioides* and fumonisin accumulation in South Africa. Plant Disease 96: 881-888.

Snyman, L.D., Kellerman, T.S., Veggelaar, R., Flett, B.C., Basson, K.M. and Schultz, R.A. 2011. Diplonine, a neurotoxin isolated from cultures of the fungus *Stenocarpella maydis* (Berk.) Sacc. that induces Diplodiosis. Journal of Agriculture and Food Chemistry 59: 9039-9044.

Solfrizzo, M., Avantaggiato, G., Carratù, M.R., Galvano, F., Pietri, A. and Visconti, A. 1998. The use of biomarkers to assess the in vivo effect of activated carbon on fumonisins fed through diets contaminated with *Fusarium moniliforme*. Revue de Medicine Veterinaire 149: 667.

Stack, M.E. 1998. Analysis of fumonisin B1 and its hydrolysis product in tortillas. Journal of AOAC International 81: 737-740.

Stanker, L. H., Merrill, P. Scotcher, M.C. and Cheng, L.W. 2008. Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA. Journal of Immunological Methods 336: 1-8.

Sulyok, M., Berthiller, F., Schumacher, R. and Krska, R. 2005. Development of a HPLC-MS/MS based method for screening the most relevant types of mycotoxins in a single chromatographic run. The World Mycotoxin Form – the third conference, Noordwijk aan Zee, the Netherlands, November 10-11. Books of Abstracts, 115 pp.

Sulyok, M., Krska, R. and Schumacher, R. 2010. Application of an LC-MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. Food Chemistry 119: 408-416.

Summerell, B.A., Leslie, J.F., Backhouse, D., Bryden, W.L. and Burgess, L.W. 2001. Fusarium: Paul E. Nelson Memorial Symposium. APS Press, St. Paul, Minnesota, 392 p.

Sutton, B.C. 1980. The Coelomycetes: Fungi Imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute. Kew, UK. 696 pp.

Tembo, L., Asea, G., Gibson, P.T. and Okori, P. 2012. Resistance breeding strategy for *Stenocarpella maydis* and *Fusarium graminearum* cob rots in tropical maize. Plant Breeding 132: 83-89.

Utian, W.H. 1973.Comparative trial of P1496, a new non-steroidal oestrogen analogue. British Medical Journal 1: 579-581.

Van der Plank, J.E. 1968. Disease resistance in plants. Academic Press, New York, USA. 206 pp.

Van Egmond, H.P., Schothorst, R.C. and Jonker, M.A. 2007. Regulations relating to mycotoxins in food. Analytical and Bioanalytical Chemistry 389: 147-157.

Van Loon, L.C., Rep, M. and Pieterse, C.M.J. 2006. Significance of inducible defence-related proteins in infected plants. Annual Review of Phytopathology 44: 135-162.

Van Rensburg, J.B.J. and Ferreira, M.J. 1997. Resistance of elite maize inbred lines to isolates of *Stenocarpella maydis* (Berk.) Sutton. South African Journal of Plant and Soil 14: 89-92.

Van Rensburg, J.B.J. and Flett, B.C. 2010. A review of research achievements on maize stem borer, *Busseola fusca* (Fuller) and Diplodia ear rot caused by *Stenocarpella maydis* (Berk. Sutton). South African Journal of Plant and Soil 27: 74-80.

Van Rensburg, J.B.J., Rossouw, J.D. and Deventer, C.S.V. 2003. New generation maize inbred lines resistant to Diplodia ear rot, caused by Stenocarpella maydis (Berk) Sutton. South African Journal of Plant and Soil 20: 127.

Van Wees, S.C.M., Van der Ent, S. and Pieterse, C.M.J. 2008. Plant immune responses triggered by beneficial microbes. Current Opinion in Plant Biology 11: 443-448.

Velluti, A., Marin, S., Bettucci, L., Ramos, A.J. and Sanchis, V. 2000. The effect of fungal competitionof maize grain by *Fusarium moniliforme*, *F.proliferatum* and *F. graminearum* and on fumonisin B1 and zearalenone formation. International Journal of Food Microbiology 59: 59-66.

Velluti, A., Marin, S., Gonzalez, R., Ramos, A.J. and Sanchis, A.J. 2001. Fumonisin B1, zearalenone and deoxynivalenol production by *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum* in mixed cultures on irradiated maize grains. Journal of the Science of Food and Agriculture 81: 88-94.

Viger, B., Reid, L.M., Dwyer, L.M., Stewart, D.W., Sinha, R.C., Arnason, J.T. and Butler, G. 2001. Maize resistance to gibberella ear rot: symptoms, deoxynivalenol, and yield. Canadian Journal of Plant Pathology 23: 99-105.

Vlot, A.C., Dempsey, D.A. and Klessig, D.F. 2009. Salicylic acid, a multifaceted hormone to combat disease. Annual Review of Phytopathology 47: 177-206.

Stellenbosch University http://scholar.sun.ac.za

WHO. 2006. Mycotoxins in African foods: Implications to food safety and health. AFRO Food Safety Newsletter. World Health Organization Food Safety (FOS). Online publication: http://www.afro.who.int/des/ (17 October 2013).

Waalwijk, C., Koch, S.H., Ncube, E., Allwood, J., Flett, B.C., de Vries, I. and Kema, G. H. J. 2008. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. World Mycotoxin Journal 1: 39-47.

Wagacha, J.M. and Muthomi, J.W. 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. International Journal of Food Microbiology 124: 1-12.

Walters, D., Walsh, D., Newton, A. and Lyon, G. 2005. Induced resistance for plant disease control: Maximizing the efficacy of resistance elicitors. Phytopathology 95: 1368-1373.

Walters, D.R. and Fountaine, J.M. 2009. Practical application of induced resistance to plant diseases: An appraisal of effectiveness under field conditions. Journal of Agricultural Science 147: 523-535.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T. and Merrill, A.H. 1991. Inhibition of sphingolipid biosynthesis by fumonisins: implications for diseases associated with *Fusarium moniliforme*. Journal of Biological Chemistry 266: 14486-14490.

Wang, J.S., Luo, H., Billam, M., Wang, Z., Guan, H., Tang, L., Goldston, T., Afriyie-Gyawu, E., Lovett, C., Griswold, J., Brattin, B., Taylor, R.J., Huebner, H.J. and Phillips, T.D. 2005. Short-term safety evaluation of processed calcium montmorillonite clay (NovaSil) in humans. Food Additives and Contaminants 22: 270-279.

Warfield, C.Y. and Davis, R.M. 1996. Importance of the husk covering on the susceptibility of corn hybrids to Fusarium ear rot. Plant Disease 80: 208-210.

White, D. 1999. Fusarium kernel or ear rot. Compendium of Corn Diseases. American Phytopathological Society Press, St. Paul, USA. 21 pp.

Williams, W.P., Ozkan, S., Ankala, A. and Windham, G.L. 2011. Ear rot, aflatoxin accumulation, and fungal biomass in maize after inoculation with Aspergillus flavus. Field Crops Research 120: 196-200.

Williams, W.P., Windham, G.L. and Buckley, P.M. 2003. Enhancing maize germplasm with resistance to aflatoxin contamination. Toxin Reviews 22: 175-193.

Wiser, W.J., Kramer, H.H. and Ullstrup, A.J. 1960. Evaluating inbred lines of corn for resistance to Diplodia ear rot. Agronomy Journal 52:624-626.

Stellenbosch University http://scholar.sun.ac.za

Woloshuk, G.L. and Fakhoury, A. 2000. Novel inhibitor for controlling Aspergillus flavus growth and aflatoxin production on corn. Proceedings of the 2000 Aflatoxin/Fumonisin Workshop. U.S. Department of Agriculture Res. Serv., Washington, DC., 103.

Wu, F. 2006. Mycotoxin reduction in Bt corn: Potential economic, health, and regulatory impacts. Transgenic Research 15: 277-289.

Wu, F. 2007. Measuring the economic impacts of *Fusarium* toxins in animal feeds. Animal Feed Science and Technology 37: 363-374.

Young, H. C.1943. The toothpick method of inoculating corn for ear and stalk rots. Phytopathology 33: 16.

Yu, J., Cleveland, T.E., Nierman, W.C. and Bennett, J.W. 2005. *Aspergillus flavus* genomics: Gateway to human and animal health, food safety, and crop resistance to diseases. Revista Iberoamericana de Micología 22: 194-202.

Table 1. Regulatory limits for mycotoxins in feed and food as issued by the United States Food and Drug Administration (Adapted from NGFA, 2011).

	Current mycotoxin limits			
Food and Feed	**Aflatoxin B_1 (ppb)**	**Deoxynivalenol (ppm)**	**Zearalenone (ppm)**	**Fumonisin B_1+B_2+B_3 (ppm)**
Human foods	20	1	0.5	2 - 4
Infant formulae	0.5	*	*	*
Animal feeds	20 - 300	5 - 10	0.5	5 - 100

ppb, parts per billion; ppm, parts per million

* None existent

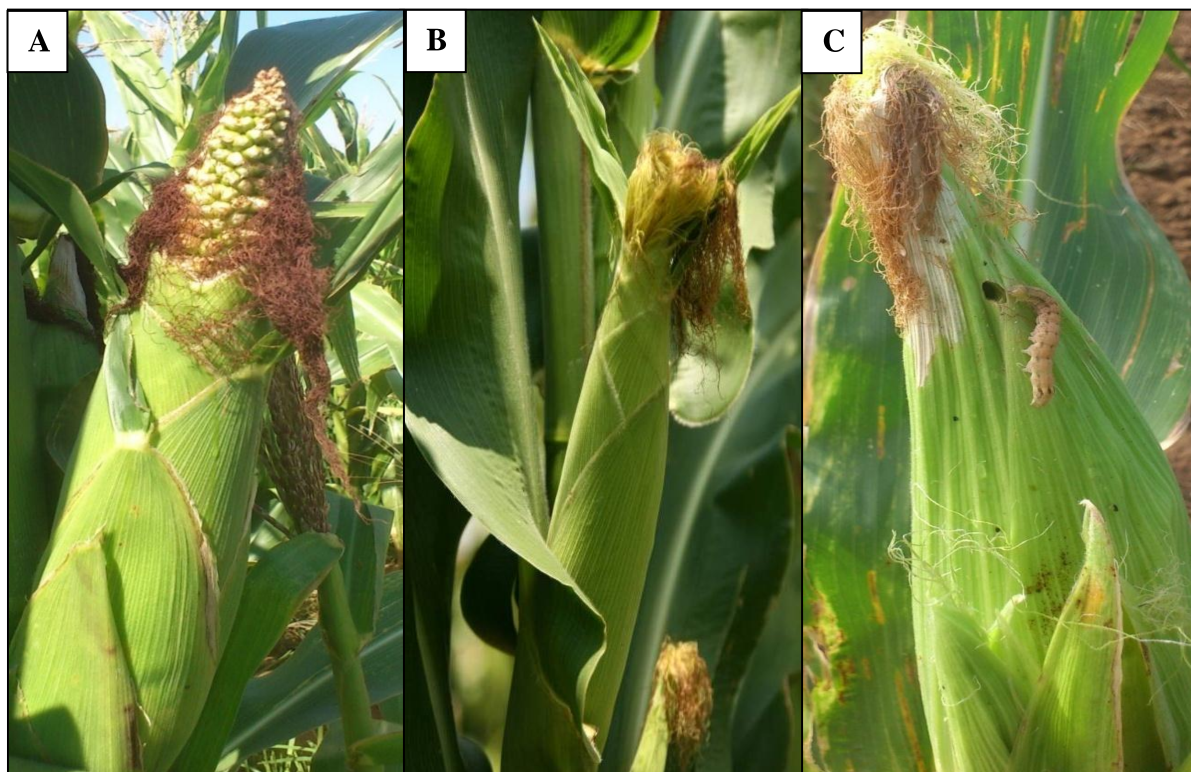


Fig. 1. Poor husk coverage (A), pendant maize ears (B) and insect feeding-damage (C) render maize plants more susceptible to Fusarium ear rot disease development.

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 2

Multi-site screening for resistance in South African maize inbred lines to Fusarium ear rot and fumonisin contamination

ABSTRACT

Fusarium verticillioides is a common pathogen of maize, causing Fusarium ear rot worldwide. Aside from reducing yield and grain quality, *F. verticillioides* produces fumonisins, a group of mycotoxins which are harmful to both human and animal health. The planting of resistant cultivars can be an efficient approach to reduce ear rot diseases and minimize the risk of economic losses and mycotoxin accumulation in maize. The objective of this study, therefore, was to evaluate 11 genetically diverse maize inbred lines as potential sources of resistance to Fusarium ear rot and fumonisin contamination under different production systems and environmental conditions. Inbred lines were artificially inoculated with a cocktail of *F. verticillioides* isolates MRC 826, GCI 316 and GCI 790 just after anthesis, in field trials planted at Potchefstroom, Vaalharts, Cedara, Makhatini and Buffelsvallei in 2011 and 2012. Following harvest, maize ears were evaluated using visual disease severity rating, followed by the quantification of fungal concentration and total fumonisin (B_1 + B_2 + B_3) content by means of quantitative real-time PCR and liquid chromatography tandem mass spectrometry, respectively. Genotype main effect and genotype by environment interaction (GGE), as well as additive main effects and multiplicative interaction analyses revealed a significant ($P \leq 0.01$) genotype x environment interaction for visual symptoms, fungal biomass and toxin rating. Additionally, the GGE biplot technique was used to identify resistant inbred lines at target-environments. Inbred lines CML390, CML444, US2540W, and R0424W consistently showed low Fusarium ear rot severity (≤ 5%), fungal biomass (≤ 0.1 ng μl^{-1}) and accumulated fumonisin levels (≤ 5 ppm) at most test locations. Moreover, inbred lines R0424W, CML390 and CML444 were well adapted under Makhatini and Cedara conditions while inbred line US2540W performed better at Vaalharts and Potchefstroom. These resistant lines could be included in local conventional breeding programmes to improve local maize genotypes' resistance to *F. verticillioides* and the fumonisins it produces.

INTRODUCTION

With an ever increasing human population, the security and safety of accessible food sources demand top priority. Maize is the most widely cultivated crop in Southern Africa and serves as a staple diet to millions of people (Asiedu, 1989). However, sustainable production of maize is compromised by the infection of maize grain with fungal pathogens in the field and during storage. One of the more serious diseases of maize is Fusarium ear rot (FER) caused by *Fusarium proliferatum, Fusarium subglutinans* and *Fusarium verticillioides* (Munkvold, 2003). Of these, *F. verticillioides* is most often associated with FER. Apart from reducing yield and grain quality, *F. verticillioides* also produces toxic secondary metabolites, known as fumonisins (Munkvold, 2003).

Fumonisins are a group of structurally related polyketide-derived chemicals with the most prolific and thoroughly studied member being FB_1 (Rheeder *et al*., 2002). Contamination of maize with fumonisins presents a challenging problem as contamination results in serious illness and immune suppressions in humans and livestock (Marasas, 2001). While desirable, prevention of fumonisin contamination of foods and feeds is not possible since their production in field maize is largely a matter of uncontrollable events (Munkvold, 2003). However, certain practices, such as the "farm to fork" policy of the Codex Alimentarius Commission, are available to reduce fumonisin levels (CAC, 2003). The planting of resistant and partially resistant maize cultivars, as well as cultivars improved by introduction of maize-derived and/or foreign genes will in future form an important strategy to reduce ear rot diseases and mycotoxin contamination of grain (Munkvold, 2003).

Various degrees of resistance to FER and fumonisins exists among maize inbred lines and hybrids (Warren, 1978; Hoenisch and Davis, 1994; Balconi *et al.*, 2004; Desjardins *et al.*, 2005; Palaversic *et al.*, 2008). As resistant genotypes adapted to all environments are rare to find, it is crucial to continuously search for new resistant sources to use in breeding programmes. Such an approach would include multi-location field testing of breeding lines to estimate genotype x environment effects and to identify genotypes with stable resistance to disease and mycotoxin contamination. In a recent investigation by Small *et al.* (2012), genetically diverse maize inbred lines were evaluated in South Africa. From this study, nine inbred lines were identified for further testing in the current study over 2 years and multiple locations in South Africa. Inbred lines that proved to be resistant over all of these locations would provide breeders with material to cross with agronomic superior female lines, especially since genetic engineering does not yet enjoy widespread public acceptance.

MATERIALS AND METHODS

Plant material

In the 2007/2008 and 2008/2009 growing seasons, a total of 24 maize inbred lines, selected by the South African Agricultural Research Council Grain Crops Institute (ARC-GCI) for their genetic discrepancy and good agronomic traits, were evaluated for resistance to *F. verticillioides* and the fumonisin it produces (Small *et al.*, 2012). Based on the results of this study, nine inbred lines (Table 1) were selected for further evaluation under diverse geographical, climatic and irrigation conditions. Additionally, two inbred lines (R2565Y and R0544W) of South African origin were included in the trial as susceptible checks (Table 1). The inbred lines comprised of white and yellow maize with a normal to high-lysine protein content and were evaluated at five field sites in maize-producing areas in South Africa.

Field sites

Field trials were planted in the 2010/2011 and 2011/2012 maize seasons at Potchefstroom (grid ref.: 26˚73'S, 27˚07'E; altitude, 1349 m), Buffelsvallei (grid ref.: 26˚48'S, 26˚61'E; altitude, 1383 m), Vaalharts (grid ref.: 27˚95'S, 24˚83'E; altitude, 1180 m), Cedara (grid ref.: 29˚54'S, 30.26'E; altitude, 1068 m) and Makhatini (grid ref.: 22˚39'S, 32˚17'E; altitude, 77 m) (Fig. 1). Based on their micro- and macroclimates, these five sites can be divided into western and eastern locations. Western experimental locations such as Potchefstroom, Buffelsvallei and Vaalharts are situated in the drier and warmer parts of the maize-production area whereas eastern localities such as Cedara and Makhatini are situated in areas of high humidity. Site-specific (ARC reference numbers: 30649, 30693, 30142, 30817 and 30729) weather data including temperature, radiation, humidity, wind velocity and total rainfall were obtained from the ARC-Institute for Soil Water and Climate’s meteorology office.

Land preparations

Agricultural practices applied at each locality were in line with common practices at each locality. Experimental lands were sprayed with pre-emergence herbicide flumetsum/S-metolachlor at 630 g L^{-1} (Bateleur Gold EC) and the post-emergence herbicide halosulfuron-methyl at 750 g kg^{-1} (Servian 75 WG/Cyprex WP) to manage weeds such as *Cyperus esculentus* and *Cyperus rotundus*. Trials were fertilised using a rate of 150 kg ha^{-1} (30) + 0.5 Zn at Potchefstroom, Vaalharts, Cedara and Buffelsvallei, and 2:3:4 (30) + 0.5 Zn at Makhatini before planting. Maize seeds were hand planted (two seeds per planting hole) in

double-row plots that were 10 m long, and 3 weeks after emergence were thinned to 33 plants per row. The intra-row spacing was 0.3 m with an inter-row spacing of 1 m. The trials were planted using a randomised block design and were replicated three times. The Potchefstroom trials were conducted under dryland conditions with moisture stress being monitored and overhead irrigation applied when necessary. The trials in Makhatini and Cedara were irrigated via overhead and central pivot systems, respectively, whereas the trials in Vaalharts were flood irrigated on a weekly basis. At the sixth leaf stage, top dressing was applied at trials planted at Buffelsvallei and Makhatini with LAN 28 at a rate of 150 kg ha^{-1} and 250 kg ha^{-1}, respectively. The same broadcast method was followed for field trials at the Potchefstroom (100 kg ha^{-1}), Vaalharts (100 kg ha^{-1}) and Cedara (980 kg ha^{-1}) localities using LAN 28 at the eight leaf stage. For control of stalk borer infestation, field trials at Potchefstroom, Vaalharts and Buffelsvallei were treated with the insecticide beta-cyfluthrin at 0.5 g kg^{-1} (Bulldock 0.05 GR) at the 10th to 12th leaf stage and trials at Makhatini and Cedara were treated with Carbaryl at 25 g kg^{-1} (Kombat GR) at the 12th leaf stage. Both insecticides were applied (40 g per 50 m) manually into the whorl of the maize plants.

Production of fungal inoculum

The conidial suspension for the artificial inoculation of field trials was prepared according to the protocol used by Small *et al*. (2012). A cocktail of aggressive *F. verticillioides* isolates was inoculated and consisted of isolates MRC 826, provided by Dr. Vismer from the Medical Research Council – Programme on Mycotoxins and Experimental Carcinogenesis unit (MRC-PROMEC, Tygerberg, South Africa) and isolates GCI 316 and GCI 790, provided by Dr. Janse van Rensburg from the ARC-GCI (Potchefstroom, South Africa). *Fusarium verticillioides* isolates GCI 316 and GCI 790 were originally isolated from infected maize in Ndwedwe (KwaZulu-Natal) and Rushof (Northern Cape) respectively. In contrast, the well characterized MRC 826 was collected from infected maize in the Transkei region (Eastern Cape) and is known to produce unsurpassed high levels of FB_1 (Rheeder *et al*., 2002). These isolates have been preserved at -80°C in the culture collection of the Department of Plant Pathology at Stellenbosch University.

Artificial inoculation of maize ears

The maize lines planted in the field trials were artificially inoculated with *F. verticillioides* isolates to ensure proper selection of genotypes with high resistance, because previous studies have indicated that insufficient disease development occurs under natural infection (Papst *et*

Stellenbosch University http://scholar.sun.ac.za

al., 2007; Blandino *et al*., 2009; Small *et al*., 2012). Maize ears were inoculated using a cattle vaccinator fitted with an 18 G × 1.5" (1.20 × 38 mm) Terumo needle (sterile, non-toxic, non-pyrogenic)(Fig. 2). Liquid spore suspensions (2 ml) were injected down the silk channel of all ears and plants at 50% silk-emergence, based on the silk channel inoculation method described by Afolabi *et al*. (2007). The inoculation procedure and timing were the same for each field location.

Disease severity

At physiological maturity, (kernel moisture content of 18%) ears were hand-harvested, bulked by plot and de-husked in order to quantify the severity of ear rot development on each ear. Disease severity was evaluated by estimating the percentage of each ear surface covered by visible damage of infection (Fig. 3), such as white streaking radiating from the cap of the kernels or white-pinkish mycelial growth in between the kernels alongside stalk borer channels (Clements *et al.*, 2004).

Grain processing

Following disease quantification, ears were mechanically threshed and bulked separately for each field plot. Subsequently, a sample of 250 g was taken from each bulked sample and ground using a coffee grinder that was provided by the ARC-Plant Protection Research Institute in Stellenbosch. The grinder was thoroughly cleaned with high pressure air between each sample to avoid cross contamination. A Philips blender (400 W, 1.75 L) was further used to grind the maize into a fine powder after which a 2- and 5-g sub-samples of the ground grain from each plot was weighed into a 50-ml Falcon tube (BD Biosciences, Durham, USA) and kept at -20°C until used for DNA and fumonisin extractions, respectively.

Quantification of *Fusarium verticillioides* in maize grain

Genomic DNA extractions: Fusarium verticillioides isolate MRC 826 was used as a positive control to produce DNA standards for the absolute quantification of this ear rot pathogen in maize samples. The fungus was grown in 100 ml potato dextrose broth (PDB) incubated in a 250-ml Erlenmeyer flask at 25°C on a rotary shaker. After 2 weeks, mycelium was harvested by filtration through two layers of sterile cheesecloth, washed twice with autoclaved water and freeze-died. Freeze-dried mycelia were then stored at -20°C until genomic DNA was extracted with the DNeasy® Plant Mini kit (QIAGEN) using the CTAB/PVP lysis method due to its high reproducibility (Boutigny *et al*., 2012).

DNA from milled maize samples (2 g) were extracted using the same commercial kit and protocol described by Boutigny *et al*. (2012). A NanoDrop ND-1000 Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) was employed to verify the quantity of the DNA whilst the purity of the DNA was evaluated by comparing absorbance ratios A260/A280 and A260/A230. Good quality DNA was diluted to a concentration of 10 ng μl^{-1} for the detection and absolute quantification of *F. verticillioides* in the maize samples using quantitative real-time PCR (qRT-PCR).

Quantitative detection of F. verticillioides in maize using qRT-PCR: Due to the subjective nature of visual ratings and the ability of *F. verticillioides* to be present in symptomless material, the amount of fungal material present in the grain was determined in qRT-PCR assays as described in Boutigny *et al*. (2012). The qRT-PCR assays contained one replicate of each maize sample, a negative control containing no template DNA, and standard pathogen DNA that was diluted 16-fold in maize DNA (10 ng μl^{-1}) free of *F. verticillioides* infection. Regression equations of standard curves created to detect *F. verticillioides* in the maize samples were highly significant ($R^2 > 0.99$). Slopes were within the acceptance criterion (m = -3.4) and efficiencies ranged from 95 to 97%. The detection limit of the qRT-PCR assays was found to be between 5.48 ng μl^{-1} and 0.016 ng μl^{-1}. The presence of inhibitors in pathogen DNA as well as intra- and inter run variability of the qRT-PCR assays were not tested as these techniques have been previously validated in the same laboratory by Boutigny *et al*. (2012).

Fumonisin analysis

Sample preparation: A 5-g sub-sample of the stored milled maize from each plot per trial was accurately weighed (to the nearest 0.01 g) into 50-ml Falcon tubes and stored at -20°C. Fumonisin extractions were carried out following the method established by Small *et al*. (2012).

Following preparation of an extraction buffer consisting of 70% AR grade methanol and 30% Milli-Q H_2O (Millipore, Bedford, MA, USA), 20 ml was added to each sample (4 ml:1 g) using the Fast pipette (Labnet International Inc., Edison, USA). Tubes were shaken vigorously and fumonisins were extracted by placing the tubes on a rotary shaker at an angle for 30 min at 25°C and 200 rpm. The tubes were subsequently centrifuged at 4°C for 10 min at 4000 rpm after which 2 ml of the clear extract was filtered through a 0.20 µm nylon filter disk and placed in the fridge overnight. After centrifugation for 10 min at maximum rpm,

Stellenbosch University http://scholar.sun.ac.za

extracts were diluted in 2-fold by adding 900 µl of supernatant to 900 µl of Milli-Q H_2O in a glass vial. Caps were then fitted onto the vials and the vials were vortexed.

Liquid chromatographic tandem mass spectrometry (LC-MS/MS) analysis: Standards of FB_1 (10 mg), FB_2 (10 mg) and FB_3 (1 mg), > 95% pure, were obtained from the MRC-PROMEC unit at Tygerberg in South Africa and prepared as described by Small *et al*. (2012). Standards were combined in a 50-ml stock solution with final concentrations of 200, 200, and 20 µg ml^{-1} for FB_1, FB_2 and FB_3, respectively. From the 50-ml stock solution, volumes of 1 ml were aliquoted into 2-ml Eppendorf tubes and dried in a flow cabinet overnight. The dried standards were then stored at -20°C.

A dried standard aliquot was reconstituted with 1 ml acetonitrile/Milli-Q H_2O (50/50, v/v) on the day of analysis and served as a calibration standard solution. Furthermore, the calibration standard solution was diluted to produce a series of standards that was included in each run. Unkowns were plotted on the standard curve to calculate total levels of fumonisin ranging from 0.05 to 20 ppm for FB_1 and FB_2, and between 0.005 and 2 ppm for FB_3.

The concentration of fumonisins in the contaminated maize samples was determined by the Central Analytical Facility at Stellenbosch University. The solutions were thoroughly vortexed and 5 µl injected into the LC-MS/MS system. Samples with results exceeding the standard curve limits were subjected to further dilution using Milli-Q H_2O before being re-analysed. For confirmation, about 20% of the samples were analysed in duplicate. Minimum detectable limits for FB_1, FB_2 and FB_3 were 0.02, 0.002 and 0.02 ppm, respectively. The recovery rates ranged between 60 and 65% for each of the compounds analysed (Small *et al*., 2012).

Statistical analysis

Data obtained from visual ratings, fumonisin analysis and qRT-PCR was subjected to univariate analysis of variance (ANOVA) using the General linear model (GLM) procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Experimental results were combined in one ANOVA (John and Quenouille, 1977) after testing for homogeneity of variance using Levene's test (Levene, 1960). The Shapiro-Wilk test was performed to test for normality (Shapiro and Wilk, 1965) and variables were Ln(x+1) transformed to stabilise the variance and improve normality where needed (Snedecor and Cochran, 1980). Student's t-least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott, 1998) and a probability level of 5% was considered

Stellenbosch University http://scholar.sun.ac.za

significant for all significance tests. Non-parametric Pearson correlation coefficients were determined for relationships among ear rot symptoms, fungal biomass and total fumonisins using the CORR procedure in SAS based on untransformed means.

To determine the effects of field location and inbred lines on the severity of FER, fungal biomass and fumonisin accumulation in the grain, genotype main effect and genotype by environment interaction (GGE) biplots were constructed with genotype-focus and symmetrical scaling using GenStat 15th edition (Payne *et al.*, 2012). GGE biplot analysis is a valuable tool for plant breeders and researchers for assessing the performance of genotypes planted in different locations because it visually displays both entries (genotype) and testers (environment or traits) in a two-way table (Yan and Kang, 2003). This allows identification of maize inbred lines with stable resistance in different environments, comparison of their performance as well as identification of potential target-environments.

Further interpretation of genotype x environmental interactions was complemented by additive main effects and multiplicative interaction (AMMI) analysis of variance (Gauch and Zobel, 1996) that was performed using SAS statistical software version 9.2. Similar to GGE biplot analysis, the AMMI method combines traditional ANOVA and principle component analysis (PCA) graphically in a biplot where interaction PCA scores are plotted against genotype and environment means. Additionally, AMMI stability values for the individual maize genotypes are generated in a table which provides insights into the stability and relative adaptability of the inbred lines across multiple environments.

RESULTS

Artificial silk channel inoculation resulted in sufficient FER development and high concentrations of *F. verticillioides* and fumonisins in the maize lines during the two planting seasons. This allowed for adequate differentiation between resistant genotypes and susceptible checks. In the 2011/2012 season, however, only four locations were included in the multi-site screening trial as the trial planted at Buffelsvallei resulted in poor germination and was discarded. According to the ANOVA, a significant year x genotype x location interaction was observed for FER severity ($P \leq 0.01$), fungal biomass ($P \leq 0.01$) and total fumonisin contamination ($P \leq 0.01$). There was too many variation in the genotype x environment interactions and therefore it was decided to report on the main genotypic effects found across locations within each season. GGE and AMMI analyses (Table 2) were used to best explain these interactions and to identify resistant genotypes in target-environments.

Stellenbosch University http://scholar.sun.ac.za

Disease severity

The mean visual rating of FER infection of maize ears in 2011 and 2012 were 8.38 and 7.96%, respectively. FER development was significantly more severe at Cedara in 2011 (11.57%) than in 2012 (1.20%)($P \leq 0.01$) while the mean visual ratings of FER symptoms were significantly higher at Makhatini and Vaalharts in 2012 (7.51 and 8.80%, respectively) compared to the previous year (2.18 and 3.61%, respectively)($P \leq 0.01$). At Potchefstroom there was no significant difference between mean FER symptoms for the two seasons (P = 0.08).

During the 2010/2011 season, disease levels at Buffelsvallei (4.46%), Makhatini (2.18%) and Vaalharts (3.61%) did not differ significantly, but mean visual ratings of FER symptoms were significantly higher ($P \leq 0.05$) at Cedara (11.57%) and Potchefstroom (8.43%)(Table 3) than the other field sites. The inbred lines showed different responses to FER severity between locations ($P \leq 0.05$). For example, line R2565Y developed significantly (P = 0.02) more FER at Potchefstroom (23.33%) than at Vaalharts (0.25%), exemplifying the high inbred x location effect ($P \leq 0.01$). Inbred line US2540 consistently developed low levels of FER across all locations, with mean visual ratings ranging from 0.47 to 2.10% (Table 3). Differences in FER severity among inbred lines were also observed within individual field sites (Table 3). At Cedara, for instance, seven inbred lines including lines VO617Y-1, R0549W, US2540, CML390, R0424W, I-B, and R2565Y had significantly ($P \leq 0.05$) less FER when compared with lines R119W and K62R-2 (Table 3).

FER disease developed during the 2011/2012 season in the inoculated maize ears with maximum levels found at Potchefstroom and Makhatini as high as 41.67 and 26.5%, respectively (Table 3). As seen in the previous year, lines differed significantly ($P \leq 0.05$) in visual FER symptoms within and amongst locations. For instance, at Vaalharts, lines R2565Y and I-B had significantly ($P \leq 0.05$) different responses than those displaying the lowest risk in FER severity (lines K62R-2, R0549W, US2540, R0544W and CML444); and line I-B was significantly (P = 0.00) more prone to FER disease development at Vaalharts (21.89%) than at Cedara (1.31%)(Table 3).

Quantification of *Fusarium verticillioides*

The average fungal concentrations recorded for the two planting seasons were comparable (0.10 and 0.09 ng μl^{-1}). A higher average fungal biomass was observed at Cedara during the 2010/2011 season (0.16 ng μl^{-1}) when compared to the 2011/2012 season (0.05 ng μl^{-1}) while *F. verticillioides* infection was more severe at the Makhatini location in the second (0.20 ng

μl^{-1}) than the first year (0.09 ng μl^{-1})($P \leq 0.01$)(Table 4). There was no significant difference between mean fungal levels for the two seasons at Potchefstroom and Vaalharts ($P \geq 0.05$).

The individual inbred lines differed substantially in fungal biomass concentrations across locations during the 2010/2011 season ($P \leq 0.05$). For example, at Cedara, line VO617Y-1 accumulated significantly ($P = 0.00$) more *F. verticillioides* DNA (0.33 ng μl^{-1}) than at Potchefstroom (0.03 ng μl^{-1})(Table 4). This location effect was also indicated by the higher mean fungal biomass observed at Cedara (0.16 ng μl^{-1}) compared with Potchefstroom (0.06 ng μl^{-1})($P = 0.00$). Maize inbred lines showed significantly different reactions ($P \leq 0.05$) to *F. verticillioides* within each location with lines US2540, R0424W, CML444 and CML390 being the only lines that constantly contained low levels of fungal DNA (0.01 to 0.10 ng μl^{-1})(Table 4).

The significant inbred x location effect that was observed during the 2011/2012 season ($P \leq 0.01$; Table 2) manifested in the lower average fungal biomass detected at Cedara (0.05 ng μl^{-1}), Potchefstroom (0.09 ng μl^{-1}) and Vaalharts (0.06 ng μl^{-1}) than at Makhatini (0.20 ng μl^{-1}). Significant differences between the inbred lines could be determined ($P \leq 0.05$) within field sites (Table 4). At Makhatini, for instance, relatively more fungal DNA accumulated in lines US2540 and I-B ($P \leq 0.05$) while lines R0549W, CML444, CML390, K62-R, R0424W, R2565Y and R0544W had comparable responses to the growth and proliferation of *F. verticillioides* (Table 4).

Fumonisin analysis

The mean total fumonisin concentrations determined in the maize kernels were significantly higher ($P \leq 0.05$) in the 2010/2011 season (9.66 ppm) than the 2011/2012 season (6.97 ppm). A significant amount of fumonisins ($P \leq 0.01$) accumulated in the maize at Cedara and Vaalharts in 2011 (21.18 and 7.42 ppm, respectively) but not in 2012 (3.30 and 2.06 ppm, respectively) (Table 5). The significant year x location interaction ($P \leq 0.01$) was also apparent at Makhatini with a 4-fold increase in mean total fumonisin levels during the 2011/2012 season (Table 5).

During the 2010/2011 season, lines differed considerably ($P \leq 0.05$) in total fumonisin content across locations as recorded in lines VO617Y-1 and R0549W which had lower accumulated fumonisins at Buffelsvallei (1.92 and 1.32 ppm, respectively) when compared with Cedara (35.57 and 74.31 ppm, respectively) (Table 5). Additionally, the significant inbred x location interaction was also indicated by the higher mean fumonisin levels measured at Cedara (21.18 ppm) than at Buffelsvallei (9.22 ppm)($P = 0.00$). Despite

Stellenbosch University http://scholar.sun.ac.za

differences in fumonisin concentrations across locations, inbred lines R0424W, CML444 and CML390 had consistently low fumonisin contamination (≤ 5 ppm) in the grain during the 2010/2011 season, with exception of Cedara (Table 5). Significant effects of the inbred lines on fumonisin contamination were also observed at all field sites ($P \leq 0.05$). At Makhatini, for example, lines R119W (5.94 ppm) and I-B (7.81 ppm) were significantly ($P \leq 0.05$) more prone to fumonisin contamination than line R0424W (2.64 ppm)(Table 5).

During the 2011/2012 season, total fumonisin levels at Cedara (3.30 ppm), Potchefstroom (2.82 ppm) and Vaalharts (2.06 ppm) did not differ significantly ($P \geq 0.05$), but fumonisin concentrations were significantly higher at Makhatini (17.69 ppm) (Table 5). Fumonisin analysis also indicated several lines with significant different responses to fumonisins in different environments. One such an example includes line CML444 which was more susceptible ($P = 0.02$) to fumonisin accumulation at Cedara (10.82 ppm) when compared with Vaalharts (2.89 ppm). Of the 11 inbred lines tested, only lines VO617Y-1, K62R-2, US2540, R0424W, R0544W and CML390 consistently showed low accumulated fumonisin levels (≤ 5 ppm) across the locations, with exception of Makhatini (Table 5). The selected inbred lines also differed significantly in their response to fumonisin contamination at all locations ($P \leq 0.05$). For instance, at Potchefstroom, lines R2565Y, I-B, CML444 and R119W had significantly ($P \leq 0.05$) higher fumonisin levels than the other lines (VO617Y-1, K62R-2, US2540, R0549W, R0424W, R0544W and CML390) having fumonisin concentrations below the detectable limit (0.02 ppm)(Table 5).

Correlations between ear rot severity, fungal biomass and fumonisin contamination

A poor but significant relationship was established between visual and qRT-PCR data (Pearson correlation of r = 0.19; $P \leq 0.01$), as well as visual and toxin rating (r = 0.25; $P \leq 0.01$) over the 2 years. For instance, line VO617Y-1 had a FER severity rating of 0.87% and a fungal DNA concentration of 0.33 ng μl^{-1} at Cedara, and line R119W showed no FER symptoms but contained 47.2 ppm of fumonisin at Buffelsvallei during the 2010/2011 season. In contrast, a strong and significant correlation was found between toxin and qRT-PCR data (r = 0.71; $P \leq 0.01$) which is exemplified by line I-B and R119W having relatively high fumonisin (32.29 and 27.69 ppm, respectively) and *F. verticillioides* (0.56 and 0.25 ng μl^{-1}, respectively) concentrations at Makhatini in the 2011/2012 growing season.

Stellenbosch University http://scholar.sun.ac.za

Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses

Combined AMMI analysis of variance in a multi-location trial over 2 years revealed the effect of location, inbred line and inbred line x location interaction to be highly significant ($P \leq 0.01$)(Table 2). Therefore, the GGE biplot method was used to identify resistant genotypes in target-environments by ranking the inbred lines in terms of their FER severity, fungal biomass and total fumonisin content, taking the inbred line x location effect into account. The stability of the inbred lines' performance across multiple locations was confirmed by AMMI stability values (ASV)(Table 6).

GGE biplot analysis for resistance to FER severity is presented with two principle components accounting for a total of 57.5% (PC1 34.9%, PC2 22.6%) of the variance seen in the visual data (Fig. 4). The first principle component (PC1) is located on the X-axis and indicates the level of resistance exhibited by the inbred lines, with inbred lines having higher PC1 values considered lower risk to FER development than others. The second principle component (PC2) is located on the Y-axis and presents performance stability where lines with PC2 values near zero demonstrate broader adaptability (Yan and Kang, 2003). Based on their visual ranking, lines CML390, US2540, VO617Y-1, R0544W and CML444 are considered highly resistant to FER disease severity (Fig. 4). Of these, lines CML390, US2540, VO617Y-1 are relatively stable while line CML444 is unstable in performance across locations (Table 6). Inbred lines R0424W and R0544W, as well as lines I-B, R2565Y and R0549W can be placed in the "moderate risk and high stability" and "moderate risk and low stability" groups, respectively (Fig. 4; Table 6). Furthermore, inbred lines K62R-2 and R119W exhibited high susceptibility but low stability over all of the trial sites (Fig. 4; Table 6).

GGE biplot analysis also enables identification of potential target-environments (Yan and Kang, 2003). Inbred lines furthest away from the origin are connected by a straight line and form a polygon (Fig. 4). This polygon is divided into several sectors when lines radiating from the origin of the biplot intersect the sides of the polygon perpendicularly. Consequently, environments placed within a given sector are those where the inbred lines in that sector are best suited for (Yan and Kang, 2003). Six sectors can be distinguished with the largest sectors incorporating lines R2565Y and I-B that were best suited for the Makhatini location during the 2011/2012 growing season (Fig. 4). Inbred line CML444 was highly resistant at Makhatini and Buffelsvallei in 2011 and at Vaalharts in 2012 whilst line US2540 was the leading genotype at the Potchefstroom location in 2011. Although lines VO617Y-1 and

CML390 performed the best in the majority of the test locations (Vaalharts_10, Potchefstroom_11, Cedara_10 and Cedara_11), only Cedara was identified as target-environment for these inbred lines.

GGE biplot analysis for resistance to fungal biomass showed significance for the first two principle components explaining 43.7% (PC1) and 18.8% (PC2) of the variance (Fig. 5). Genotype-focus scaling of the biplot differed substantially in comparison to visual representation of resistance to FER severity (Fig. 4). This is not surprising due to poor relationship established between visual and qRT-PCR data earlier in this study. Inbred lines with the highest resistance to fungal biomass concentration include lines CML390, US2540, VO617Y-1, R0424W and CML444 with only the former line being stable in performance across locations (Fig. 5; Table 6). Additionally, lines CML390 and R0424W were consistent in their performance at Makhatini and Vaalharts during the 2 years. Conversely, lines R119W, I-B, R2565Y and K62R-2 did not perform well in the field and contained unacceptable concentrations of *F. verticillioides* (Fig. 5; Table 5).

Similar conclusions were drawn from the GGE biplot method regarding resistance to total fumonisin content (Fig. 6). It was re-iterated that inbred lines CML390, R0424W, US2540 and CML444 had the highest resistance status over the 2 years (Fig. 6). However, inbred lines CML444 and CML390 in conjunction with lines R0549W, VO617Y-1 and K62R-2 showed a less stable performance when compared with the other inbred lines (Table 6). In the largest sector, Makhatini was identified as a target-environment for lines CML444 and R0424W and the fourth largest sector which includes the Buffelsvallei_10, Potchefstroom_11 and Vaalharts_11 environments, had line US2450 as leading genotype. Furthermore, line CML444 was most resistant at Cedara and Potchefstroom during the 2010/2011 season (Fig. 6). AMMI and GGE analyses also confirmed inbred lines R119W, I-B and R2565Y to be high risk due to their durable susceptibility to fumonisin accumulation (Fig. 4; Table 6).

Weather data

The average monthly temperatures were generally similar for the 2 years at the each field site with a slight temperature increase in 2011 at Makhatini and Cedara at pollination time (March)(Fig. 7). Maximum temperatures for the 2010/2011 planting season were recorded at Makhatini (34.61°C) during grain filling (March) whereas Vaalharts reached a temperature of 36°C during seedling emergence (January) in the 2011/2012 season. Additionally, there was substantial fluctuation in mean monthly rainfall amongst locations for the same experimental

Stellenbosch University http://scholar.sun.ac.za

period except during December at Buffelsvallei where up to 222 mm of rain was recorded (Fig. 7). Total monthly rainfall was higher in 2010/2011 at Potchefstroom and Buffelsvallei at the start of the planting season (December) and over the inoculation period (February) than at the Vaalharts and Makhatini locations. At Vaalharts there was lower rainfall in 2011 and 2012 during grain filling (March) and drying (April and May) when compared to the other respective field locations (Fig. 7).

DISCUSSION

In this study, the stability of resistance in maize inbred lines to FER and fumonisin contamination, previously characterized by Small *et al*. (2012), has been confirmed in a multi-location trial over 2 years. This is of great importance, as *F. verticillioides* is the predominant fungus associated with FER of maize in South Africa (Boutigny *et al*., 2012), and because none of the cultivars commercially available in the country have immunity to FER or fumonisin accumulation (Rheeder *et al*., 1990; Schjøth *et al*., 2008). Maize genotypes with potential resistance to *F. verticillioides* and its fumonisins have also been identified in tropical parts of Africa (Afolabi *et al*., 2007), but their agronomic suitability to production conditions in South Africa has not been established.

Highly significant genotype x environment interactions were observed for the inbred lines tested. Such interactions could be the result of differential responses of a genotype to various biotic and abiotic factors such as inoculum pressure, host susceptibility, climatic conditions, insect infestation, kernel maturation, as well as pre- and postharvest handling (Munkvold, 2003). Genotype x environment interactions are of leading concern to plant breeders as these often changes the performance of the inbred lines in different locations making proper selection difficult (Sharma *et al*., 1987). For this reason it is important that the genotype x environment effect is properly understood and analysed.

Fumonisin production results from the complex interaction of several physiological stress factors such as pH, temperature, water availability or nutrient sources (Jurado *et al*., 2008). Amongst these, temperature and water stress are considered key factors modulating fumonisin production (Marín *et al*., 2010). *In planta* studies by Marín *et al*. (1999) have shown that the optimal growth conditions for *F. verticillioides* and fumonisin B_1 production are a temperature of 30°C and a water activity of 0.97, with a dramatic reduction in germination and fumonisin production below a water activity of 0.92. These findings suggest

that dry and warm weather, followed by a period of high humidity may promote infection and higher fumonisin accumulation in the grain.

The fungal and total fumonisin content in the maize were highly and positively correlated. In contrast, a poor relationship between visual ratings, *F. verticillioides* and fumonisin concentration was established as certain lines were recorded as having severe FER symptoms but low fungal biomass and total fumonisin levels. The subjective nature of visual ratings and the ability of non-fumonisin producing *Fusarium* species to induce similar disease symptoms as *F. verticillioides*, may have contributed to the lower correlations involving ear rot severity (Small *et al.*, 2012). Inbred lines with symptomless infection but with high fumonisin levels were also observed, which is in agreement with previous investigations (Clements *et al.*, 2003; Small *et al.*, 2012). Due to the poor correlations found between visual symptoms, fungal biomass and toxin rating, qRT-PCR and mycotoxin analysis are highly recommended when evaluating resistance in maize. Quantitative procedures, however, can be time-consuming and cost-prohibitive whereas visual ratings can be relatively simple when conducting large-scale trials (Eller *et al.*, 2008).

In this study, fumonisin analysis revealed that FER caused by fumonisin-producing *F. verticillioides* can reach serious proportions in certain commercial production areas of South Africa such as Cedara and Makhatini. Mean total fumonisins ranged from 6.91-74.31 ppm at Cedara in the 2010/2011 season and 8.14-32.29 ppm at Makhatini in the 2011/2012 season. These levels far exceed the 4 ppm fumonisin allowed for human and animal consumption as set by United States authorities, a limit used as guideline in the absence of South African legislation (NGFA, 2011). Given that precautionary control measures such as fertilisation, irrigation, herbicide and insecticide treatments were implemented to reduce plant stress in the field, the high fumonisin concentrations in the grain of Cedara and Makhatini could most likely be attributed to prevailing climatic conditions. Both field sites had an average temperature of 30°C during grain filling (March) which is known to favour FER development and fumonisin production (Janse van Rensburg *et al.*, 2011). Additionally, relative high rainfall during the months of April and May when the grain was drying, combined with elevated humidity due to overhead irrigation, may have allowed extended fumonisin accumulation at these field sites.

Fumonisins in maize constitute a challenge in commercial and subsistence farming systems in South Africa. Cultural approaches are currently being used, but host-plant resistance other than Bt maize has not been utilized to address the mycotoxin problem (Clements *et al.*, 2004). None of the maize inbred lines tested in the current study were

Stellenbosch University http://scholar.sun.ac.za

immune to FER severity, fungal biomass or total fumonisin accumulation (Small *et al*., 2012). However, four inbred lines including lines US2540, R0424W, CML444 and CML390 consistently showed low disease severity, fungal biomass and fumonisin levels across most locations and years. Amongst these, inbred lines R0424W and CML390 are considered most superior as they exhibited broader adaptability at Cedara and Makhatini which are situated in the "high risk" parts of the country. These lines can be used to identify sources of genetic resistance that can be introduced into high-yielding superior female lines. Resistant cultivars will give the South African maize industry a competitive advantage for markets that require high quality and safe foods. Additionally, certain lines were highly susceptible to FER development, fungal biomass and fumonisin accumulation, such as lines K62R-2, R2565Y and R119W. They could be used for comparative functional genomics/proteomics, or as control checks in future studies.

Stellenbosch University http://scholar.sun.ac.za

REFERENCES

Afolabi, C.G., Ojiambo, P.S., Ekpo, E.J.A., Menkir, A. and Bandyopadhyay, R. 2007. Evaluation of maize inbred lines for resistance to Fusarium ear rot and fumonisin accumulation in grain in tropical Africa. Plant Disease 91: 279-286.

Asiedu, J.J. 1989. Processing tropical crops: A technological approach. The Macmillan Press, London, 266 pp.

Balconi, C., Lupotto, E., Lanzanova, C., Conti, E., Gualdi, L., Mazzoleni, A., Valoti, P. and Motto, M. 2004. Screening maize genotypes for resistance to *Fusarium verticillioides* comparing *in vivo* plant response and *in vitro* bioassays. Maize Genetics Cooperation Newsletter 78: 7-8.

Blandino, M., Reyneri, A., Colombari, G. and Pietri, A. 2009. Comparison of integrated field programmes for the reduction of fumonisin contamination in maize kernels. Field Crops Research 111: 284-289.

Boutigny, A.-L., Beukes, I., Small, I., Zühlke, S., Spiteller, M., Van Rensburg, B.J., Flett, B.C. and Viljoen, A. 2012. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. Plant Pathology 61: 522-531.

CAC. 2003. Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin A, zearalenone, fumonisins and trichothecenes CAC/RCP 51-2003. Codex Alimentarius Commission. Online publication: http://www.codexalimentarius.org/input/download/standards/406/CXP_051e.pdf (29 September 2013).

Clements, M.J., Campbell, K.W., Maragos, C.M., Pilcher, C., Headrick, J.M., Pataky, J.K. and White, D.G. 2003. Influence of Cry1Ab protein and hybrid genotype on fumonisin contamination and Fusarium ear rot of corn. Crop Science 43: 1283-1293.

Clements, M.J., Maragos, C.A., Pataky, J.K. and White, D.G. 2004. Sources of resistance to fumonisin accumulation in grain and Fusarium ear and kernel rot of corn. Phytopathology 94: 251-260.

Desjardins, A.E., Plattner, R.D., Stessman, R.J., McCormick, S.P. and Millard, M.J. 2005. Identification and heritability of fumonisin insensitivity in *Zea mays*. Phytochemistry 66: 2474-2480.

Eller, M., Holland, J. and Payne, G. 2008. Breeding for improved resistance to fumonisin contamination in maize. Toxin Review 27: 371-389.

Gauch, H. and Zobel, R. 1996. AMMI analysis of yield trials. Pages 85-122 in: Genotype by environment interaction. M. Kang and H. Gauch, eds. CRC Press, Boca Raton, New York, USA.

Hoenisch, R.W. and Davis, R.M. 1994. Relationship between kernel pericarp thickness and susceptibility to Fusarium ear rot in field corn. Plant Disease 78: 517-519.

Janse van Rensburg, B., McLaren, N.W., Flett, B.C., Ncube, E., Schoeman, A. and Viljoen, A. 2011. Mycotoxigenic fungi of maize in South Africa. Proceedings of the World Congress of Microbes, Beijing, China, 29 July-3 August.

John, J.A. and Quenouille, M.H. 1977. Experiments: Design and Analysis, 2nd ed. The Garden City Press Limited, Letchworth, Hertfordshire.

Jurado, M., Marin, P., Magan, N. and González-Jaén, M.T. 2008. Relationship between solute and matric potential stress, temperature, growth, and 49 *FUM1* gene expression in two *Fusarium verticillioides* strains from Spain. Applied and Environmental Microbiology 74: 2032-2036.

Levene, H. 1960. Contributions to probability and statistics. Stanford University Press, Stanford, California, USA. 278 pp.

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: A historical perspective. Environmental Health Perspectives 109: 239-243.

Marin, P., Magan, N., Vásquez, C. and González-Jaén, M.T. 2010. Differential effect of environmental conditions on the growth and regulation of the fumonisin biosynthetic gene *FUM1* in the maize pathogens and fumonisin producers *Fusarium verticillioides* and *Fusarium proliferatum*. Federation of European Microbiological Societies 73: 303-311.

Marin, S., Magan, N., Belli, A., Ramos, A.J., Canela, R. and Sanchis, V. 1999. 2-dimensional profiles of fumonisin B1 production by *Fusarium moniliforme* and *Fusarium proliferatum* in relation to environmental factors and potential for modelling toxin formation in maize grain. International Journal of Food Microbiology 51: 159-167.

Munkvold, G.P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology 41: 99-116.

NGFA. National Grain and Feed Association. 2011. FDA mycotoxin regulatory guidance: A guide for grain elevators, feed manufacturers, grain processors and exporters. 5 pp.

Ott, R.L. 1998. An Introduction to Statistical methods and data analysis. Duxbury Press, Belmont, California, USA. 837 pp.

Stellenbosch University http://scholar.sun.ac.za

Palaversic, B., Ivic, D., Vragolovic, A., Jukic, M., Buhinicek, I. and Cvjetkovic, B. 2008. Screening maize for resistance to *Fusarium verticillioides* ear rot. Cereal Research Communications 36: 151-152.

Papst, C., Zellner, J., Venkataratnam, S. and Eder, J. 2007: Fusarium-Problematik bei Ko¨ rnermais (*Zea mais* L.). Gesunde Pflanzen 59: 7-16.

Payne, R., Murray, D.A., Harding, S.A., Baird, D.B. and Soutar, D.M. 2012. GenStat for Windows (15th ed.) Introduction. VSN International, Hemel Hempstead. SAS. 2000. SAS/STAT Users Guide, Version 8, First Edition, Volume 2. SAS Institute Inc., Cary, NC, USA.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S. and Van Schalkwyk, D.J. 1990. Reaction of South African maize cultivars to ear inoculation with *Fusarium moniliforme*, *F. graminearum* and *Diplodia maydis*. Phytophylactica 22: 213-218.

Rheeder, J.P., Marasas, W.F.O. and Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* species. Applied Environmental Microbiology 68: 2101-2105.

Schjøth, J.E., Tronsmo, A.M. and Sundheim, L. 2008. Resistance to *Fusarium verticillioides* in 20 Zambian maize hybrids. Journal of Phytopathology 156: 470-479.

Shapiro, S.S. and Wilk, M.B. 1965. An analysis of Variance Test for Normality (complete samples). Biometrika 52: 591-611.

Sharma, R.C., Smith, E.L. and McNew, R.W. 1987. Stability of harvest index and grain yield in winter wheat. Crop Science 27: 104-108.

Small, I.M. 2010. Resistance in maize to *Fusarium verticillioides* and fumonisin. MSc thesis, University of Stellenbosch, Stellenbosch, South Africa.

Small, I.M., Flett, B.C., Marasas, W.F.O., McLeod, A., Stander, M. A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticillioides* and fumonisin accumulation in South Africa. Plant Disease 96: 881-888.

Snedecor, G.W. and Cochran, W.G. 1980. Statistical Methods, 7th ed. Iowa State University Press, Ames, USA. 478 pp.

Warren, H.L. 1978. Comparison of normal and high-lysine maize inbreds for resistance to kernel rot caused by *Fusarium moniliforme*. Phytopathology 68: 1331-1335.

Yan, W. and Kang, M. 2003. GGE Biplot Analysis: A graphical tool for breeders, geneticists and agronomists. CRC Press, Boca Raton, Florida, USA. 271 pp.

Stellenbosch University http://scholar.sun.ac.za

Table 1. Maize inbred lines evaluated for resistance to Fusarium ear rot severity, fungal biomass and fumonisin accumulation during multi-site field trials in the 2010/2011 and 2011/2012 seasons.

Inbred line identity	Origin	Resistance: FER/fumonisin*
VO617Y-1	ARC-GCI-South Africa	Resistant
K62R-2	ARC-GCI-South Africa	Intermediate
R2565Y	ARC-GCI-South Africa	Susceptible
R0549W	ARC-GCI-South Africa	Resistant
US2540	ARC-GCI-South Africa	Resistant
R0424W	ARC-GCI-South Africa	Intermediate
R0544W	ARC-GCI-South Africa	Susceptible
I-B	ARC-GCI-South Africa	Intermediate
CML444	CIMMYT-Zimbabwe	Resistant
R119W	ARC-GCI-South Africa	Resistant
CML390	CIMMYT-Zimbabwe	Resistant

ARC-GCI, Agricultural Research Council Grain Crops Institute

CIMMYT, International Maize and Wheat Improvement Centre

*As characterized by Small *et al.* (2012)

Stellenbosch University http://scholar.sun.ac.za

Table 2. AMMI analysis of variance (ANOVA) for resistance to Fusarium ear rot severity, fungal biomass and fumonisin accumulation in 11 maize inbred lines tested in nine environments in South Africa during the 2010/2011 and 2011/2012 seasons.

		FER severity[1]			Fungal biomass[2]			Fumonisins[3]		
Source of variation	*Df*	*MS*	*F value*	*P > F*	*MS*	*F value*	*P > F*	*MS*	*F value*	*P > F*
Total	296	9.72	*	*	10.03	*	*	9.75	*	*
Treatment	98	17.69	2.78	0.00	21.35	4.37	0.00	20.10	3.95	0.00
Environment	8	0.00	*	*	0.00	*	*	0.00	*	*
Block	18	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Genotype	10	45.41	7.14	0.00	79.61	16.32	0.00	63.25	12.44	0.00
Interaction	80	16	2.51	0.00	16.20	3.32	0.00	16.72	3.28	0.00
IPCA	17	23.28	3.66	0.00	25.19	5.16	0.00	28.10	5.52	0.00
IPCA	15	22.01	3.46	0.00	24.57	5.03	0.00	25.02	4.92	0.00
Residual	48	11.54	1.81	0.00	10.40	2.13	0.00	10.09	1.98	0.00
Error	180	6.36	*	*	4.88	*	*	5.08	*	*

[1] Percentage of maize ears covered with visual symptoms of Fusarium ear rot

[2] Absolute concentrations of *Fusarium verticillioides* DNA (ng μl^{-1})

[3] Fumonisin content = Total of $FB_1 + FB_2 + FB_3$ (ppm)

Stellenbosch University http://scholar.sun.ac.za

Table 3. Evaluation of 11 maize inbred lines for resistance to Fusarium ear rot in a multi-location field trial in South Africa during the 2010/2011 and 2011/2012 growing seasons.

	Fusarium ear rot severity (%)[1,2,3]									
Inbred line	**Buffelsvallei**		**Cedara**		**Makhatini**		**Potchefstroom**		**Vaalharts**	
	2011	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*
VO617Y-1	1.15 c-e	-	0.87 d	2.43 ab	2.04 b	9.71 a-c	2.17 d-g	0.00 c	1.21 de	6.32 a-d
R0549W	1.08 c-e	-	5.30 b-d	0.91 b	0.44 b	3.23 bc	14.43 a-c	0.00 c	5.34 a-c	2.42 cd
US2540	0.47 de	-	2.10 cd	0.00 b	1.22 b	2.67 c	0.75 e-g	1.20 bc	1.62 c-e	0.98 d
CML444	5.15 b	-	9.34 a-c	1.71 ab	0.79 b	26.50 a	0.05 g	2.96 bc	1.10 de	1.14 cd
R119W	0.00 e	-	36.51 a	1.53 b	1.64 b	15.72 ab	7.69 b-d	41.67 a	13.56 a	8.09 a-c
CML390	2.47 b-d	-	7.05 bc	0.50 b	0.44 b	5.08 bc	0.48 fg	0.00 c	0.14 e	6.59 a-d
K62R-2	21.43 a	-	33.51 a	9.94 a	0.47 b	4.56 bc	30.55 a	3.01 b	10.15 ab	4.23 b-d
R0424W	2.04 b-d	-	2.00 cd	0.99 b	1.49 b	5.94 a-c	5.29 cd	2.20 bc	3.21 b-d	15.61 ab
I-B	5.15 b	-	4.41 cd	1.31 b	1.74 b	3.69 bc	4.32 c-e	1.34 bc	1.28 c-e	21.89 a
R2565Y	6.49 b	-	3.97 cd	0.85 b	12.50 a	3.41 bc	23.33 ab	3.01 b	0.25 e	26.03 a
R0544W	3.60 bc	-	22.15 ab	1.79 ab	1.22 b	2.13 c	3.69 c-f	0.00 c	1.85 c-e	3.53 b-d
Mean	4.46	-	11.57	1.20	2.18	7.51	8.43	5.04	3.61	8.80

[1] Percentage of maize ears covered with visual symptoms of Fusarium ear rot

[2] Mean of disease severity for three field plots

[3] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)

Stellenbosch University http://scholar.sun.ac.za

Table 4. Evaluation of 11 maize inbred lines for resistance to fungal biomass in a multi-location field trial in South Africa during the 2010/2011 and 2011/2012 growing seasons.

Fungal biomass (ng µl^{-1})[1,2]										
Inbred line	**Buffelsvallei**		**Cedara**		**Makhatini**		**Potchefstroom**		**Vaalharts**	
	2011	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*
VO617Y-1	0.02 de	-	0.33 a	0.02 bc	0.13 a	0.18 a-c	0.03 c-e	0.01 c	0.05 cd	0.05 c-e
R0549W	0.02 de	-	0.28 ab	0.02 c	0.08 a	0.08 c	0.14 a	0.03 c	0.31 a	0.03 de
US2540	0.01 e	-	0.06 d	0.03 bc	0.10 a	0.33 a	0.02 de	0.02 c	0.05 cd	0.02 de
CML444	0.04 de	-	0.08 d	0.13 a	0.06 a	0.13 bc	0.00 e	0.07 bc	0.05 cd	0.04 de
R119W	0.81 a	-	0.27 a-c	0.08 a-c	0.11 a	0.25 b	0.06 bc	0.13 a-c	0.12 b	0.12 a-c
CML390	0.03 de	-	0.09 cd	0.03 bc	0.09 a	0.09 c	0.01 e	0.01 c	0.01 d	0.03 de
K62R-2	0.15 c	-	0.18 a-d	0.09 ab	0.09 a	0.17 cd	0.05 b-d	0.25 a	0.11 bc	0.01 e
R0424W	0.04 de	-	0.10 b-d	0.05 bc	0.06 a	0.12 bc	0.05 b-d	0.02 c	0.03 d	0.04 de
I-B	0.07 d	-	0.12 b-d	0.04 bc	0.11 a	0.56 a	0.07 b	0.09 a-c	0.16 b	0.13 ab
R2565Y	0.16 c	-	0.15 b-d	0.02 c	0.08 a	0.14 bc	0.13 a	0.20 ab	0.10 bc	0.15 a
R0544W	0.26 b	-	0.12 b-d	0.04 bc	0.06 a	0.10 bc	0.05 b-d	0.16 a-c	0.03 d	0.08 b-d
Mean	0.15	-	0.16	0.05	0.09	0.20	0.06	0.09	0.09	0.06

[1] Mean absolute concentrations of *Fusarium verticillioides* DNA for three field plots

[2] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)

Stellenbosch University http://scholar.sun.ac.za

Table 5. Evaluation of 11 maize inbred lines for resistance to fumonisin accumulation in a multi-location field trial in South Africa during the 2010/2011 and 2011/2012 growing seasons.

Fumonisin (ppm)[1,2,3]										
Inbred line	**Buffelsvallei**		**Cedara**		**Makhatini**		**Potchefstroom**		**Vaalharts**	
	2011	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*	*2011*	*2012*
VO617Y-1	1.92 de	-	35.57 b	1.51 c-f	4.91 a-c	24.23 ab	5.44 ab	0.00* c	5.18 ef	3.60 a
R0549W	1.32 e	-	74.31 a	4.14 bc	4.43 a-c	10.69 cd	5.54 ab	0.00* c	13.54 b	0.58 c
US2540	1.62 de	-	6.91 f	0.54 f	3.88 bc	23.09 a-c	4.11 b	0.00* c	4.88 d-f	0.98 a-c
CML444	4.68 c	-	8.02 ef	10.82 a	3.33 bc	10.74 b-d	0.17 d	6.69 b	5.07 e	2.89 ab
R119W	47.21 a	-	27.75 cd	5.81 ab	5.94 ab	27.69 a	4.72 ab	9.91 a	10.58 c	3.20 ab
CML390	3.12 cd	-	7.86 ef	3.88 b-d	3.05 bc	8.14 d	1.59 c	0.00* c	3.88 g	1.02 a-c
K62R-2	4.78 c	-	22.19 b-d	2.78 b-e	4.84 a-c	15.01 a-d	4.26 b	0.00* c	7.70 d	0.89 bc
R0424W	2.71 c-e	-	8.98 ef	1.30 d-f	2.64 c	10.57 cd	3.86 b	0.00* c	4.18 fg	1.58 a-c
I-B	5.08 c	-	8.57 ef	2.18 b-f	7.81 a	32.29 a	4.86 ab	6.98 b	17.01 a	3.60 a
R2565Y	4.53 c	-	12.80 de	0.93 ef	3.90 a-c	14.95 a-d	7.51 a	7.43 ab	9.62 cd	2.44 a-c
R0544W	24.48 b	-	20.01 cd	2.40 b-e	4.72 a-c	17.23 a-d	3.82 b	0.00* c	4.12 fg	1.86 a-c
Mean	9.22	-	21.18	3.30	4.49	17.69	4.17	2.82	7.42	2.06

[1] Fumonisin content = Total of $FB_1 + FB_2 + FB_3$
[2] Mean fumonisin concentration for three field plots
[3] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)
* Below detectable limit

Stellenbosch University http://scholar.sun.ac.za

Table 6. AMMI stability values (ASV) and ranking orders for Fusarium ear rot severity, fungal biomass and fumonisin contamination of the 11 maize inbred lines tested in nine environments in South Africa during the 2010/2011 and 2011/2012 seasons.

	FER severity[1]		**Fungal biomass**[2]		**Fumonisins**[3]	
Inbred line #	*ASV*	*Rank*	*ASV*	*Rank*	*ASV*	*Rank*
VO617Y-1	0.66	3	2.64	11	2.32	9
R0549W	1.57	7	2.32	10	2.96	10
US2540	0.84	4	1.03	6	1.27	4
CML444	2.60	11	1.69	7	3.67	11
R119W	2.14	9	0.77	5	1.16	3
CML390	0.95	5	0.61	4	2.16	7
K62R-2	1.47	6	0.13	1	2.28	8
R0424W	0.21	1	0.13	2	0.36	1
I-B	1.63	8	0.15	3	1.84	6
R2565Y	2.45	10	2.11	8	1.43	5
R0544W	0.38	2	2.32	9	0.89	2

[1] Percentage of maize ears covered with visual symptoms of Fusarium ear rot

[2] Absolute concentrations of *Fusarium verticillioides* DNA (ng μl^{-1})

[3] Fumonisin content = Total of $FB_1 + FB_2 + FB_3$ (ppm)

Stellenbosch University http://scholar.sun.ac.za

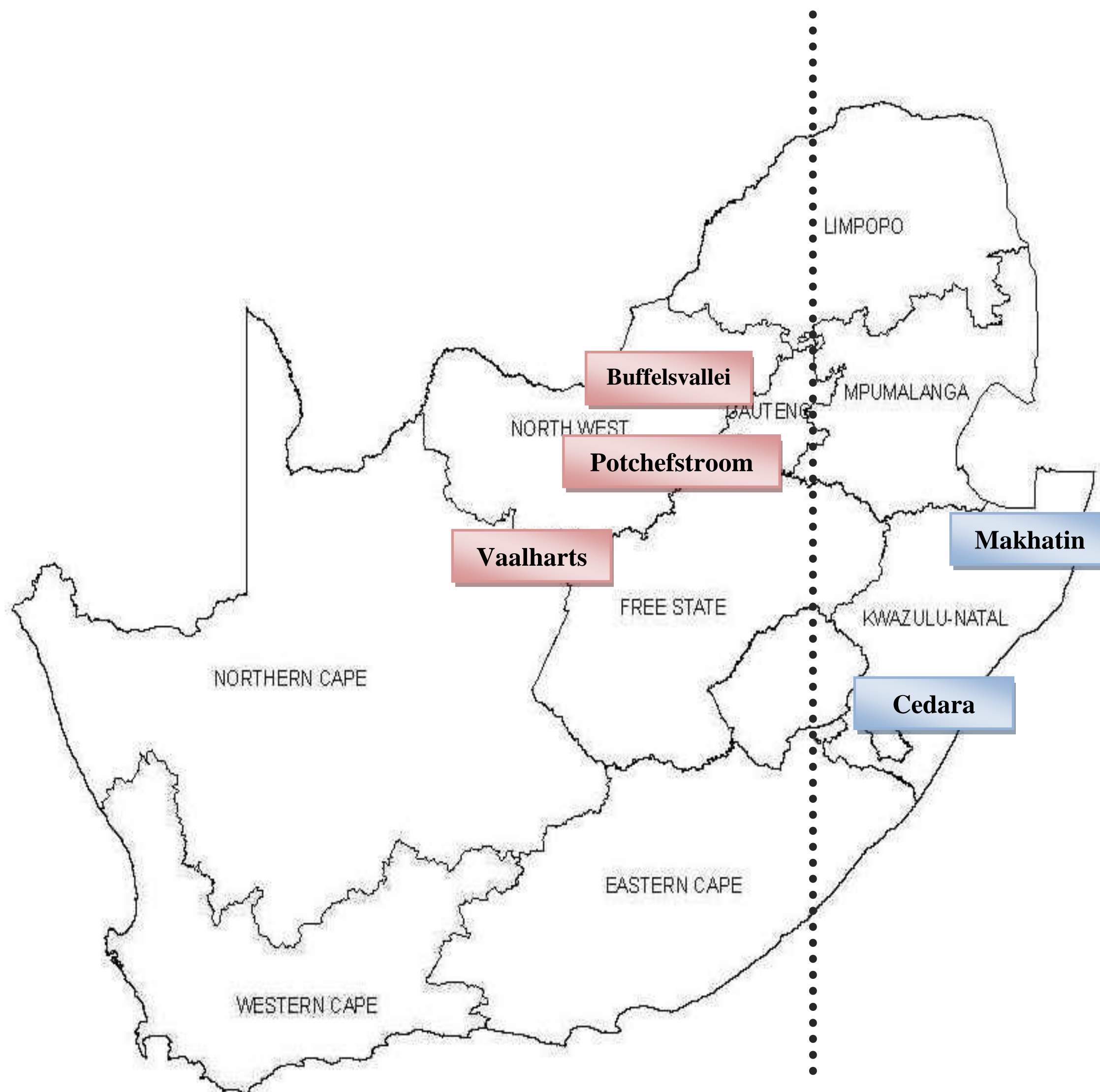


Fig. 1. The five localities in South Africa where maize samples were collected for the quantitative detection of *Fusarium verticillioides* and the fumonisins it produces. The dotted line divides the country into western and eastern localities.

Stellenbosch University http://scholar.sun.ac.za

Fig. 2. Artificial silk channel inoculation at Blister (R2) stage.

Fig. 3. Graphic representation of Fusarium ear rot disease assessment (Courtesy: M. Vermeulen).

Stellenbosch University http://scholar.sun.ac.za

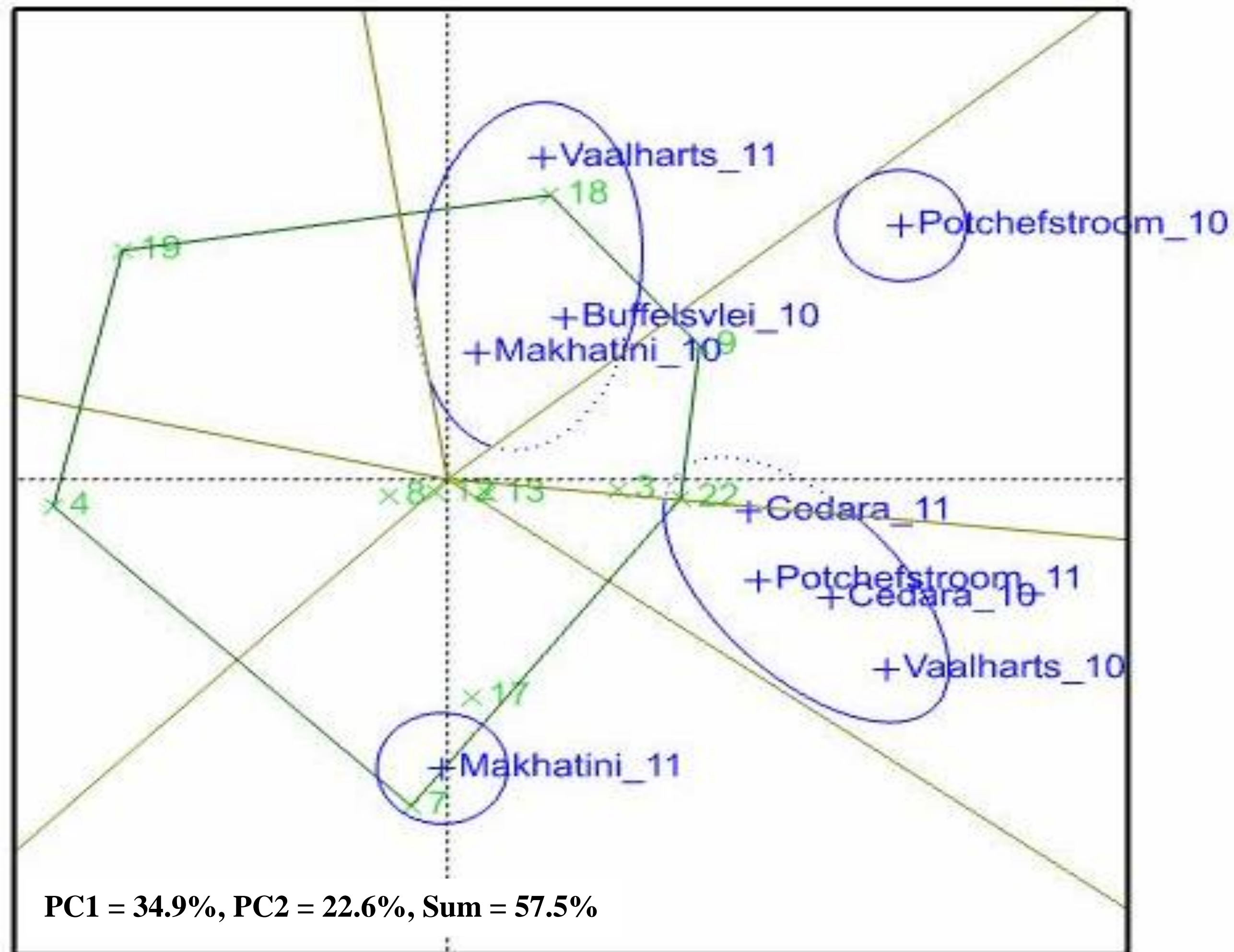


Fig. 4. Genotype main effect + genotype by environment interaction (GGE) biplot showing Fusarium ear rot severity of 11 maize inbred lines tested in nine locations in South Africa during the 2010/2011 and 2011/2012 growing seasons. Line 3: VO617Y-1; line 4: K62R-2; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 12: R0424W; line 13: R0544W; line 17: I-B; line 18: CML444; line 19: R119W; line 22: CML390.

Stellenbosch University http://scholar.sun.ac.za

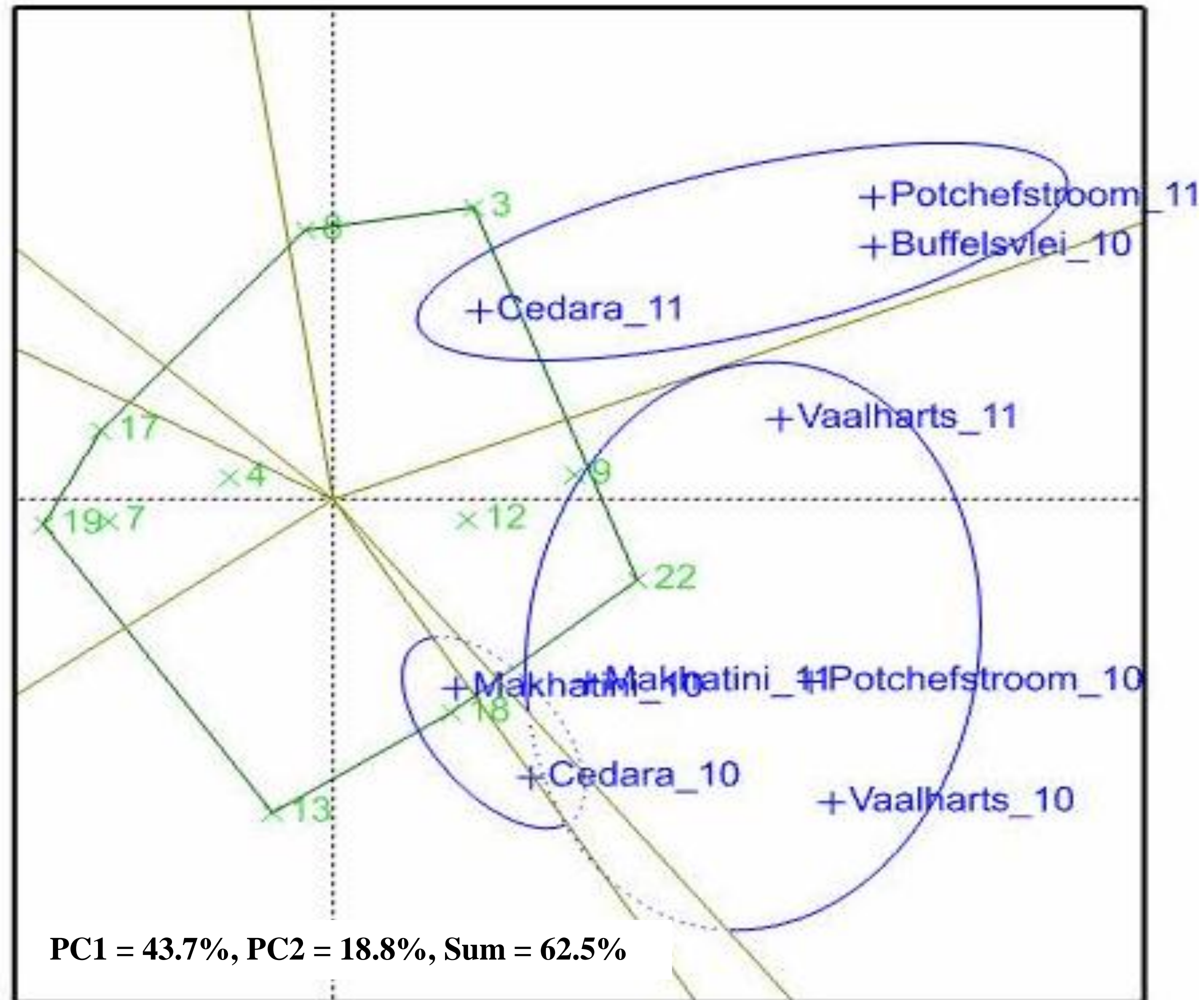


Fig. 5. Genotype main effect + genotype by environment interaction (GGE) biplot showing fungal biomass of 11 maize inbred lines tested in nine locations in South Africa during the 2010/2011 and 2011/2012 growing seasons. Line 3: VO617Y-1; line 4: K62R-2; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 12: R0424W; line 13: R0544W; line 17: I-B; line 18: CML444; line 19: R119W; line 22: CML390.

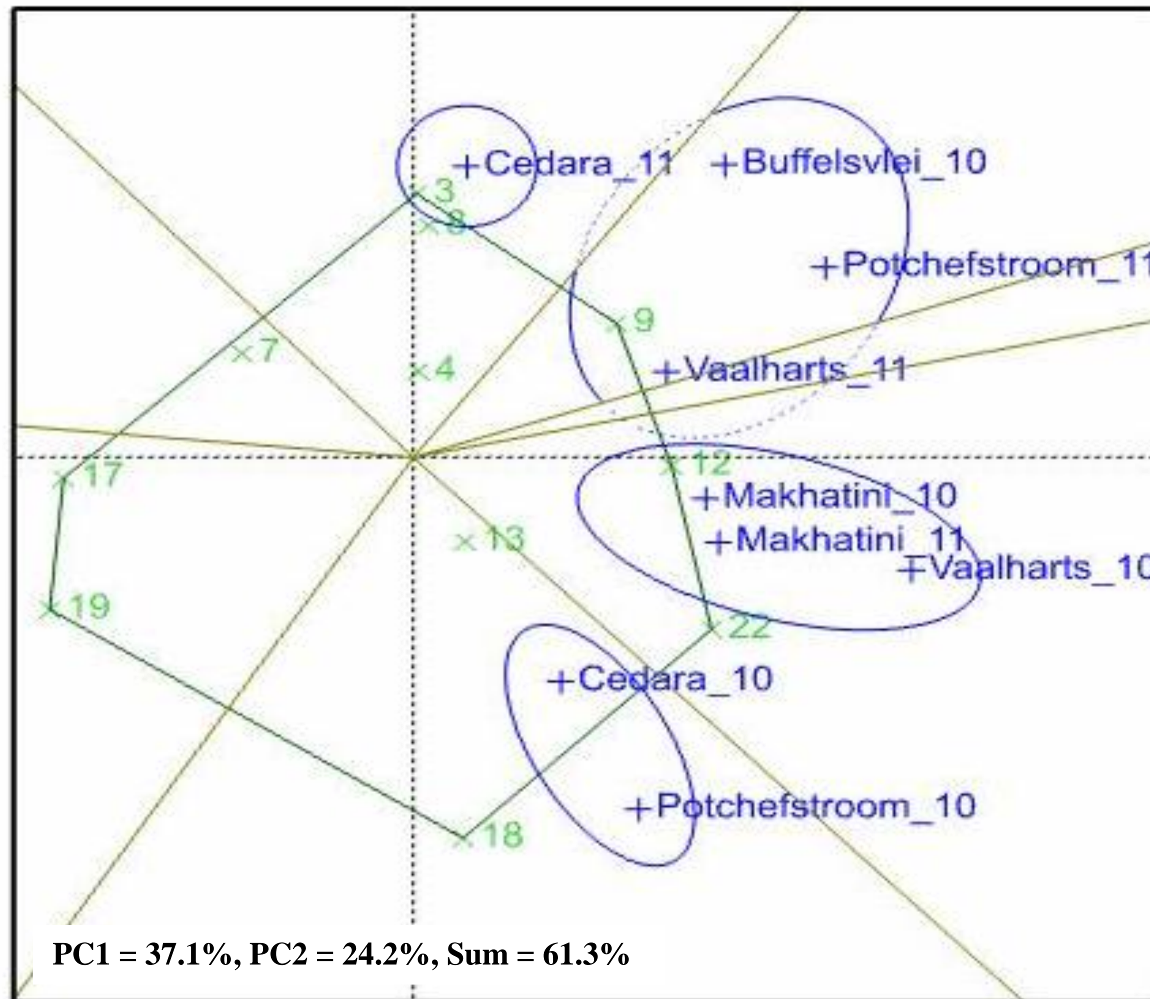


Fig. 6. Genotype main effect + genotype by environment interaction (GGE) biplot showing total fumonisin accumulation of 11 maize inbred lines tested in nine locations during the 2010/2011 and 2011/2012 seasons. Line 3: VO617Y-1; line 4: K62R-2; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 12: R0424W; line 13: R0544W; line 17: I-B; line 18: CML444; line 19: R119W; line 22: CML390.

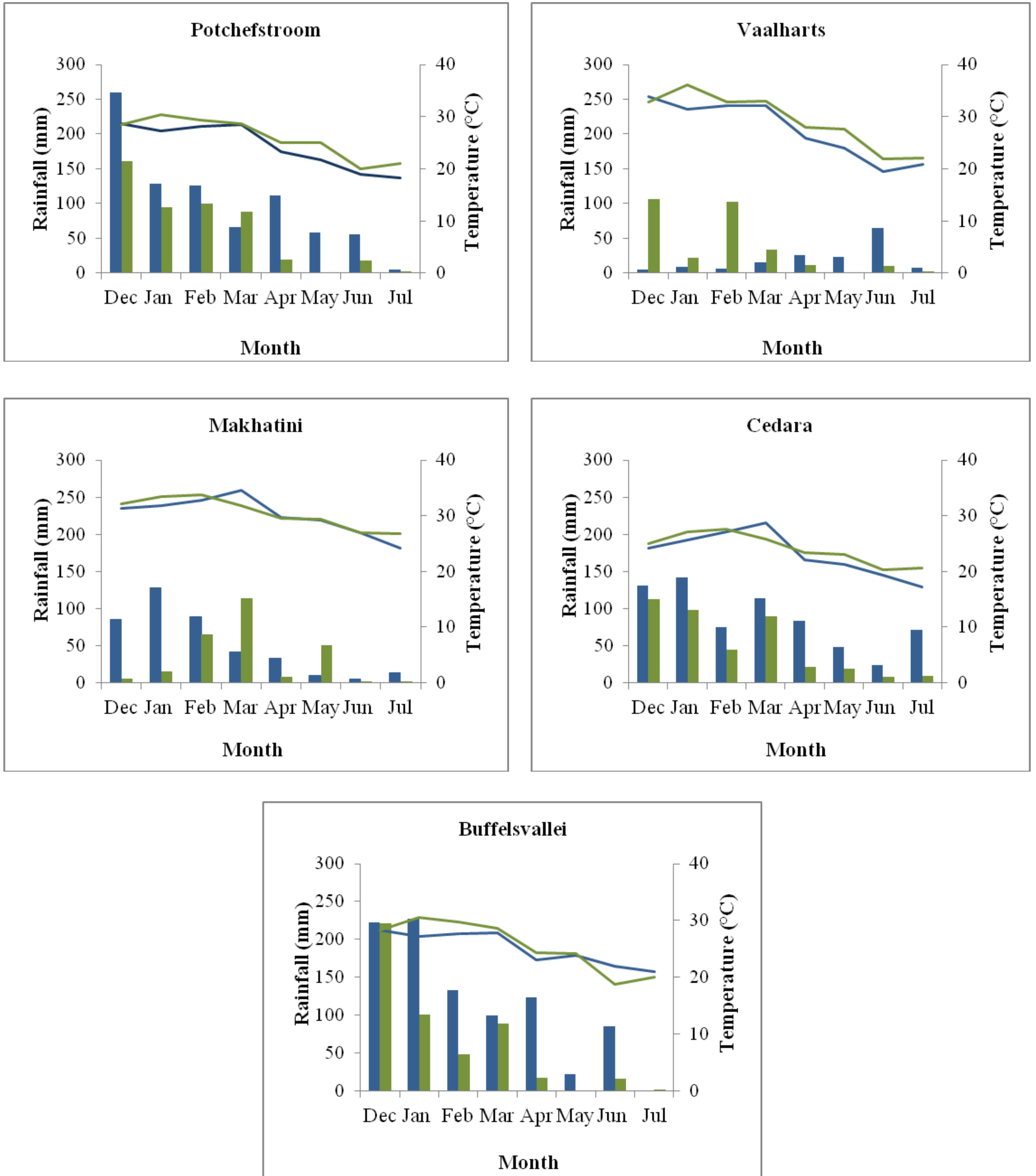


Fig. 7. Weather data recorded from Agricultural Research Council's weather stations for the 2010/2011 (blue) and 2011/2012 season (green) at the respective field locations. Bar and line series indicate total monthly rainfall and mean monthly temperature, respectively, for the two planting seasons.

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 3

Evaluation of Fusarium ear rot-resistant maize inbred lines as potential sources of resistance to other major ear rot diseases and associated mycotoxin contamination

ABSTRACT

Mycotoxin contamination of maize grain regularly occurs in countries where climatic conditions favour the growth and proliferation of ear rot fungi. The threat of these mycotoxins becomes most important when the food supply is limited, and animals and humans are forced to consume mycotoxin-infected food. Host-resistance offers the most effective means for control of mycotoxins and their producers, commonly known as maize ear rot fungi. As maize cultivars and breeding lines with resistance to multiple ear rot pathogens have not been identified in South Africa, Fusarium ear rot-resistant inbred lines were evaluated at multiple locations for their potential to resist Gibberella ear rot (GER), Aspergillus ear rot (AER) and Diplodia ear rot (DER) as well as their associated mycotoxins. The inbred lines were artificially inoculated to ensure adequate inoculum pressure. Following harvest, maize ears were visually evaluated for ear rot and concentrations of fungal biomass and mycotoxins, respectively, determined by quantitative real-time PCR (qRT-PCR) and liquid chromatography tandem mass spectrometry assays. Visual disease ratings correlated poorly with both qRT-PCR and mycotoxin data. Consequently, mycotoxin data from the GER and AER trials, and qRT-PCR data from the DER trials, were subjected to genotype main effect and genotype by environment interaction, as well as additive main effects and multiplicative interaction analyses. Although significant genotype x environment interactions were observed in the field, inbred lines CML444 and CML390 showed to be resistant while line R119W was highly susceptible to ear rot infection and mycotoxin contamination across the tested locations. These inbred lines could be used for future studies on host-pathogen interaction, plant resistance responses, and to develop molecular markers to aid in marker-assisted selection.

Stellenbosch University http://scholar.sun.ac.za

INTRODUCTION

Demand for maize grain is projected to increase with 45% by 2020 (James, 2008). This increase translates to 852 million metric tonnes of maize, of which the majority will be destined for food consumption in developing countries − particularly Sub-Saharan Africa. The only way that these countries can meet their maize needs by 2020 is to increase the productivity of croplands (James, 2008). This presents a daunting challenge to emerging and subsistence farmers in South Africa, who have limited access to the necessary resources required to overcome the multitude of biotic and abiotic constraints (Bänziger and De Meyer, 2002). Among the biotic constraints, ear rot diseases can cause huge yield losses and deterioration in grain quality, and in some years, the entire crop can be destroyed (Rao and Ristanovic, 1986).

Economically important ear rot diseases of maize include Fusarium ear rot (FER), Gibberella ear rot (GER), Aspergillus ear rot (AER) and Diplodia ear rot (DER), which are caused by fungal species of the genera *Fusarium, Aspergillus* and *Stenocarpella*, respectively (Afolabi *et al*., 2007). The distribution of the different ear rot fungi in maize fields varies according to year, agro-ecological zones, climatic factors, cultivar susceptibility as well as agricultural practices (Logrieco *et al*., 2002). Warm, dry weather is conducive to FER profileration (caused by *Fusarium verticillioides*)(Munkvold, 2003) whereas AER (caused by *Aspergillus flavus*) is typically associated with drought stress and humid temperatures as kernels mature (Afolabi, 2007). GER (caused by *Fusarium graminearum sensu lato*) tends to be more severe in cooler areas with elevated precipitation during grain filling (Boutigny *et al*., 2012) while DER (caused by *Stenocarpella maydis*) requires warm and wet conditions after silk emergence (Van Rensburg and Flett, 2010). Once environmental conditions are conducive to infection, the development of ear rot diseases becomes worrisome to maize producers and traders, not only because it compromises the economic value of the grain, but also because ear rot fungi deposit mycotoxins in their hosts (Marasas, 2001).

Several diseases that are fatal to humans and livestock have been associated with mycotoxins. (Richard and Payne, 2002). Despite their omnipresence in maize-based foods and feeds, the management of mycotoxins has only focused on those that pose a serious threat to society. These include the fumonisins primarily produced by *F. verticillioides*, the aflatoxins produced by *A. flavus* and the type B trichothecenes (TCT B) and zearalenone (ZEA) which are produced by *F. graminearum* s.l. (Boutigny *et al*., 2011). A recent study conducted by Snyman *et al*. (2011) reported that *S. maydis* produces diplonine, a neurotoxin

that causes symptoms similar to diplodiosis in cattle and sheep. However, standards of this toxin are still to be commercialized and linked to harmful effects in humans (Barros *et al*., 2008; Snyman *et al*., 2011). Food-borne mycotoxins that are considered to be of greatest importance in west-central Africa and south-eastern United States are the aflatoxins (Afolabi *et al*., 2007; Henry *et al*., 2009), whereas the fumonisins, type B trichothecenes and zearalenone are of particular concern because of its prevalence in Southern African maize (Boutigny *et al*., 2012).

Host resistance is widely regarded to be the most effective strategy for the control of ear rot pathogens and their mycotoxins (Russell *et al*., 2010). However, the development of resistant cultivars has been difficult due to i) high environmental effects; ii) ineffective disease screening methods, iii) unwanted traits coupled with highly resistant germplasm and iv) the polygenetic nature of resistance. As global warming threatens to increase the range and severity of plant diseases in the near future, research is aimed at finding genetic markers to accelerate the pace at which breeders can incorporate stable resistance into hybrid plants (Russell *et al*., 2010).

Quantitative trait loci (QTL) studies by Robertson-Hoyt *et al*. (2007) revealed that some of the genes involved in resistance to FER and AER, as well as their associated mycotoxins are identical or genetically linked. Additionally, studies by Martin *et al*. (2012) yielded strong evidence of a fixed QTL for GER, FER and reduced mycotoxin concentrations. These studies imply that breeding for resistance against one type of ear rot pathogen and its mycotoxin may lead to similar responses for another ear rot pathogen and its mycotoxin. Both constitutive and induced kernel proteins inhibitory to *A. flavus* and its aflatoxins have been characterized (Chen *et al*., 1999; Chen *et al*., 2001) and a few major genes associated with resistance to GER have been identified (Reid *et al*., 1994; Ali *et al*., 2005; Yuan *et al*., 2008). Additionally, Van Rensburg and Ferreira (1997) and Van Rensburg *et al.* (2003) identified maize inbred lines with enhanced resistance to DER, but no attempt to map the genes underlying such resistance had been made.

Maize inbred lines with desirable traits and good combinability have been shown to exhibit resistance to FER and fumonisin accumulation (Small *et al*., 2012). The objective of this study, therefore, was to evaluate whether local FER and fumonisin-resistant maize inbred lines also have resistance to AER, GER and DER at multiple field sites. Inbred lines possessing such resistance would be valuable for the development of multiple ear rot and mycotoxin resistant hybrids that can be grown by farmers to ensure safe and high quality maize grain.

Stellenbosch University http://scholar.sun.ac.za

MATERIAL AND METHODS

Plant material

Seven maize inbred lines with diverse genetic backgrounds, previously evaluated for resistance to FER and fumonisin accumulation, were screened for resistance to AER, GER and DER and associated mycotoxin contamination under local growing conditions. Additionally, two inbred lines (I137tnW and R2565y) previously characterized by Small *et al*. (2012) were included in the study as susceptible checks (Table 1).

Field sites

Separate field trials, one for each of the ear rot diseases under investigation, were planted in December 2012 at Potchefstroom (North West Province) and Vaalharts (Northern Cape Province), and in February 2013 at Makhatini (KwaZulu-Natal Province). The first two locations are situated in the drier and warmer areas of South Africa while the third is situated in a hot area with relative high humidity (Chapter 2). Site-specific weather data was obtained from the ARC's weather stations.

Agricultural practices at the field locations were similar to those described in Chapter 2 with slight modifications. Field trials were irrigated on a regular basis to mitigate drought stress. Elevated humidity caused weeds to grow dense in between trials planted at Vaalharts as these trials were not treated with herbicides. The trial layout was a randomised block design, using three replications (33 plants per row) per trial. Furthermore, a split plot design was used for the DER trials where one row was artificially inoculated and the other left untreated in order to determine the level of natural infection in the vicinity (Flett and McLaren, 1994).

Production of fungal inoculum

The conidial suspension for the artificial inoculation of the GER trials was prepared according to the protocol used by Reid *et al*. (1996). Three local isolates of *F. graminearum* s.l. (M0002, M0010 and M0100), were used as inoculum. These isolates were originally isolated from infected maize ears in Warden (Free State province), Delmas (Mpumalanga province) and Ventersdorp (North West province), respectively (Boutigny *et al*., 2011). Isolates were incubated separately for 2 weeks in potato dextrose broth (PDB), the resulting macroconidial suspensions adjusted to 3×10^4 conidia ml^{-1} and stored at 4°C for 1 or 2 days.

Stellenbosch University http://scholar.sun.ac.za

The inoculum was produced by combining the three spore suspensions in equal proportions prior to inoculation.

Inoculum for the AER trials was prepared using a modified procedure of Henry *et al.* (2009). Three toxigenic strains of *A. flavus* (MRC 3951, MRC 3952 and MRC 3954) were obtained from the Medical Research Council's Programme on Mycotoxins and Experimental Carcinogenesis unit (MRC-PROMEC, Tygerberg, South Africa) and grown separately on sterile maize kernels in 250-ml Erlenmeyer flasks. Each flask contained 50 g of maize kernels, firstly soaked in 25 ml distilled water overnight and then autoclaved for 40 min. The inoculated kernels were incubated for 2 weeks at 30°C after which the conidia were washed from the maize kernels with a 2% Tween 20 solution and filtered through two layers of sterile cheesecloth into a 500-ml Schott bottle. The conidial concentration was determined with a haemocytometer, adjusted to 5 x 10^5 conidia ml^{-1} and refrigerated at 4°C for 1 or 2 days before inoculations. The inoculum was produced by combining the three spore suspensions in equal proportions prior to inoculation.

Ground maize kernels infected with aggressive cultures of *S. maydis* was prepared accordingly to the protocol described by Flett and McLaren (1994) at the ARC-GCI (Potchefstroom, South Africa) to serve as inoculum for the DER field trials. Maize kernels (400 ml) in clean jars (500 ml) were soaked in tap water for 1 day after which the water was decanted and 30 ml of Fries Bosal medium (Chambers, 1987) was added. The jars were then autoclaved for 30 min on 2 consecutive days. Each jar was inoculated with *S. maydis* mycelia and incubated at 28ºC for 60 days. Following incubation, the infected kernels were removed, air-dried for 5 days and milled to a fine powder. Inoculum was stored in a cool room (± 6ºC) prior to use (Flett and McLaren, 1994).

Artificial inoculation of maize ears

GER and AER trials were inoculated 7 days after mid-silk with *A. flavus* and *F. graminearum* s.l. cocktails, respectively, using the silk channel inoculation method (Chapter 2). Conversely, the DER field trials were inoculated by placing 5 g of *S. maydis* inoculum into the apical whorl of each plant at approximately 2 weeks before anthesis (Moremoholo *et al.* 2010). The inoculation procedure and timing were the same for each location. After field inoculations, irrigation was applied to facilitate disease development.

Stellenbosch University http://scholar.sun.ac.za

Disease severity

The inoculated maize ears were harvested from the field at 18% kernel moisture and subjected to visual ratings to determine disease severity. Disease screening for GER and AER was conducted by estimating the percentage of each ear covered by apparent symptoms of infection (Mukanga *et al.,* 2011)(Table 2). Symptoms of DER were observed by removing kernels from the base and tip of the ear due to assess "hidden diplodia" (Nowell, 1997) (Table 2). *Stenocarpella maydis*-infected ears of the inoculated and uninoculated treatments were expressed as a percentage of the total number of ears harvested in each row of each plot (Rossouw *et al*., 2002).

After visual assessment of disease severity, the maize grain was milled to fine particles (Chapter 2). Ground maize samples from each plot were weighed into 2-g and 5-g sub-samples and stored at -20°C for quantification of fungal biomass and mycotoxin content, respectively.

Development of species-specific primers for *Stenocarpella maydis*

Fungal isolates: A total of 41 *S. maydis* isolates were selected for this study. These comprised of 30 isolates recovered from naturally infected maize ears exhibiting DER in the Potchefstroom field trial during the 2011/2012 season and an additional 11 isolates obtained from maize collected from different maize-producing areas in South Africa provided by Prof. Bradley Flett (ARC-GCI, Potchefstroom). Single spore isolations were made after which *S. maydis* isolates were subcultured and grown on malt extract agar (MEA)(MERK, Gauteng, South Africa) at 25°C under near-UV radiation (12h/day) for 2 weeks.

Genomic DNA extractions, PCR amplification and sequencing: Genomic DNA was extracted from mycelia established on MEA plates by using the Wizard® SV Genomic DNA Purification kit (Promega Corporation, Madison, USA). Primer sets ITS1 and ITS4 (White *et al*., 1990), EF_1-728F and EF_1-986R (Carbone and Kohn, 1999) and Bt2a and Bt2b (Glass and Donaldson, 1995) were used to amplify the internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF1-α) and β-tubulin (β-tub) gene regions, respectively.

All PCR amplification was performed in a Veriti Thermal Cycler 9902 (Applied Biosystems, Singapore) in a total reaction volume of 40 µl. The PCR assays consisted of 1 x reaction buffer, 1.5 mM $MgCl_2$, 0.4 mM of each primer, 0.2 µM of each dNTP's, 1 unit of BioTaq DNA polymerase (5 U μl^{-1}) and 30 ng genomic DNA to amplify the ITS gene region. PCR reaction concentrations were similar for the amplification of the TEF1-α while the

Stellenbosch University http://scholar.sun.ac.za

magnesium concentration in the β-tub PCR assay was increased to 2 mM $MgCl_2$. PCR amplification conditions for the ITS and TEF1-α gene region were as follows: an initial denaturation temperature of 94°C for 5 min, followed by 35 cycles of denaturation temperature of 94°C for 30 s, primer annealing at 50°C for 45 s, primer extension at 72°C for 1 min and a final extension step at 72°C for 5 min. PCR amplification of the β-tub gene region was carried out with an initial denaturation temperature of 94°C for 5 min, followed by 35 cycles of denaturation temperature of 94°C for 45 s, primer annealing at 60°C for 45 s, primer extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

Amplified PCR products were separated by electrophoresis at 75 V for 60 min on a horizontal 1% agarose gel (w/v) in 1 X Tris-acetate EDTA (TAE) running buffer (40 mM Tris base, 11.4 ml of acetic acid glacial, 2 mM EDTA disodium salt, pH 8) and visualized under UV light (GeneSnap v6.08, SynGene, Cambridge, UK) following GRGreen staining. Subsequently, PCR fragments were purified and concentrated according to the manufacturer's instructions using a PCR cleanup kit (MSB® Spin PCRapace, STRATEC Molecular GmbH, Berlin, Germany). Purified PCR products were sequenced in both directions by the Central Analytical Facility at Stellenbosch University and edited using the Geneious Pro version 5.4.6 software (Biomatters Ltd, Auckland, New Zealand). The nucleotide sequences of the ITS, TEF1-α and β-tub gene regions generated in this study were assembled separately and added to other sequences of *S. maydis*, its closely related species *S. macrospora* as well as other maize ear rot fungi that were retrieved from GenBank (http://www.ncbi.nlm.nih.gov)(Table 3). Sequences were aligned using Geneious Pro v.5.4.6 software and gene regions unique to *S. maydis* were visually identified.

Primer design: Species-specific primers were designed using Primer3 Input v.4.0.0 software (Untergrasser *et al*., 2012). Primers of sufficient GC contents, length and high annealing temperatures were chosen (> 55°C), thereby preventing co-amplification of non-specific DNA targets. Additionally, the resulting amplicon size was restricted to 100 base pairs (bp) for further evaluation under qRT-PCR conditions. Visual inspection of primer pairs based on the ITS sequences showed that they were not suitable for the species-specific amplification. However, primer sets Maydis1 F (5'-CTTGTCACATCACCCCTCCT-3') and Maydis1 R (5'-CCCAGGCATACTTGAAGGAA-3') and Maydis2 F (5'-CAGCACATGCCT TCCTTTCT-3') and Maydis2 R (5'-AGGAGGGGTGATGTGACAAG-3') were designed on the TEF1-α sequences to amplify a 161-bp and 196-bp PCR product, respectively. Primers Maydis3 F (5'-CAGCTCCAAGCCTACCACTG-3') and Maydis3 R (5'-GTGCTCGCCA

GAGATGGT-3') were designed based on the β-tub gene region to amplify a 104-bp PCR product. Primers were synthesized by Whitehead Scientific (Pty) Ltd in Cape Town, South Africa, reconstituted with 0.1 x TE buffer (pH 7.5) and made up to stock solutions of 100 mM.

Specificity of the primer sets to S. maydis: The designed primer sets were blasted against the National Centre of Biotechnology Information (NCBI) database to determine their specificity to the target genes. No homologies were found with any published nucleotide sequences including pathogenic ear rot fungi. PCR assays with the designed primer pairs were also tested on genomic DNA extracted from three *S. maydis* isolates as well as a range of unrelated fungal species known to frequently infect maize (Fig. 1). In order to avoid false negative results, PCR assays were conducted three times. The PCR reaction mixtures for the amplification of the TEF1-α and the ß-tubulin gene regions was as previously described and conducted in 25-µl reaction volumes. The amplification conditions for the TEF1-α gene region was as previously described and the optimized PCR thermal profile for the amplification of the ß-tubulin gene region consisted of an initial denaturation at 96°C for 5 min, followed by 30 cycles consisting of denaturation at 96°C for 30 s, primer annealing at 63°C for 30 s, primer extension at 72°C for 90 s and a final extension step at 72°C for 7 min.

After amplification, the PCR products were loaded onto a horizontal 2% agarose gel (w/v), separated by electrophoresis at 40 V for 90 min and stained with GRGreen. PCR products were then visualized under UV light and images of the gel were digitally captured using a GeneSnap v.6.08 (SynGene, Cambridge, UK) camera system.

Quantification of *F. graminearum* s.l., *A. flavus* and *S. maydis* in maize grain

Genomic DNA extractions: Fusarium graminearum isolate NRRL 28439, provided by Dr. O'Donnell from the Department of Agriculture-Agriculture Research Service (Peoria, USA); *S. maydis* isolate BF 10, provided by Prof. Flett from the ARC-GCI (Potchefstroom, South Africa) and *A. flavus* isolate MRC 3951, provided by Dr. Rheeder from the MRC-PROMEC unit (Tygerberg, South Africa) were utilized to generate DNA standards for the detection and quantification of these fungi in infected maize samples. These isolates are now maintained in the culture collection of the Department of Plant Pathology at Stellenbosch University.

Isolates NRRL 28439 and BF 10 were incubated on a rotary shaker at 25°C for 2 weeks. Following incubation, mycelium was harvested by filtration through two layers of sterile cheesecloth, washed twice with autoclaved water and freeze-died. The isolate of *A. flavus*

Stellenbosch University http://scholar.sun.ac.za

was grown on potato dextrose agar (PDA) at 30°C for 1 week after which the mycelia were scraped off into 2-ml Eppendorf tubes and freeze-dried. Freeze-dried material was stored at -20°C until DNA extractions were carried out.

Genomic DNA of the fungal isolates and milled maize samples was extracted with the DNeasy Plant Mini Kit (QIAGEN) using the protocols described by Boutigny *et al.* (2012). The DNA concentration of each maize sample was determined in duplicate using the NanoDrop ND-1000 Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). High quality DNA was obtained and diluted to a concentration of 10 ng μl^{-1} for the absolute quantification of the respective ear rot fungi in quantitative real-time PCR (qRT-PCR) assays.

Quantitative detection of maize ear rot fungi using qRT-PCR: The primers used for the detection of *F. graminearum* s.l. were FgramB379 fwd/FgramB411 (Nicolaisen *et al*., 2009) while Af2 fwd/Af2 rev were used for the detection of *A. flavus* (Mideros *et al.,* 2009) and Maydis3 fwd/Maydis3 rev for the detection of *S. maydis.*

All qRT-PCR assays were performed on a Rotor-gene TM 6000 (Corbett Life Science) in a total reaction volume of 25 µl consisting of 1 x SensiMix SYBR (Quantace), 200 nM of each primer and 20 ng μl^{-1} template DNA. For the amplification of *A. flavus* in maize samples, the assay employed 200 nM of the forward primer, 75 nM of the reverse primer and 10 ng μl^{-1} template DNA. The cycling conditions for the amplification of *F. graminearum* s.l. consisted of an initial denaturation step at 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s; 60ºC for 15 s and 72ºC for 15 s. The melting curve was set at 72ºC to 95ºC, rising by 1˚C each step (Boutigny *et al*., 2012) and gain optimization performed at the first acquisition. The cycling conditions for the amplification of *S. maydis* were the same as for *F. graminearum* s.l. with an annealing termperature of 64ºC. The cycling conditions for the amplification of *A. flavus* 95ºC for 10 min; 35 cycles of 59ºC for 30 s; 72ºC for 30 s, and a melting analysis of 72ºC to 95ºC, rising by 1ºC each step (Mideros *et al*., 2009). Gain optimization was performed at the first acquisition.

To test for linearity and presence of inhibitors, a 4-fold dilution series was prepared for each pathogen and contained purified fungal DNA ranging from 25 ng μl^{-1} to 24 pg μl^{-1} mixed in pure maize DNA at 10 ng μl^{-1}. Each standard sample was run in triplicate and standard curves were generated by plotting the threshold cycle values (C_t) against the logarithm of known DNA concentrations (measured by the Nanodrop). Criteria for DNA quality acceptance are a slope between -3.1 and -3.6 and efficiency between 0.90 and 1.1

Stellenbosch University http://scholar.sun.ac.za

(Mideros *et al.,* 2009). The standard curves produced for *F. graminearum* s.l. and *S. maydis* had a linear relation (R^2) above 0.99, efficiency of 0.98 and a slope of -3.3, whereas the standard curve for *A. flavus* had linearity above 0.99, efficiency of 0.92 and slope of -3.5 when only considering DNA concentrations from 10 to 0.01 ng μl^{-1} (due to inhibition at higher pathogen concentration). Inhibition was evaluated by extrapolating the C_t value of the undiluted sample with the equation calculated by linear regression and comparing it with the measured C_t value. The presence of inhibitors is indicated with ΔC_t above 0.5 (Boutigny *et al*., 2012). Additionally, assays containing one replicate of each maize sample, non-template control and triplicate standard DNA (diluted 16-fold) were carried out and the equation of R^2 used to transform C_t values into fungal biomass concentrations.

The robustness of real-time quantification for *F. graminearum* s.l. in contaminated grain has previously been validated in the same laboratory (Boutigny *et al*., 2012). Nonetheless, qRT-PCR reactions were also performed to determine intra- and inter-run variability of assays and to test reproducibility of the method used to extract DNA from maize infected with *S. maydis* and *A. flavus*. Four different maize samples were selected to accommodate the largest variation in contamination with *S. maydis.* Firstly, DNA was extracted from four independent sub-samples of each of these four samples of ground maize kernels and, secondly, three independent runs were performed for each maize sample and triplicates included in each run. These experiments were repeated with maize contaminated with *A. flavus.* For *S. maydis* and *A. flavus*, triplicate qRT-PCR on the same extracted DNA preparation within a single run showed marginal variation in C_t values (mean SD_{Sm} = 0.08, n_{Sm} = 60; mean SD_{Af} = 0.07, n_{Af} = 60) whereas larger variation was found between separate runs (mean SD_{Sm} = 0.20, n_{Sm} = 12; mean SD_{Af} = 0.19, n_{Af} = 12). Therefore, it can be concluded that the DNA extraction method and qRT-PCR assays used to assess *S. maydis* and *A. flavus* in artificially inoculated maize samples from field studies were highly reproducible.

Mycotoxin analysis

Milled maize (5 g) from the GER and AER trials were subjected to liquid chromatographic tandem mass spectrometry (LC-MS/MS) analysis as described in Chapter 2, with some modifications. The mycotoxins were extracted from milled maize (5 g) sub-samples as follows: 20 ml methanol extraction buffer (70% meOH:30% Milli-Q H_2O) was added to each sample and the suspension shaken at 200 rpm in an incubator/shaker set at 25°C for 30 min. The samples were then centrifuged at 4000 rpm at 4°C for 10 min. A sterile syringe was used to remove 2 ml clear extract that was filtered through a 0.20 µm nylon filter into a 2-ml

Eppendorf tube. The samples were placed at 4ºC overnight after which they were centrifuged for 10 min at 14 000 rpm before transferring 1.8 ml clear extract to LC-MS/MS glass vials (undiluted).

Standards of deoxynivalenol (DON)(5 mg), 3-acetyl-deoxynivalenol (3-A-DON)(5 mg), 15-acetyl-deoxynivelanol (15-A-DON)(5 mg), nivalenol (NIV)(5 mg) were obtained from Tega Marketing (Gauteng, South Africa), and standards of ZEA (10 mg) and an aflatoxin B and G mixture (AFB_1 and AFG_1 = 28.75 µg; AFB_2 and AFG_2 = 8.3 µg) were obtained from Sigma-Aldrich (Aston Manor, South Africa). TCT B (DON, A-DON and NIV) and ZEA were reconstituted in their own original glass vials with 1 ml acetonitrile (ACN). The vials were closed, well shaken and the respective mycotoxins left to fully dissolve. Each standard was then transferred in equal amounts into a 500-µl Eppendorf tube and used as stock solution. The final concentrations for TCT B and ZEA in the combined stock solution were 500 and 1000 ppm, respectively. A stock solution for aflatoxins was obtained by reconstitution of dried standards with 5 ml ACN, that had a final concentration of 5.75 and 1.66 µg $µl^{-1}$ for AFB_1/AFG_1 and AFB_2/AFG_2, respectively. Since aflatoxins are light sensitive, the 5-ml stock solution was aliquoted in volumes of 1 ml into 2-ml Eppendorf tubes and kept in the dark at 4 °C.

On the day of analysis, stock solutions were diluted with 70% methanol to generate calibration curves which were included in each run of the LC-MS/MS. Unkowns were plotted on the standard curve to calculate total levels of TCT B and ZEA ranging from 0.032 and 100 ppm for DON, A-DON and NIV, and between 0.064 and 200 ppm for ZEA. The detection range for the aflatoxins was 0.015 to 383 ppb for AFB_1/AFG_1, and between 0.004 and 110 ppb for AFB_2/AFG_2.

Multi-mycotoxin analysis was conducted at the Central Analytical Facility at Stellenbosch University. Solutions were thoroughly vortexed and 5 µl injected into the LC-MS/MS system. For samples with results above the standard curve limits (aflatoxins have high carry-over) an appropriate dilution was made using Milli-Q H_2O, and the samples re-analysed. Approximately 20% of the samples were run in duplicate for confirmation. Minimum limits of quantification for DON, NIV, A-DON, ZEA, AFB_1/AFB_2 and AFG_1/AFG_2 were 0.010, 0.100, 0.010 ppm, 0.5, 0.1 and 0.2 ppb, respectively. Diplonine concentrations potentially present in the DER maize samples were not quantified in this study as calibration standards for this mycotoxin are not commercially available.

Stellenbosch University http://scholar.sun.ac.za

Statistical analysis

Data obtained from the GER, AER and DER trials, were separately subjected to univariate analysis of variance using the General linear model (GML) procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Experimental results were combined in one ANOVA (John and Quenouille, 1977) after testing for homogeneity of variance using Levene's test (Levene, 1960). The Shapiro-Wilk test was performed to test for normality (Shapiro, 1965) and the Student's t-least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott, 1998). Non-parametric Pearson correlation coefficients were determined for relationships among ear rot severity, fungal biomass and mycotoxin contamination using the CORR procedure in SAS based on untransformed means (Chapter 2).

To determine the effects of inbred x location interaction on mycotoxin contamination, data from the GER and AER trials were subjected to genotype main effect and genotype by environmental interaction (GGE) biplot analysis using GenStat 15th edition (Payne *et al*., 2012). Visual representation of the GGE biplot was further supported with additive main effects and multiplicative interaction (AMMI) analysis of variance (Gauch and Zobel, 1996) performed in SAS version 9.2 (Chapter 2).

The consistency of the inbred lines reactions towards DER and *S. maydis* infection were established using Spearman rank correlations of the six (three locations x two treatments) regression points. Regression analyses with the model $Y = AX^b$ were used to determine the relationship between infection potential (X) of a trial site and fungal biomass within a genotype (Y). Infection potential is defined as the average fungal concentration over all inbred lines associated with a specific season, location and inoculation treatment (Flett and McLaren, 1994). Additionally, GGE and AMMI analyses were conducted to determine the inbred x location interaction effect on *S. maydis* biomass (inoculated treatment).

RESULTS

Gibberella ear rot

Disease severity, fungal biomass and mycotoxin production: After field inoculations, fungal colonization resulted in GER development and mycotoxin accumulation in all maize entries during the 2012/2013 season (Table 4). The mean GER severity for the nine inbred lines ranged from 4.70 ± 1.77% to 23.10 ± 21.9% at Potchefstroom, 1.00 ± 0.2 % to 85 ± 15% at Vaalharts, and 8.37 ± 4.53% to 28.82 ± 8.18% at Makhatini (Table 4). Mean fungal DNA

levels ranged from below 0.02 ng μl^{-1} (detectable limit) to 0.78 ± 0.19 ng μl^{-1} at Potchefstroom, 0.02 ± 0.01 ng μl^{-1} to 1.44 ± 0.19 ng μl^{-1} at Vaalharts, and 0.65 ± 0.34 ng μl^{-1} to 3.23 ± 1.32 ng μl^{-1} at Makhatini (Table 4). Mycotoxin contamination included DON, A-DON and ZEA as no NIV was detected in the maize samples. DON was detected in 96.29% of the maize samples collected (n = 81) with mean and maximum levels of 13.13 ppm and 35.29 ppm, respectively. All of the DON-contaminated samples also contained the acetylated derivative 15-A-DON (mean: 0.74 ppm, maximum: 3.01 ppm) while none of the samples contained 3-A-DON. ZEA was detected in 48.14% of the samples investigated with an average concentration of 1.13 ppm. According to the ANOVA, a significant inbred x location interaction was observed for both GER severity and fungal biomass ($P \leq 0.01$), but not for associated mycotoxin contamination ($P = 0.06$). GGE and AMMI (Table 5) analyses were used to best explain these interactions.

There were no significant differences between inbred lines evaluated for GER severity at Makhatini ($P = 0.75$). At the Potchefstroom and Vaalharts locations, however, the inbred lines differed significantly in their responses to GER severity ($P \leq 0.01$). At Vaalharts, for example, lines R2565Y, R119W and I137tnW exhibited significantly more GER symptoms when compared to the other inbred lines ($P \leq 0.05$)(Table 4). Additionally, the influence of location on disease development was exemplified by line R2565Y, having the highest disease severity at Vaalharts (85.00%) and the lowest at Makhatini (8.37%)(Table 4).

The individual inbred lines responded differently to *F. graminearum* s.l. infection within each location ($P \leq 0.05$), with lines VO617Y-1, US2540, CML444, R119W and CML390 being significantly more susceptible at Makhatini than at the other two field sites ($P \leq 0.05$). This location effect also manifested in the higher average *F. graminearum* s.l. concentration found at Makhatini (1.20 ng μl^{-1}) when compared with Potchefstroom (0.23 ng μl^{-1}) and Vaalharts (0.33 ng μl^{-1})(Table 4).

The higher average mycotoxin concentration found at Makhatini (20.54 ppm)(Table 4) was not surprising as fungal biomass and mycotoxin content (TCT B + ZEA) have been indicated to correlate well (Boutigny *et al.*, 2012). Similar mean mycotoxin levels observed at Potchefstroom (11.67 ppm) and Vaalharts (12.83 ppm) were merely co-incidental as significant differences between inbred lines were recorded at both locations (Table 4). At Makhatini, for instance, lines VO617Y-1(37.43 ppm) and R119W (25.32 ppm) accumulated significantly more mycotoxins than the other seven inbred lines evaluated ($P \leq 0.05$)(Table 4). Some individual inbred lines also differed significantly in their mycotoxin levels across locations (Table 5). For instance, lines VO617Y-1, US2540, CML444, CML390 and

CML182 had significantly higher mycotoxin concentrations at Makhatini ($P \leq 0.05$) than the other two field sites (Table 5).

Correlations between ear rot severity, fungal biomass and mycotoxin contamination: Despite fungal growth and mycotoxin contamination being present in all of the samples that showed symptoms of GER, a significant but poor correlation was established between visual and qRT-PCR data (Pearson correlation of r = 0.32; $P \leq 0.01$), as well as between visual and toxin rating (r = 0.53; $P \leq 0.01$). This finding is exemplified by line R2565Y which had a visual rating of 23.10% and *F. graminearum* s.l. concentration below the detectable limit (0.02 ng μl^{-1}) at Potchefstroom, or line US2540 having a GER severity of 13.04% while still containing 22.58 ppm of mycotoxins (TCT B + ZEA) at Makhatini (Table 4). Conversely, a strong and significant relationship was observed between the toxin and qRT-PCR results (r = 0.70; $P \leq 0.01$) which is supported by lines R2565Y and R119W having the highest mycotoxin (38.47 and 21.48 ppm, respectively) and *F. graminearum* s.l. (1.44 and 0.45 ng μl^{-1}, respectively) concentrations at Vaalharts in the 2012/2013 growing season (Table 4).

Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses: From the findings in the current study and in Chapter 2, it is palpable that visual ratings of disease severity should not be used as selection criteria for ear rot resistance and reduced mycotoxin contamination. Although expensive, mycotoxin analysis provides an alternative means for resistance screening in maize germplasm that is not only time-effective but also reliable. Therefore, the nine lines were ranked in terms of their total mycotoxin concentration to rid the problems encountered with normality and homogeneity of variance. Results were then combined in a GGE biplot (Fig. 2) for visual summary of stable resistance towards *F. graminearum* s.l. and its mycotoxins (TCT B + ZEA). The consistency of the inbred lines' performance across the multiple locations was also supported by AMMI stability values (ASV)(Table 6).

The GGE biplot is based on principle component analysis where the first two principle components were significant at $P = 0.01$ level, and cumulatively contributed to 91.3% of the genotypic variance (Fig. 2). This finding indicates that the inbred x location interaction is very complex. PC1 is located on the X-axis and across its value is estimated resistance, while PC2 is located on the Y-axis and presents performance stability of the inbred lines (Yan and Kang, 2003). For selection, the best inbred lines are considered those with high resistance to *F. graminearum* s.l. and stable performance across locations.

The genotypes were grouped into six sectors with the largest sector incorporating all three test environments (Fig. 2). Based on their toxin ranking and AVS, inbred lines CML390, CML182 and CML444 were constantly resistant to mycotoxin accumulation across locations (Fig. 2; Table 6). The rest of the inbred lines evaluated were not suited for any target-environment as they performed less than average. For example, line US2540 exhibited high resistance but low stability and lines VO617Y-1 and I137tnW exhibited low stability and moderate resistance (Fig. 2; Table 6). Furthermore, lines R119W, R2565Y and R0549W had low stability and low resistance to mycotoxin accumulation (Fig. 2; Table 6).

Aspergillus ear rot

Disease severity, fungal biomass and aflatoxin production: Following artificial silk channel inoculation, disease development was generally low with a higher AER severity recorded at Makhatini (4.97%) than at Potchefstroom (1.21%) and Vaalharts (0.62%)(Table 7). In addition, *A. flavus* and aflatoxin concentrations determined in the maize samples were also low (Table 7). Average fungal biomass content ranged between 0.3 ± 0.13 ng μl^{-1} and 0.31 ± 0.01 ng μl^{-1} at Potchefstroom, < 0.3 ng μl^{-1} (detectable limit) and 1.26 ± 0.75 ng μl^{-1}at Vaalharts, and from < 0.3 ng μl^{-1} to 0.3 ± 0.01 ng μl^{-1} at Makhatini. The mean total aflatoxin levels ranged from < 0.1 ppb (detectable limit) to 0.1 ± 0.02 ppm at Potchefstroom, and < 0.1 ppb to 0.18 ± 0.15 ppm at Vaalharts. No aflatoxins accumulated in the individual inbred lines at the Makhatini site (Table 7). The ANOVA indicated a significant inbred x location effect for AER severity, fungal biomass and aflatoxin contamination ($P \leq 0.01$). These interactions were explained by GGE and AMMI analyses (Table 8).

Although low disease severity percentages were recorded, significant difference could be determined for the AER trials at all the field locations ($P \leq 0.01$). At Makhatini, lines CML390 (9.28%) and CML444 (0.68%) differed significantly ($P \leq 0.05$) in their visual ratings whereas line R119W (4.91%) exhibited significantly more AER symptoms at Potchefstroom when compared to the other inbred lines ($P \leq 0.05$)(Table 7). At Vaalharts, inbred lines VO617Y-1, R119W, CML390, CML182 and I137tnW (0.05 to 0.19%) had significantly less AER than lines R2565Y, R0549W, US2540 and CML444 (0.58 to 2.50%) displaying the highest disease severities ($P \leq 0.05$) (Table 7). Significant differences in response of inbred lines to AER also occurred amongst field locations. At Makhatini, for instance, lines CML390 (9.28%), CML182 (6.37%), R119W (7.81%), R2565Y (4.32%) and I137tnW (7.11%) were significantly more prone to disease development than at Potchefstroom and Vaalharts ($P \leq 0.05$). Maize inbred lines VO617Y-1, R2565Y, R0549W,

Stellenbosch University http://scholar.sun.ac.za

US2540 and CML444 were the only lines constantly having low visual ratings (0.12 to 4.32%) across the tested locations (Table 7).

At Makhatini, no significant difference between inbred lines for mean fungal content was observed ($P = 0.39$)(Table 7). However, the individual inbred lines differed significantly in their response to fungal colonization at Potchefstroom and Vaalharts ($P \leq 0.01$). For instance, at the Potchefstroom lines R2565Y, R0549W, CML444, CML390 and CML182 (0.03 ng μl^{-1}) accumulated lower fungal DNA than lines VO617Y-1, US2540, R119W and I137tnW (0.04 to 0.31 ng μl^{-1}) (Table 7). In contrast, lines R2565Y (1.26 ng μl^{-1}) and R0549W (0.76 ng μl^{-1}) displayed the highest *A. flavus* concentrations in the Vaalharts trail, exemplifying the highly significant inbred x location interaction ($P \leq 0.01$)(Table 8).

Aflatoxins were only detected in maize lines tested at Vaalharts and Potchefstroom with genotypic differences in aflatoxin accumulation being significant at both locations ($P \leq 0.01$). At Potchefstroom, for example, lines VO617Y-1 (0.02 ppm) and R119W (0.10 ppm) recorded higher aflatoxin levels compared to the other inbred lines (Table 7). A significant inbred x location effect was observed for aflatoxin contamination ($P = 0.00$) despite lines R0549W, US2540, CML444, CML390 and CML182 having aflatoxin levels below the detectable limit of 0.1 ppb at both the Potchefstroom and Makhatini locations (Table 7).

Correlations between ear rot severity, fungal biomass and aflatoxin contamination: Certain lines recorded a high disease severity but low fungal concentrations, such as line US2540 which had a disease severity of 2.5% and *A. flavus* concentration of 0.04 ng μl^{-1} at Vaalharts; or a high disease severity and low aflatoxin content, indicated by line CML390 with AER symptoms of 9.28% and no aflatoxin contamination (< 0.1 ppb) at Makhatini (Table 7). It was, therefore, not surprising that the visual and qRT-PCR data as well as the visual and toxin data did not correlate well (Pearson correlation of $r = -0.21$; $P = 0.06$ and $r = -0.16$; $P = 0.16$). Conversely, the fungal biomass and aflatoxin results correlated moderately well ($r = 0.59$; $P \leq 0.01$).

Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses: GGE biplot analysis of total aflatoxin content revealed that PC1 and PC2 accounted for 78.3% and 21.6%, respectively, explaining a total of 100% variation (Fig. 3). Two groups of environments could be distinguished (Fig. 3). The first group includes the Makhatini and Potchefstroom environments with inbred lines CML182 and CML444 as the resistant genotypes and the second group includes the

Vaalharts location with line CML390 having the lowest aflatoxin levels. Conversely, inbred lines R119W and VO617Y-1 are not suited for any environment as they were classified as the susceptible genotypes in this study (Fig. 3). None of these resistant or susceptible lines were consistent in their performance across the field sites (Table 6) and therefore it is required that the identified target-environments are verified within multi-year experiments.

Diplodia ear rot

Disease severity and fungal biomass: With three exceptions, artificial inoculation did not significantly increase the level of DER found in the inoculated maize samples when compared with the natural infection at the field locations (P = 0.19)(Table 9). There were, however, significant differences observed between inoculated and uninoculated lines in mean *S. maydis* biomass (P = 0.02). Inoculated treatment means for disease severity and fungal concentration reached maximum levels, respectively, of 44.14% and 0.85 ng μl^{-1} at Potchefstroom, 32.80% and 0.41 ng μl^{-1} at Vaalharts, and 6.67% and 0.08 ng μl^{-1} at Makhatini (Table 9) which could allow for proper discrimination between susceptible and resistant genotypes. A significant inbred x location interaction was observed for fungal biomass (P = 0.02), but not for DER severity (P = 0.05)(Table 10).

While most of the inoculated inbred lines (lines VO617Y-1, US2540, CML444, CML390 and I137tnW) showed comparable responses to DER across locations ($P \geq 0.05$), significant differences in the reactions of some inbred lines were evident within trial sites (Table 9). For example, at Potchefstroom, lines R119W (43.88%) and I137tnW (44.14%) exhibited considerably more severe DER symptoms than lines VO617Y-1 (6.43%) and CML390 (4.79%) whereas at the Vaalharts location, lines US2540 (2.13%) and CML390 (8.77%) had significantly less ear rot than those displaying the highest severities (lines R2565Y, R0549W and I137tnW)(28.07 to 32.80%)(Table 9).

The inoculated inbred lines differed significantly from each other in their fungal biomass amounts at Potchefstroom (P = 0.03) and Makhatini (P = 0.04) but not at Vaalharts (P = 0.61) (Table 9). For example, at Potchefstroom lines R0549W (0.85 ng μl^{-1}) and R119W (0.74 ng μl^{-1}) developed significantly more *S. maydis* DNA ($P \leq 0.05$) than lines VO617Y-1 (0.02 ng μl^{-1}), R2565Y (0.10 ng μl^{-1}), CML444 (0.05 ng μl^{-1}) and CML390 (0.02 ng μl^{-1}). The resistance of the lines to fungal colonisation over locations were also inconsistent as indicated by a significant inbred x location effect (P = 0.02)(Table 10) and poor spearman rank correlation. Rankings ranged from -0.91 to 0.56, with only one of a possible 15 pair combinations (three locations x one season x two treatments/$\left(\frac{6:}{4:2:}\right)$) positively correlated (r >

0.7)(data not shown).

Correlations between ear rot severity and fungal biomass: Primer sets Maydis1 F/Maydis1 R and Maydis2 F/Maydis2 R routinely amplified DNA from *S. maydis* and *F. graminearum* s.l., *F. verticillioides*, *F. subglutinans* and *F. proliferatum* (data not shown). On the other hand, primer set Maydis3 F/Maydis3 R was specific for *S. maydis* (Fig. 1), and was thus used for quantitative detection of *S. maydis* in maize grain.

The relationship found between DER severity and fungal biomass in inoculated treatments was significant but poor (Pearson correlation of $r = 0.37$; $P \leq 0.01$). This could be explained by certain lines, such as lines R2565Y and CML390, having similar infection levels (± 0.23 ng μl^{-1}) but different disease severities (32.80% and 8.77%, respectively) at Vaalharts during the 2012/2013 growing season (Table 9).

Regression analysis: qRT-PCR data (inoculated and uninoculated treatments) for each inbred line were used in the regression model $Y = AX^b$, where Y = mean fungal biomass recorded in each line and X = the mean infection level of a trial site (infection potential). This served to explain the true nature of a genotype's resistance status in the presence of location effects (Flett and McLaren, 1994). Inbred lines were characterized according to their B parameter where $B = \pm 1$ indicated moderate resistance (intermediate); $B < 1$ indicated resistance and $B > 1$ indicated susceptibility to *S. maydis* infection (Table 11). Lines below the regression line (X=Y) in Fig. 4, such as lines VO617Y-1, R2565Y, CML444 and CML390 were regarded as the resistant genotypes and lines above regression, such as lines R0549W and R119W were regarded the susceptible genotypes. Additionally, lines US2540, CML390 and I137tnW were moderately resistant due to their close proximity to linear regression (Fig. 4). To support this finding, confidence limits were fitted to regression lines but mostly overlapped which prevented reliable selection of resistant and susceptible genotypes (data not shown). For this reason, GGE and AMMI analyses were carried out on the qRT-PCR data from the inoculated DER field trials.

Genotype main effect, genotype by environment interaction (GGE) and additive main effects and multiplicative interaction (AMMI) analyses: The partitioning of the GE effect through biplot analysis showed that PC1 and PC2 accounted for 52.5% and 37.2% of the GGE sum of squares, respectively, explaining a total of 89.7% variation (Fig. 5). Inbred lines with the lowest accumulated *S. maydis* DNA concentrations included lines VO617Y-1 > CML390 >

R2565Y > CML444 (Fig. 5). Amongst these, lines VO617Y-1 and CML444 are more desirable because lines R2565Y and CML390 were very unstable in their performance as indicated by their ASV values (Table 6). Furthermore, lines VO617Y-1 and CML390 had Potchefstroom and Vaalharts as target-environments while lines R2565Y and CML444 were best suited for the Makhatini location (Fig. 5). The other five inbred lines performed poorly across locations, with lines US2540, CML182 and I137tnW being moderately resistant whereas lines R0549W and R119W are sensitive towards *S. maydis* infection (Fig. 5). These results are in agreement with the observations earlier made with regression analysis (Fig. 4).

Weather data

Average monthly temperatures were slightly lower at Potchefstroom than at Vaalharts during the growing season (December to July)(Fig. 6). In contrast, Makhatini was notably warmer between March and July when compared to the other two field sites (Fig. 6). This period represents the seedling emergence (March), inoculation (May) and grain filling (June) stages of maize production at Makhatini as the planting date for the Makhatini trials were postponed to February 2013. Mean monthly rainfall was higher at Potchefstroom at the beginning of the season (December) and over the pollination (March) and drying periods (April) than at Vaalharts (Fig. 6). At the Makhatini location, an average rainfall of 16.60 mm was recorded between February and September, which is considerably less when compared with Potchefstroom (83.38 mm) and Vaalharts (70.49 mm)(Fig. 6).

DISCUSSION

Among the different strategies available, plant breeding offers the best opportunity to reduce mycotoxin contamination in grain crops effectively. Paucity in the literature on adequate ear rot-resistance in maize germplasm, supports that the current study is of great importance as it represents the most comprehensive evaluation of local genotypes' resistance to multiple infections by mycotoxigenic fungi to date. These inbred lines were chosen by plant breeders from ARC-Grain Crops Institute (Potchefstroom, South Africa) for their good combinability and agronomic features, and are therefore, ideal for use in plant breeding programmes.

Artificial inoculation of FER-resistant inbred lines resulted in high GER severities as well as TCT B (DON + A-DON) and ZEA concentrations (38.29 and 6.76 ppm, respectively) which are greater than the maximum tolerable levels allowed by United States Food and Drug Administration (NGFA, 2011). High TCT B and ZEA levels found in maize samples are not

Stellenbosch University http://scholar.sun.ac.za

unlikely since FER-resistant material prevented the antagonistic abilities of *F. verticillioides* in the field, which in turn could have allowed growth and proliferation of *F. graminearum* s.l. (Picot *et al*., 2012). The strong and significant correlation found between mycotoxin and fungal content support the notion that DON and its acetylated forms may play a role in fungal pathogenicity (Harris *et al*., 1999).

AER severity, fungal biomass and aflatoxin contamination was expectedly low at all three test locations. It could be argued that the climate in South Africa is unfavourable for *A. flavus* growth and aflatoxin production, making resistance screening difficult. Correlated responses of the lines for FER and AER resistance are, however, in accordance with previous studies (Robertson-Hoyt *et al*., 2007; Henry *et al*., 2009) and suggest that common resistance mechanisms may function for the two diseases.

Stenocarpella maydis has been an important ear rot pathogen of maize in South Africa since the early 1980's when the breeding line B73 was extensively used. Commercial companies, therefore, screen hybrids annually before release to prevent susceptible hybrids from entering the local the market (Schoeman and Flett, 2012). Artificial inoculation in this study did not significantly increase the level of DER found in the inoculated maize samples when compared with natural infection at the field sites. This could imply that artificial inoculation might not be necessary when screening for DER-resistance. However, early harvesting of maize ears may have prevented proper disease development, which could explain the low percentage of visual symptoms. Artificial inoculation thus remains important to ensure suitable selection of DER-resistant material because several reports have indicated that natural epidemics of *S. maydis* do not occur every year (Du Toit and Nordier, 1989; 1990; 1991; Flett and McLaren; 1994).

Since *S. maydis* infection is not limited to the surface of maize kernels and visual ratings may result in inaccurate results (Barros *et al*., 2008), a quick and reliable method to detect *S. maydis in planta* was important to develop. The species-specific primer set developed in this study (Maydis3 fwd/Maydis3 rev) proved to be highly specific to distinguish *S. maydis* from other maize ear rot pathogens, which justified its use for quantification of this fungus in the maize samples. However, it should be further tested against all other *Stenocarpella* and related species before being used as a general diagnostic tool.

The significant differences in disease severity, fungal biomass and mycotoxin levels observed between trials could be attributed to variation in environmental factors such as temperature and humidity (Picot *et al*., 2010). Additionally, poor husk protection, droopy ears, slow ear drydown, water logging and feeding-damage of birds and insects are known to

increase GER severity (Munkvold, 2003), whereas kernel damage may also result in higher AER severity in the field (Narasaiah *et al*., 2006). Higher DER incidences have been reported in maize monocultures where no or limited tilling practices are applied (Flett *et al*., 1998). In the current study, mean *F. graminearum* s.l. levels and total mycotoxin contamination (TCT B + ZEA) were significantly more severe at Makhatini when compared with the other field sites. This could not be explained by weather data since the temperature and rainfall amounts of the field sites were similar after silking, the period most critical for infection (Munkvold, 2003). Water logging and feeding-damage of birds recorded in the field and during disease evaluation could possibly explain the extended mycotoxin contamination at Makhatini (Munkvold, 2003). A higher average *A. flavus* and aflatoxin concentration was further recorded at Vaalharts in spite of having a lower ear rot rating compared to Potchefstroom and Makhatini. *Aspergillus flavus* is regarded as a storage pathogen, but may produce aflatoxins in the field under stressful conditions such as water- and nutrient deficiency (Narasaiah *et al*., 2006; Reverberi *et al*., 2008). Despite the low rainfall recorded during the season at each location, the trials were irrigated weekly, thereby excluding drought stress as explanation. It is therefore speculated that the enhanced aflatoxin production was merely a response to plant stress due to the competition with weeds at Vaalharts as no herbicide treatments were applied. A slightly higher rainfall at Vaalharts and Potchefstroom during silking (March), combined with increased relative humidity due to irrigation, could explain the higher *S. maydis* infection found at these two locations (Schoeman and Flett, 2012).

The low correlation coefficients found for both visual and qRT-PCR rating, and visual and toxin rating, highlighted the importance of mycotoxin analysis in routine screening for resistance to ear rot pathogens. However, quantification of mycotoxin concentrations in maize samples is cost-prohibitive and requires certain finesse. More efficient selection systems are therefore being sought to replace traditional phenotype-based selection systems. Marker-assisted selection has been proposed by many authors who studied the complex nature of heritability and correlations of AER and GER diseases and reduced mycotoxin contamination (Ali *et al*., 2005; Brooks *et al.* 2005; Martin *et al*., 2011; Martin *et al*., 2012).

In summary, none of the maize inbred lines in this study were completely resistant to *F. graminearum* s.l., *A. flavus* and *S. maydis* and subsequent mycotoxin contamination. Line R119W appeared to be highly susceptible while lines CML444 and CML390 were the only genotypes with good resistance to infection and multi-mycotoxin accumulation. Even so, none of these candidates were stable in their performance across the test locations and should be evaluated in multi-year experiments to further confirm their level of resistance. The

Stellenbosch University http://scholar.sun.ac.za

existence of common resistance gene areas for the different ear rot fungi warrants future investigation. This might require QTL analysis following crosses between highly resistant (for example line CML390) and susceptible (for example line R119W) inbred lines.

Stellenbosch University http://scholar.sun.ac.za

REFERENCES

Afolabi, C.G., Ojiambo, P.S., Ekpo, E.J.A., Menkir, A. and Bandyopadhyay, R. 2007. Evaluation of maize inbred lines for resistance to Fusarium ear rot and fumonisin accumulation in grain in tropical Africa. Plant Disease 91: 279-286.

Ali, M.L., Taylor, J.H., Jie, L., Sun, G., William, M., Kasha, K.J., Reid, L.M. and Pauls, K.P. 2005. Molecular mapping of QTLs for resistance to Gibberella ear rot, in corn, caused by *Fusarium graminearum*. Genome 48: 521-533.

Bänziger, M. and De Meyer, J. 2002. Collaborative maize variety development for stress-prone environments in Southern Africa. Pages 269-296 in: Farmers, scientists and plant breeding: integrating knowledge and practice. D.A. Cleveland and D. Soleri, eds. CABI Publishing, Wallingford, UK.

Barros, E., Crampton, M., Marais, G. and Lezar, S. 2008. A DNA-based method to quantify *Stenocarpella maydis* in maize. Maydica 53: 125-129.

Boutigny, A.-L., Beukes, I., Small, I., Zühlke, S., Spiteller, M., Van Rensburg, B.J., Flett, B.C. and Viljoen, A. 2012. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. Plant Pathology 61: 522-531.

Boutigny, A.-L., Ward, T.J., Van Coller, G.J., Flett, B.C., Lamprecht, S.C., O'Donnell, K. and Viljoen, A. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48: 914-920.

Brooks, T.D., Williams, W.P., Windham, G.L., Willcox, M.C. and Abbas. H.K. 2005. Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. Crop Science 45: 171-174.

Carbone, I. and Kohn, L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

Chambers, K.R. 1987. Ability of fungal isolates from maize and sorghum to infect roots and reduce seedling emergence of two maize hybrids. Plant disease 71: 736-739.

Chen, Z.-Y., Brown, R.L., Cleveland, T.E., Damann, K.E. and Russin, J.S. 2001. Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. Journal of Food Protection 64: 1785-1792.

Chen, Z.-Y., Brown, R.L., Russin, J.S., Lax, A.R. and Cleveland, T.E. 1999. A maize trypsin inhibitor with antifungal activity inhibits *Aspergillus flavus* α-amylase. Phytopathology 89: 902-907.

Stellenbosch University http://scholar.sun.ac.za

Du Toit, W., and Nordier, G.A. 1989. National cultivar trials with commercial maize cultivars. Department of Agricultural Development, Republic of South Africa.

Du Toit, W., and Nordier, G.A. 1990. National cultivar trials with commercial maize cultivars. Department of Agricultural Development, Republic of South Africa.

Du Toit, W., and Nordier, G.A. 1991. National cultivar trials with commercial maize cultivars. Department of Agricultural Development, Republic of South Africa.

Flett, B.C. and McLaren., N.W. 1994. Optimum disease potential for evaluating to *Stenocarpella maydis* ear rot in corn hybrids. Plant Disease 78: 597-589.

Flett, B.C., McLaren, N.W. and Wehner, F.C. 1998. Incidence of ear rot pathogens under alternating corn tillage practices. Plant Disease 82: 781-784.

Gauch, H. and Zobel, R. 1996. AMMI analysis of yield trials. Pages 85-122 in: Genotype by environment interaction. M. Kang and H. Gauch, eds. Boca Raton CRC Publishing, New York, USA.

Glass, N.L. and Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied Environmental Microbiology 6: 1323-1330.

Harris, L.J., Desjardins, A.E., Plattner, R.D., Nicholson, P., Butler, G., Young, J.C., Weston, G., Proctor, R.H. and Hohn, T.H. 1999. Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. Plant Disease 83: 954-960.

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

James, C. 2008. Global Status of Commercialized Biotech/GM Crops: 2008. Ithaca, NY, ISAAA. 11 pp.

John, J.A. and Quenouille, M.H. 1977. Experiments: Design and Analysis, 2nd ed. The Garden City Press Limited, Letchworth, Hertfordshire, UK.

Levene, H. 1960. Contributions to probability and Statistics. Stanford University Press, Stanford, California, 278 pp.

Logrieco, A., Mule`, G., Moretti, A. and Bottalico, A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. European Journal of Plant Pathology 108: 597-609.

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: A historical perspective. Environmental Health Perspectives 109: 239-243.

Stellenbosch University http://scholar.sun.ac.za

Martin, M., Miedaner, T., Dhillon, B.S., Ufermann, U., Kessel, B., Ouzunova, M., Schipprack, W. and Melchinger, A.E. 2011.Colocolization of QTL for Gibberella ear rot resistance and low mycotoxin contamination in early European maize. Crop Science 51: 1935-1945.

Martin, M., Miedaner, T., Schwegler, D.D., Kessel, B., Ouzunova, M., Dhillon, B.S., Schipprack, W.,.Utz, H.F. and Melchinger, A. E. 2012. Comparative quantitative trait loci mapping for Gibberella ear rot resistance and reduced deoxynivalenol contamination across connected maize populations. Crop Science 52: 32-43.

Mideros, S.X., Windham, G.L., Williams W.P. and Nelson, R.J. 2009. *Aspergillus flavus* biomass in maize estimated by quantitative real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. Plant Disease 93: 1163-1170.

Moremoholo, L., Shimelis H. and Mashela, P.W. 2010. Yield response and Stenocarpella ear rot reaction among maize inbred lines and top cross hybrids. Euphytica 174: 231-238.

Mukanga, M., Derera, J. and Tongoona, P. and Laing, M.D. 2011. Farmer's perceptions and management of maize ear rots and their implications for breeding for resistance. African Journal of Agricultural Research 6: 4544-4554.

Munkvold, G.P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopathology 41: 99-116.

Narasaiah, K.V., Sashidhar, R.B. and Subramanyam, C. 2006. Biochemical analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. Mycopathologia 162: 179-178.

NGFA. National Grain and Feed Association. 2011. FDA mycotoxin regulatory guidance: A guide for grain elevators, feed manufacturers, grain processors and exporters. 5pp.

Nicolaisen, M., Suproniene, S., Nielsen, L.K., Lazzaro, I., Spliid, N.H. and Justesen, A.F. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. Journal of Microbiological Methods 76: 234-240.

Nowell, D.C. 1997. Studies on ear rot and grey leaf spot of maize in South Africa. PhD thesis. University of Natal, Pietermaritzburg, South Africa.

Ott, R.L. 1998. An Introduction to Statistical methods and data analysis. Duxbury Press, Belmont, California, USA. 837 pp.

Payne, R., Murray, D.A., Harding, S.A., Baird, D.B. and Soutar, D.M. 2012. GenStat for Windows (15th ed.) Introduction. VSN International, Hemel Hempstead. SAS. 2000. SAS/STAT Users Guide, Version 8, First Edition, Volume 2. SAS Institute Inc., Cary, NC, USA.

Stellenbosch University http://scholar.sun.ac.za

Picot, A., Barreau, C., Pinson-Gadais, L., Caron, D., Lannou, C. and Richard-Forget, F., 2010. Factors of the *Fusarium verticillioides*-maize environment modulating fumonisin production. Critical Reviews in Microbiology 36: 221-231.

Picot, A., Hourcade-Marcolla, D., Barreau, C., Pinson-Gadais, L., Caron, D., Richard-Forget, F. and Lannou, C. 2012. Interactions between *Fusarium verticillioides* and *Fusarium graminearum* in maize ears and consequences for fungal development and mycotoxin accumulation. Plant Pathology 61: 140-151.

Rao, K.N. and Ristanovic, L.D. 1986. The Maize Pathology Program in Zambia. A proceedings of the first eastern, central and Southern Africa regional maize workshop, Lusaka, Zambia, March 10-17. (Book of Abstr.) 259 pp.

Reid, L.M., Hamilton, R.I. and Mather, D.E. 1996. Screening maize for resistance to Gibberella ear rot. Agriculture and Agri-Food Canada; Technical Bulletin 196-5E.

Reid, L.M., Mather, D.E., Bolton, A.T. and Hamilton, R.I. 1994. Evidence for a gene for silk resistance to *Fusarium graminearum* Schw. ear rot of maize. Journal of Heredity 85:118-121.

Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., Camera, E., Fabbri, C., Picardo, M., Fanelli, C. and Fabri, A.A. 2008. Modulation of anti-oxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the *ApyapA* gene. Eukaryotic Cell 7: 988-1000.

Richard, J.L. and Payne, G.A. 2002. Mycotoxins: Risks in Plant, Animal and Human Systems. Task Force report Council for Agricultural Science and Technology, Ames, Iowa, USA. 103 pp.

Robertson-Hoyt, L.A., Betran, J., Payne, G.A., White, D.G., Isakeit, T., Maragos, C.M., Molnar, T.L. and Holland, J.B. 2007. Relationships among resistances to Fusarium and Aspergillus ear rots and contamination by fumonisin and aflatoxin in maize. Phytopathology 97: 311-317.

Rossouw, J. D., van Rensburg, J. B. J. and van Deventer, C. S. 2002. Breeding for resistance to ear rot of maize caused by *Stenocarpella maydis* (Berk.) Sutton. Evaluation of selection criteria. South African Journal of Plant Soil 19: 182-187.

Russell, R., Paterson, M. and Lima, N. 2010. How will climate change affect mycotoxins in food? Food Research International 43: 1902-1914.

Shapiro, S.S. and Wilk, M.B. 1965. An analysis of Variance Test for Normality (complete samples). Biometrika 52: 591-611.

Stellenbosch University http://scholar.sun.ac.za

Schoeman, A. and Flett, B.C. 2012. Diplodia and stalk rot of maize in the spotlight. SA Grain. Online publication: www.grainsa.co.za/diplodia-ear-and-stalk-rot-of-maize-in-the-spotlight. (20 December 2013).

Small, I.M., Flett, B.C., Marasas, W. F. O., McLeod, A., Stander, M. A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticillioides* and fumonisin accumulation in South Africa. Plant Disease 96: 881-888.

Snyman, L.D., Kellerman, T.S., Veggelaar, R., Flett, B.C., Basson, K.M. and Schultz, R.A. 2011. Diplonine, a neurotoxin isolated from cultures of the fungus *Stenocarpella maydis* (Berk.) Sacc. that induces Diplodiosis. Journal of Agriculture and Food Chemistry 59: 9039-9044.

Untergrasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G. 2012. Primer3 – new capabilities and interfaces. Nucleic Acids Research 40: e115.

Van Rensburg, J.B.J. and Ferreira, M.J. 1997. Resistance of elite maize inbred lines to isolates of *Stenocarpella maydis* (Berk.) Sutton. South African Journal of Plant and Soil 14: 89-92.

Van Rensburg, J.B.J. and Flett, B.C. 2010. A review of research achievements on maize stem borer, Busseola fusca (Fuller) and Diplodia ear rot caused by *Stenocarpella maydis* (Berk. Sutton). South African Journal of Plant and Soil 27: 74-80.

Van Rensburg, J.B.J., Rossouw, J.D. and Deventer, C.S.V. 2003. New generation maize inbred lines resistant to Diplodia ear rot caused by *Stenocarpella maydis* (Berk) Sutton. South African Journal of Plant and Soil 20: 127.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols: a guide to methods and applications. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds. Academic Press, San Diego, U.S.A.

Yan, W. and Kang, M. 2003. GGE Biplot Analysis: A graphical tool for breeders, geneticists and agronomists. CRC Press, Boca Raton, Florida, USA. 271 pp.

Yuan, J., Ali, M.L., Taylor, J.H, Liu, J., Sun, G., Liu, W., Masilimany, P., Gulati-Sakhuja, A. and Pauls, K.P. 2008. A guanylyl cyclase-like gene is associated with Gibberella ear rot resistance in maize (*Zea mays* L). Theoretical and Applied Genetics 116: 465-479.

Table 1. Maize inbred lines evaluated for resistance to Gibberella, Aspergillus and Diplodia ear rot and associated mycotoxin contamination in a multi-location trail in South Africa during the 2012/2013 season.

Inbred line identity	Origin	Resistance: FER/Fumonisin	Resistance: GER/Trichothecenes B/ Zearalenone	Resistance: DER/Diplonine	Resistance: AER/Aflatoxin
VO617Y-1[A]	ARC-GCI-South Africa	Resistant	Unknown	Unknown	Unknown
R2565Y[A]	ARC-GCI-South Africa	Susceptible	Unknown	Unknown	Unknown
R0549W[A]	ARC-GCI-South Africa	Resistant	Unknown	Unknown	Unknown
US2540[A]	ARC-GCI-South Africa	Resistant	Unknown	Unknown	Unknown
CML444[A]	CIMMYT-Zimbabwe	Resistant	Unknown	Unknown	Unknown
R119W[A]	ARC-GCI-South Africa	Susceptible	Unknown	Unknown	Unknown
CML390[A]	CIMMYT-Zimbabwe	Resistant	Unknown	Unknown	Unknown
CML182[B]	CIMMYT-Zimbabwe	Resistant	Unknown	Unknown	Unknown
I137tnW[B]	ARC-GCI-South Africa	Susceptible	Unknown	Unknown	Unknown

ARC-GCI, Agricultural Research Council Grain Crops Institute

CIMMYT, International Maize and Wheat Improvement Centre

[A] Maize inbred lines evaluated for resistance to FER and fumonisin accumulation during field trials (Chapter 2)

[B] Maize inbred lines evaluated for resistance to FER and fumonisin accumulation during field and glasshouse trials in Small *et al*. (2012)

Stellenbosch University http://scholar.sun.ac.za

Table 2. Visible symptoms of the major ear rot diseases of maize in Sub-Saharan Africa (Adapted from Mukanga *et al*., 2011).

Kernel discoloration	Nature of damage	Ear rot disease	Fungal species
Yellow-green grain	Localized rotten kernels with insect mining in between kernels	Aspergillus ear rot	*A. flavus* *A. parasiticus*
Dense white or greyish brown (latent infection) mold all over the grain, sometimes with black pycnidia	Lightweight, bleached, shrunken kernels. Rotting from the base upwards	Diplodia ear rot	*S. maydis* *S. macrospora*
Pale pink, reddish brown or lavender grain surface with white streaks	Rotten grain scattered all over the cob Rotting from ear tip downwards	Fusarium ear rot	*F. verticillioides* *F. proliferatum* *F. subglutinans*
Reddish or pink mycelia covering the grain	Rotten grain Rotting from ear tip downward	Gibberella ear rot	*F. graminearum sensu lato*
Powdery blue-green growth on and in between kernels	Localized bleached and streaked kernels	Penicillium ear rot	*P. oxalicum* *P. chrysogenum* *P. cyclopium* *P. funiculosum*

Table 3. Genbank accession numbers for gene sequences used to design species-specific primers for the quantification of *Stenocarpella maydis* in maize.

		Genbank accession numbers		
Species	**Strain number**	**ITS**	**TEF1-α**	**β-tub**
Aspergillus flavus	290499/60	AY677676	-	-
	-	AB008415	-	-
A. parasiticus	-	JX857815	-	-
Fusarium graminearum sensu lato	92 R	JQ363729	-	-
	ITEM 8504	-	JN687920	-
	ICMP 15495	-	-	EU490257
F. proliferatum	MTCC 9690	HM245296	-	-
	MRC 8550	-	GU564309	GU564312
F. subglutinans	MD 29	JQ886413	-	-
	NRRL 22016	-	HM057336	-
	ATCC 38016	-	-	AB587056
F. verticillioides	HTITV 50	KF010170	-	-
	5 SNO	-	GU5642961	-
	MRC 7817	-	-	AF366546
	K 351	-	-	KF484464
Penicillium oxalicum	YLJ 87	JX231010	-	-
P. viridicatum	IBT 5273	AJ005482	-	-

Stellenbosch University http://scholar.sun.ac.za

Phomopsis viticola	Pho CT1L	-	-	HQ586930
Phaecytostroma ambiguum	CPC 16775	FR748034	FR748066	-
	CPC 16776	FR748035	FR748067	-
	CPC 17072	FR748037	FR748069	-
Stenocarpella macrospora	CBS 117560	FR748048	-	-
	CPC 11863	FR748049	-	-
S. maydis	CBS 117558	FR748051	FR748080	-
	CBS 117559	FR748052	FR748081	-
	CPC 16779	FR748055	FR748084	-
	CPC 16781	FR748056	FR748085	-
	CPC 16784	FR748059	FR748088	-

Stellenbosch University http://scholar.sun.ac.za

Table 4. Evaluation of nine maize inbred lines for resistance to Gibberella ear rot in a multi-location field trial in South Africa during the 2012/2013 growing season.

Inbred line	**Potchefstroom**			**Vaalharts**			**Makhatini**		
	GER severity[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]	*GER severity*[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]	*GER severity*[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]
VO617Y-1	10.64 a-c	0.20 b-d	6.09 bc	8.98 de	0.14 b-d	6.71 c-e	28.82 a	3.25 a	37.43 a
R0549W	15.30 a-c	0.32 b	15.27 b	11.56 d	0.24 b-d	12.79 b-d	28.01 a	0.76 bc	20.65 b
US2540	9.02 a-c	0.30 b	7.65 bc	12.09 d	0.06 cd	4.50 de	13.04 a	0.94 bc	22.58 b
CML444	11.62 a-c	0.24 bc	7.47 bc	8.30 de	0.10 cd	5.26 c-e	11.65 a	0.88 bc	16.71 b
CML390	4.70 c	0.06 cd	2.86 c	1.00 e	0.02 d	0.25 e	21.02 a	0.89 bc	12.83 b
CML182	7.53 bc	0.15 b-d	3.99 c	12.41 d	0.20 b-d	10.35 c-e	12.27 a	0.65 c	16.30 b
R119W	19.89 ab	0.78 a	37.49 a	40.11 b	0.45 b	21.48 b	22.19 a	1.83 b	25.32 a
R2565Y	23.10 a	0.02 d	10.98 bc	85.00 a	1.44 a	38.47 a	8.37 a	0.72 bc	19.53 b
I137tnW	12.84 a-c	0.02 d	13.28 bc	26.63 c	0.36 bc	15.70 bc	19.07 a	0.92 bc	13.53 b
Mean	12.74	0.23	11.67	22.79	0.33	12.83	18.27	1.20	20.54

[1] Percentage of maize ears covered with visual symptoms of Gibberella ear rot

[2] Absolute concentrations of *Fusarium graminearum sensu lato* DNA (ng μl^{-1})

[3] Mycotoxin content = DON + A-DON + ZEA (ppm)

[4] Mean values for three field plots

[5] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)

Stellenbosch University http://scholar.sun.ac.za

Table 5. AMMI analysis of variance (ANOVA) for resistance to Gibberella ear rot severity, fungal biomass and mycotoxin accumulation in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season.

		GER severity[1]			Fungal biomass[2]			Mycotoxins[3]		
Source of variation	*Df*	*MS*	*F value*	*P >F*	*MS*	*F value*	*P >F*	*MS*	*F value*	*P >F*
Total	80	6.75	*	*	6.75	*	*	6.74	*	*
Treatment	26	11.41	2.25	0.00	15.30	5.17	0.00	12.55	2.83	0.00
Environment	2	0.00	*	*	0.00	*	*	0.00	*	*
Block	6	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Genotype	8	17.91	3.53	0.00	19.02	6.43	0.00	25.07	5.65	0.00
Interaction	16	9.58	1.89	0.04	15.36	5.19	0.00	7.86	1.77	0.06
IPCA	9	14.95	2.95	0.00	19.11	6.46	0.00	10.61	2.39	0.02
IPCA	7	2.67	0.52	0.80	10.53	3.56	0.00	4.33	0.97	0.45
Residual	0	*	*	*	*	*	*	*	*	*
Error	48	5.06	*	*	2.95	*	*	4.43	*	*

[1] Percentage of maize ears covered with visual symptoms of Gibberella ear rot

[2] Absolute concentrations of *Fusarium graminearum sensu lato* DNA (ng μl^{-1})

[3] Mycotoxin content = Total of DON + A-DON + ZEA (ppm)

Table 6. AMMI stability values (ASV) and ranking orders for trichothecenes B, zearalenone and aflatoxin contamination and *Stenocarpella maydis* biomass of the nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season.

	Trichothecenes B and Zearalenone[1]		**Aflatoxins**[2]		***S. maydis* biomass**[3]	
Inbred line	*ASV*	*Rank*	*ASV*	*Rank*	*ASV*	*Rank*
VO617Y-1	4.31	9	1.31	6	1.42	4
R0549W	1.99	5	0.58	2	0.95	3
US2540	3.55	8	1.03	5	1.74	6
CML444	0.81	2	1.34	7	0.41	1
CML390	1.37	3	1.53	8	4.70	9
CML182	0.71	1	0.99	4	1.70	5
R119W	1.43	4	1.86	9	2.20	7
R2565Y	3.43	7	0.83	3	2.69	8
I137tnW	2.64	6	0.19	1	0.71	2

[1] Mycotoxin content = Total of DON + A-DON + ZEA (ppm)

[2] Mycotoxin content = Total of AFB_1 + AFB_2 (ppm)

[3] Absolute concentrations of *Stenocarpella maydis* DNA (ng μl^{-1})

Stellenbosch University http://scholar.sun.ac.za

Table 7. Evaluation of nine maize inbred lines for resistance to Aspergillus ear rot in a multi-location field trial in South Africa during the 2012/2013 growing season.

Inbred line	**Potchefstroom**			**Vaalharts**			**Makhatini**		
	AER severity[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]	*AER severity*[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]	*AER severity*[1,4,5]	*Fungal biomass*[2,4,5]	*Mycotoxin content*[3,4,5]
VO617Y-1	0.19 c	0.14 b	0.02 b	0.12 c	0.00* d	0.10 ab	2.20 bc	0.00* b	0.00* a
R0549W	1.69 bc	0.03 d	0.00* c	1.03 b	0.76 ab	0.04 ab	3.79 a-c	0.00* b	0.00* a
US2540	1.69 b	0.04 c	0.00* c	2.50 a	0.04 cd	0.17 a	3.20 bc	0.03 ab	0.00* a
CML444	0.58 bc	0.03 d	0.00* c	0.58 b	0.11 cd	0.04 ab	0.68 c	0.00* ab	0.00* a
CML390	0.17 c	0.03 d	0.00* c	0.17 c	0.26 b-d	0.00* b	9.28 a	0.03 a	0.00* a
CML182	0.11 c	0.03 d	0.00* c	0.11 c	0.00* d	0.00* b	6.37 ab	0.00* ab	0.00* a
R119W	4.91 a	0.31 a	0.10 a	0.05 c	0.55 b-d	0.12 ab	7.81 ab	0.00* b	0.00* a
R2565Y	0.82 bc	0.03 d	0.01 c	0.86 b	1.26 a	0.18 ab	4.32 a-c	0.00* b	0.00* a
I137tnW	0.76 bc	0.13 b	0.01 c	0.19 c	0.66 a-c	0.08 ab	7.11 a-c	0.00* ab	0.00* a
Mean	1.21	0.08	0.02	0.62	0.40	0.08	4.97	0.00	0.00

[1] Percentage of maize ears covered with visual symptoms of Aspergillus ear rot

[2] Absolute concentrations of *Aspergillus flavus* DNA (ng μl^{-1})

[3] Mycotoxin content = $AFB_1 + AFB_2$ (ppm)

[4] Mean values for three field plots; *Below detectable limit

[5] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)

Table 8. AMMI analysis of variance (ANOVA) for resistance to Aspergillus ear rot severity, fungal biomass and aflatoxin accumulation in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season.

		AER severity[1]			Fungal biomass[2]			Aflatoxins[3]		
Source of variation	*Df*	*MS*	*F value*	*P >F*	*MS*	*F value*	*P >F*	*MS*	*F value*	*P >F*
Total	80	6.66	*	*	6.74	*	*	4.28	*	*
Treatment	26	11.86	2.53	0.00	14.08	3.90	0.00	8.26	3.10	0.00
Environment	2	0.00	*	*	0.00	*	*	0.00	*	*
Block	6	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
Genotype	8	9.07	1.93	0.07	11.75	3.25	0.00	13.93	5.22	0.00
Interaction	16	14.74	3.14	0.00	17.00	4.71	0.00	6.47	2.42	0.00
IPCA	9	19.22	4.10	0.00	17.11	4.73	0.00	6.68	2.50	0.01
IPCA	7	8.97	1.91	0.08	16.87	4.67	0.00	6.19	2.32	0.04
Residual	0	*	*	*	*	*	*	*	*	*
Error	48	4.68	*	*	3.61	*	*	2.66	*	*

[1] Percentage of maize ears covered with visual symptoms of Aspergillus ear rot

[2] Absolute concentrations of *Aspergillus flavus* DNA (ng μl^{-1})

[3] Mycotoxin content = Total of AFB_1 + AFB_2 (ppm)

Stellenbosch University http://scholar.sun.ac.za

Table 9. Evaluation of nine maize inbred lines for resistance to Diplodia ear rot and fungal biomass in a multi-location field trial in South Africa during the 2012/2013 growing season.

Inbred line	**Potchefstroom**				**Vaalharts**				**Makhatini**			
	DER severity[1,3,4]		*Fungal biomass*[2,3,4]		*DER severity*[1,3,4]		*Fungal biomass*[2,3,4]		*DER severity*[1,3,4]		*Fungal biomass*[2,3,4]	
	A. I.	N. I.	A. I.	N. I.	A. I.	N. I.	A. I.	N. I.	A. I.	N. I.	A. I.	N. I.
VO617Y-1	6.43 b	12.69 ab	0.02 b	0.24 a-c	16.67 a-c	3.02 a	0.19 a	0.00* b	0.00 a	1.85 b	0.00* ab	0.02 a
R0549W	25.60 ab	42.11 a	0.85 a	0.30 ab	31.50 ab	19.44 a	0.41 a	0.00* b	0.00 a	1.85 b	0.02 ab	0.03 a
US2540	17.67 ab	25.53 ab	0.30 ab	0.15 a-c	2.13 c	28.68 a	0.10 a	0.02 b	0.00 a	0.00 b	0.04 ab	0.00* a
CML444	22.84 ab	11.09 b	0.05 b	0.07 a-c	12.18 a-c	26.19 a	0.17 a	0.00* b	0.00 a	1.15 b	0.00* ab	0.12 a
CML390	4.79 b	8.38 b	0.02 b	0.02 c	8.77 c	12.12 a	0.24 a	0.48 a	6.67 a	0.00 b	0.08 a	0.00* a
CML182	31.44 ab	19.64 ab	0.36 ab	0.23 a-c	14.8 a-c	11.90 a	0.33 a	0.03 b	0.00 a	10.4 a	0.00* ab	0.00* a
R119W	43.88 a	20.21 ab	0.74 a	0.15 a-c	15.15 a-c	27.78 a	0.35 a	0.02 b	0.00 a	0.00 b	0.00* ab	0.06 a
R2565Y	25.60 ab	34.55 ab	0.10 b	0.04 a-c	32.80 a	11.11 a	0.22 a	0.00* b	4.17 a	0.00 b	0.00* b	0.15 a
I137tnW	44.14 a	17.72 ab	0.38 ab	0.32 a	28.1 ab	17.39 a	0.29 a	0.00* b	1.33 a	0.00 b	0.00* ab	0.07 a
Mean	24.75	21.33	0.31	0.17	18.00	17.51	0.26	0.06	1.35	1.70	0.02	0.05

A.I., Artificial inoculation; N.I., Natural infection

[1] Percentage of total number of maize ears covered with visual symptoms of Diplodia ear rot

[2] Absolute concentrations of *Stenocarpella maydis* DNA (ng μl^{-1})

[3] Mean values for three field plots; *Below detectable limit

[4] Means followed by the same alphabetical letter in each column are not significantly different according to Student's t-least significant difference test ($P \geq 0.05$)

Stellenbosch University http://scholar.sun.ac.za

Table 10. AMMI analysis of variance (ANOVA) for resistance to Diplodia ear rot severity and fungal biomass in nine maize inbred lines tested in three environments in South Africa during the 2012/2013 season.

		DER severity[1]			Fungal biomass[2]		
Source of variation	*Df*	*MS*	*F value*	*P >F*	*MS*	*F value*	*P >F*
Total	80	4.98	*	*	6.71	*	*
Treatment	26	8.19	2.11	0.01	9.34	1.52	0.10
Environment	2	0.00	*	*	0.00	*	*
Block	6	0.00	0.00	1.00	0.00	0.00	1.00
Genotype	8	12.52	3.23	0.00	13.95	2.27	0.03
Interaction	16	7.05	1.82	0.05	8.20	1.33	0.02
IPCA	9	8.65	2.23	0.03	10.61	1.72	0.10
IPCA	7	5.01	1.29	0.27	5.10	0.83	0.56
Residual	0	*	*	*	*	*	*
Error	48	3.87	*	*	6.13	*	*

[1] Percentage of maize ears covered with visual symptoms of Diplodia ear rot (inoculated treatment)

[2] Absolute concentrations of *Stenocarpella maydis* DNA (ng μl^{-1})

Table 11. Estimates[a] for the relationship between *Stenocarpella maydis* infection potential and fungal biomass concentrations in the maize inbred lines.

Inbred line	*A* estimate	Standard error	Lower 95% CL	Upper 95% CL	*B* estimate	Standard error	Lower 95% CL	Upper 95% CL	R^2
VO617Y-1	0.23	0.25	-0.46	0.91	0.44	0.61	-1.24	2.13	0.28
R0549W	9.01	5.69	-6.79	24.81	2.09	0.50	0.70	3.48	0.96
US2540	1.90	2.33	-4.57	8.38	1.70	0.96	-0.95	4.36	0.80
CML444	0.17	0.12	-0.16	0.51	0.37	0.36	-0.63	1.37	0.37
CML390	0.19	0.30	-0.63	1.01	0.53	0.93	-2.04	3.10	0.15
CML182	1.35	0.45	0.10	2.60	1.07	0.24	0.40	1.75	0.96
R119W	27.21	14.87	-14.08	68.49	3.12	0.45	1.87	4.37	0.99
R2565Y	0.25	0.19	-0.28	0.77	0.44	0.43	-0.76	1.65	0.39
I137tnW	0.95	0.30	0.11	1.78	0.77	0.22	0.17	1.38	0.93

CL, Confidence limits fitted to regression lines

[a] For the regression exponential function $Y = AX^b$, where X is the mean fungal biomass per trial site and treatment (infection potential) and Y is the mean fungal biomass within each inbred line associated with a specific location and treatment.

Stellenbosch University http://scholar.sun.ac.za

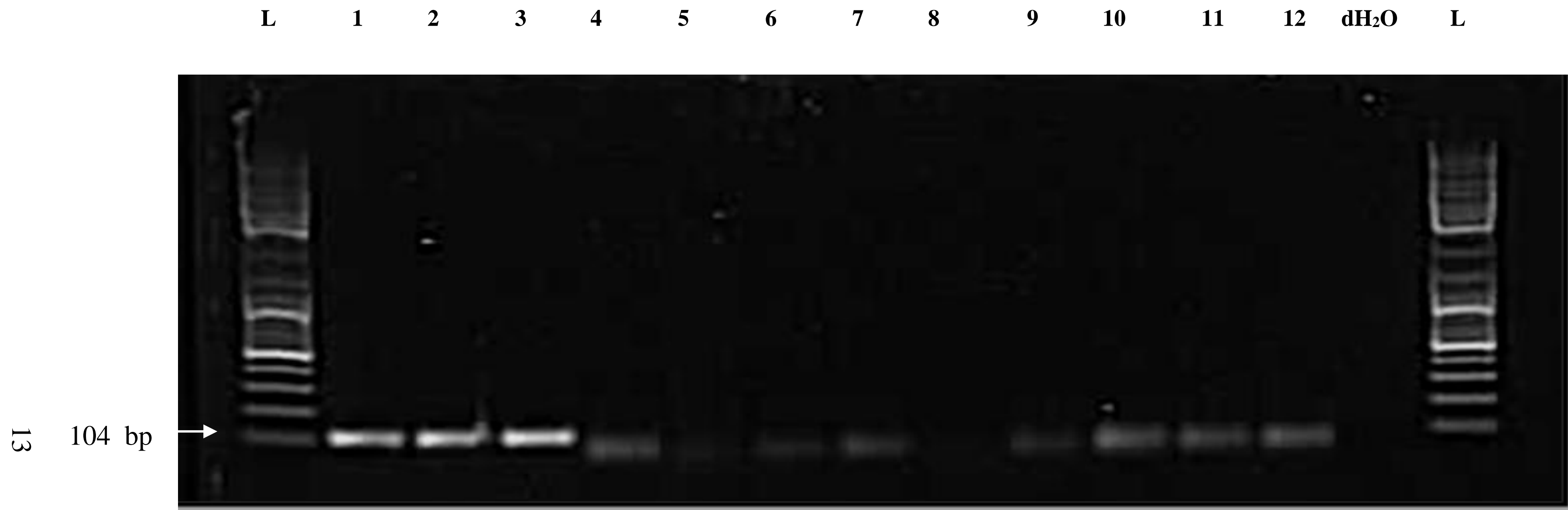


Fig. 1. Gel image representing PCR-based identification of *Stenocarpella maydis* using the primer pair Maydis3 F/Maydis3 R. Lanes 1, 2 and 3: *Stenocarpella maydis*, Lanes 4, 5, 6, 7, 8: *Fusarium graminearum sensu lato*, Lane 9: *Aspergillus flavus*, Lane 10: *Fusarium proliferatum*, Lane 11: *Fusarium subglutinans* and Lane 12: *Fusarium verticillioides*. Fine bands at the bottom of the gel indicate primer-dimer formation.

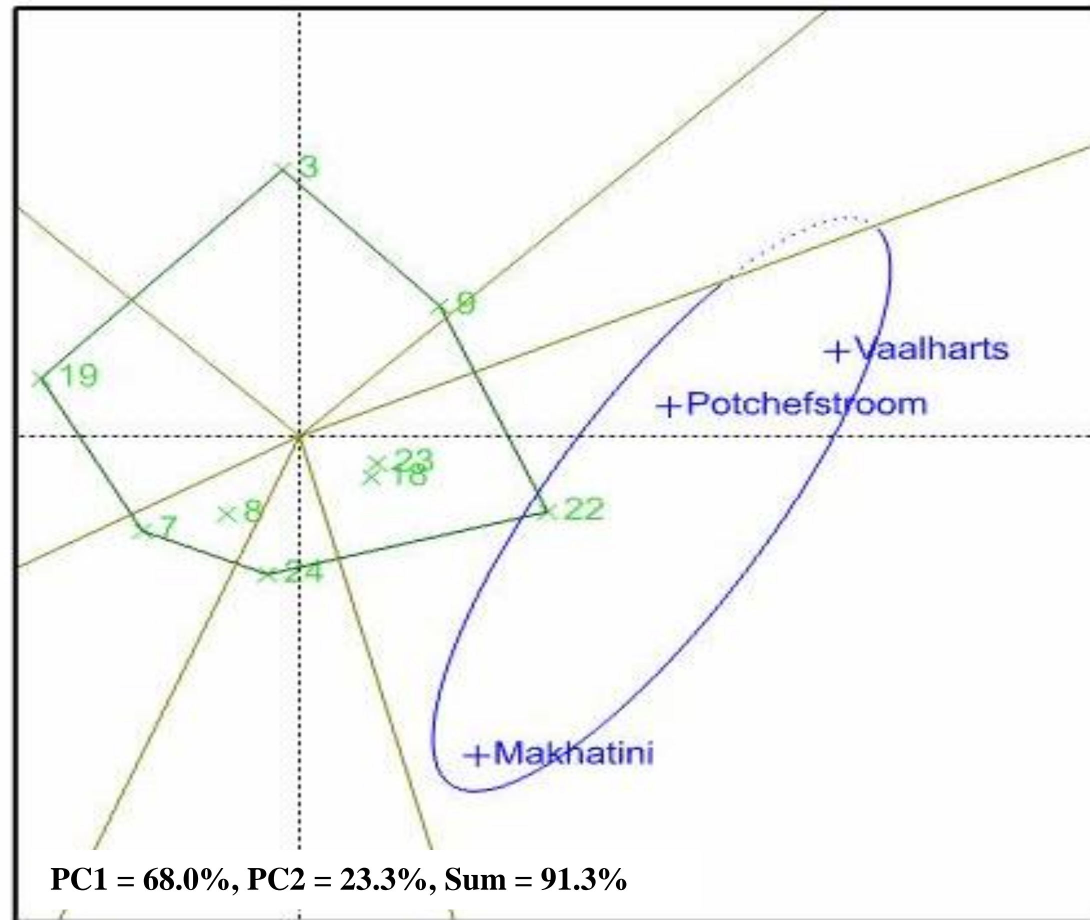


Fig. 2. Genotype main effect + genotype by environment interaction (GGE) biplot showing total mycotoxin content (TCT B + ZEA) of nine maize inbred lines tested in three locations in South Africa during the 2012/2013 growing season. Line 3: VO617Y-1; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 18: CML444; line 19: R119W; line 22: CML390; line 23: CML182; line 24: I137tnW.

Stellenbosch University http://scholar.sun.ac.za

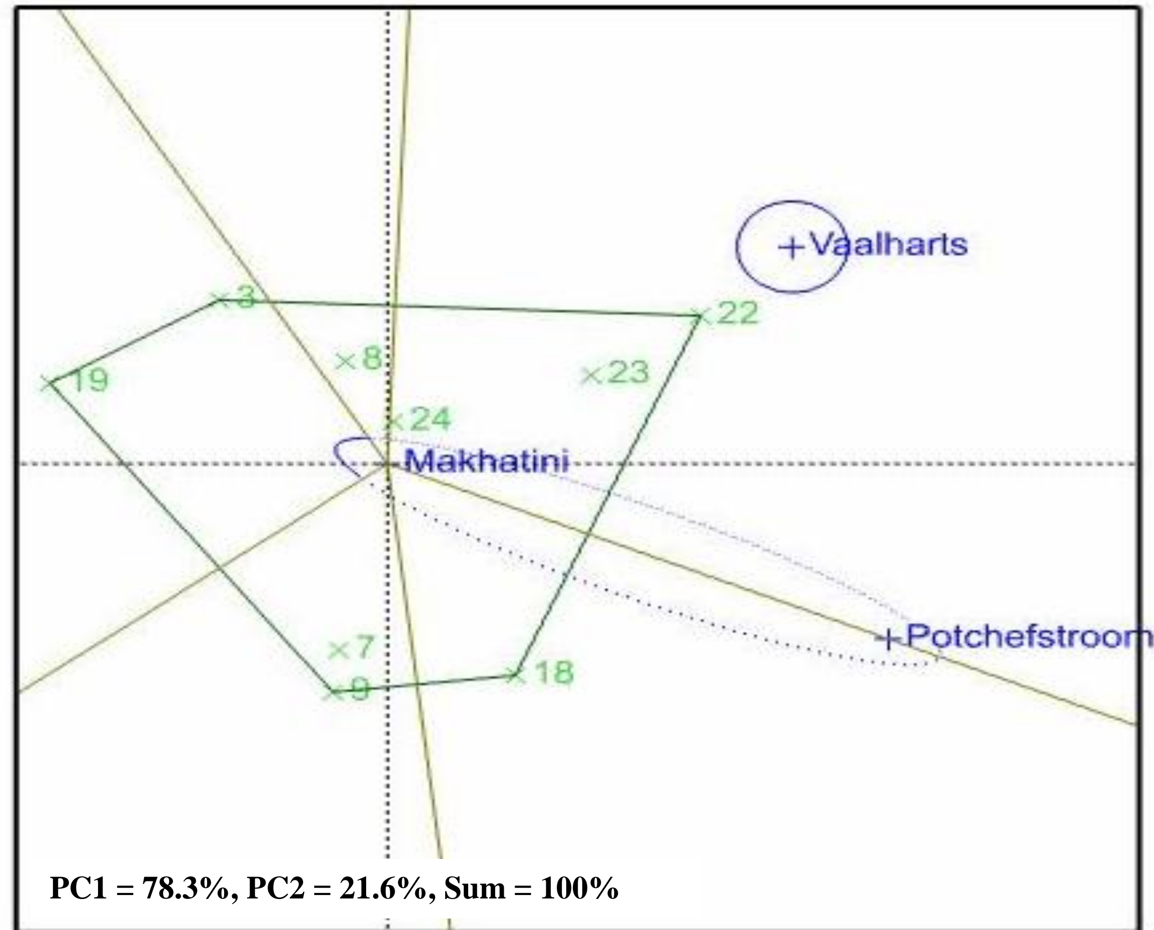


Fig. 3. Genotype main effect + genotype by environment interaction (GGE) biplot showing total aflatoxin content (AFB_1 + AFB_2) of nine maize inbred lines tested in three locations in South Africa during the 2012/2013 growing season. Line 3: VO617Y-1; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 18: CML444; line 19: R119W; line 22: CML390; line 23: CML182; line 24: I137tnW.

Stellenbosch University http://scholar.sun.ac.za

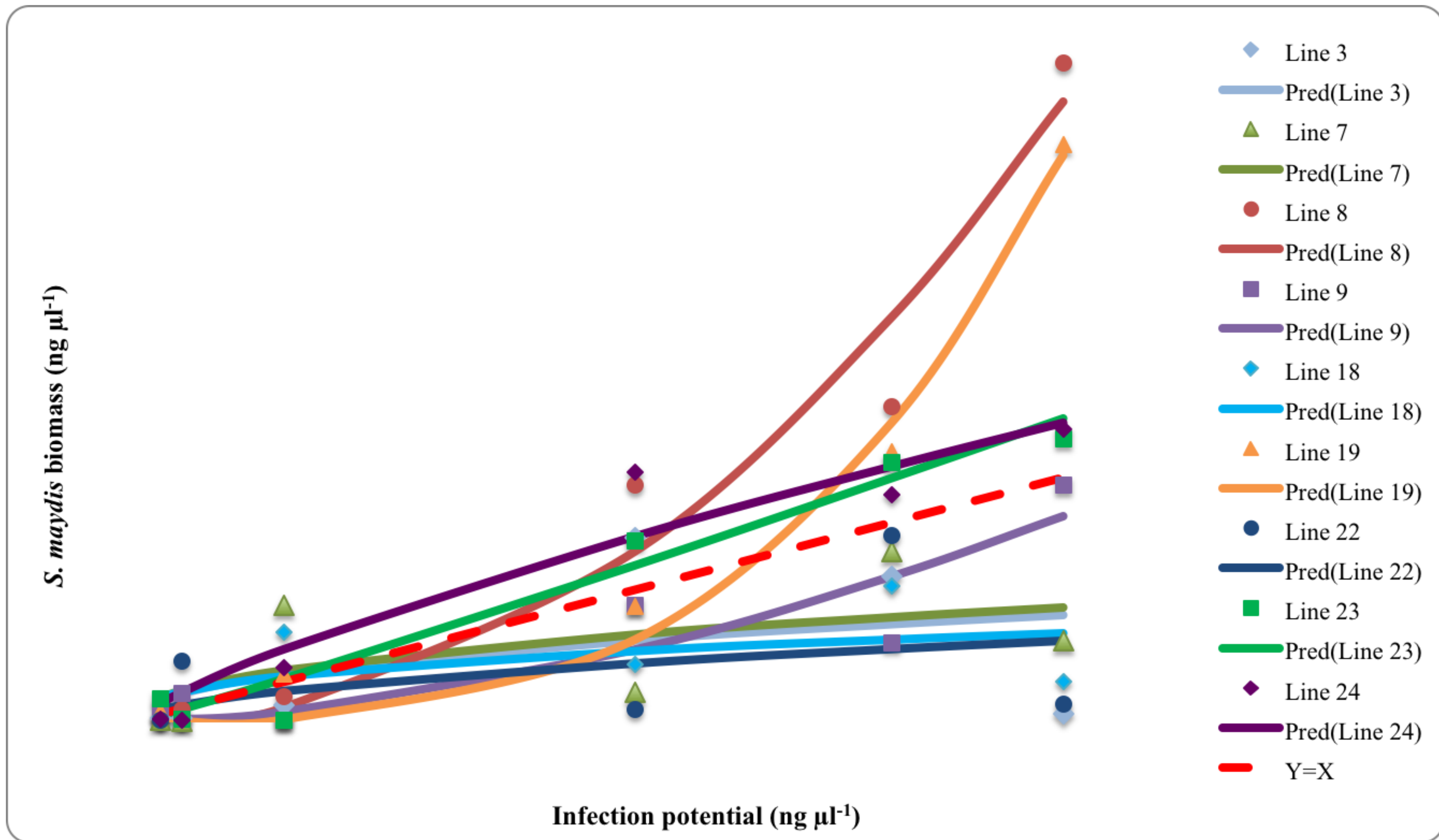


Fig. 4. Relationship between *Stenocarpella maydis* infection potential and observed fungal biomass concentrations in resistant, intermediate and susceptible maize inbred lines. Line 3: VO617Y-1; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 18: CML444; line 19: R119W; line 22: CML390; line 23: CML182; line 24: I137tnW.

Stellenbosch University http://scholar.sun.ac.za

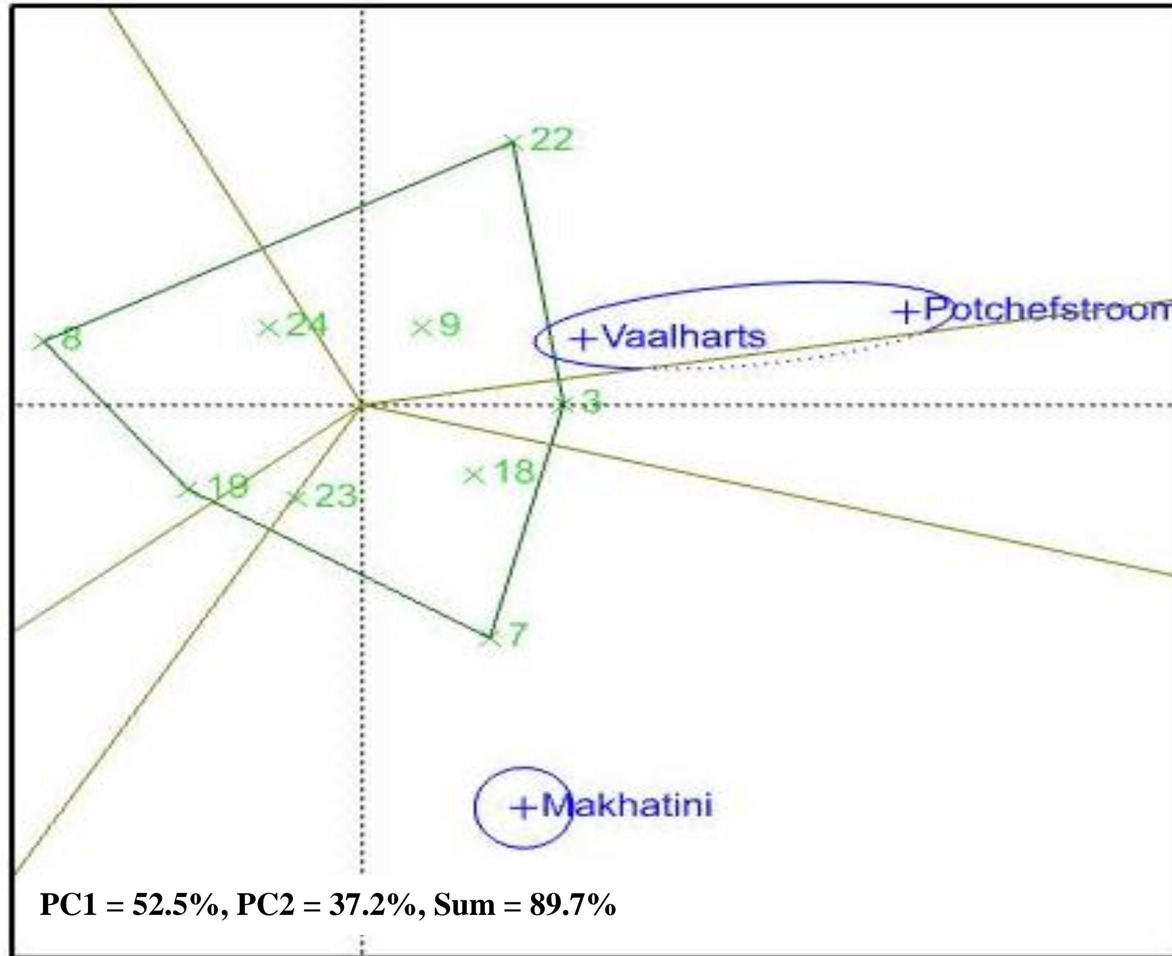


Fig. 5. Genotype main effect + genotype by environment interaction (GGE) biplot showing *Stenocarpella maydis* biomass of nine maize inbred lines tested in three locations in South Africa during the 2012/2013 growing season. Line 3: VO617Y-1; line 7: R2565Y; line 8: R0549W; line 9: US2540; line 18: CML444; line 19: R119W; line 22: CML390; line 23: CML182; line 24: I137tnW.

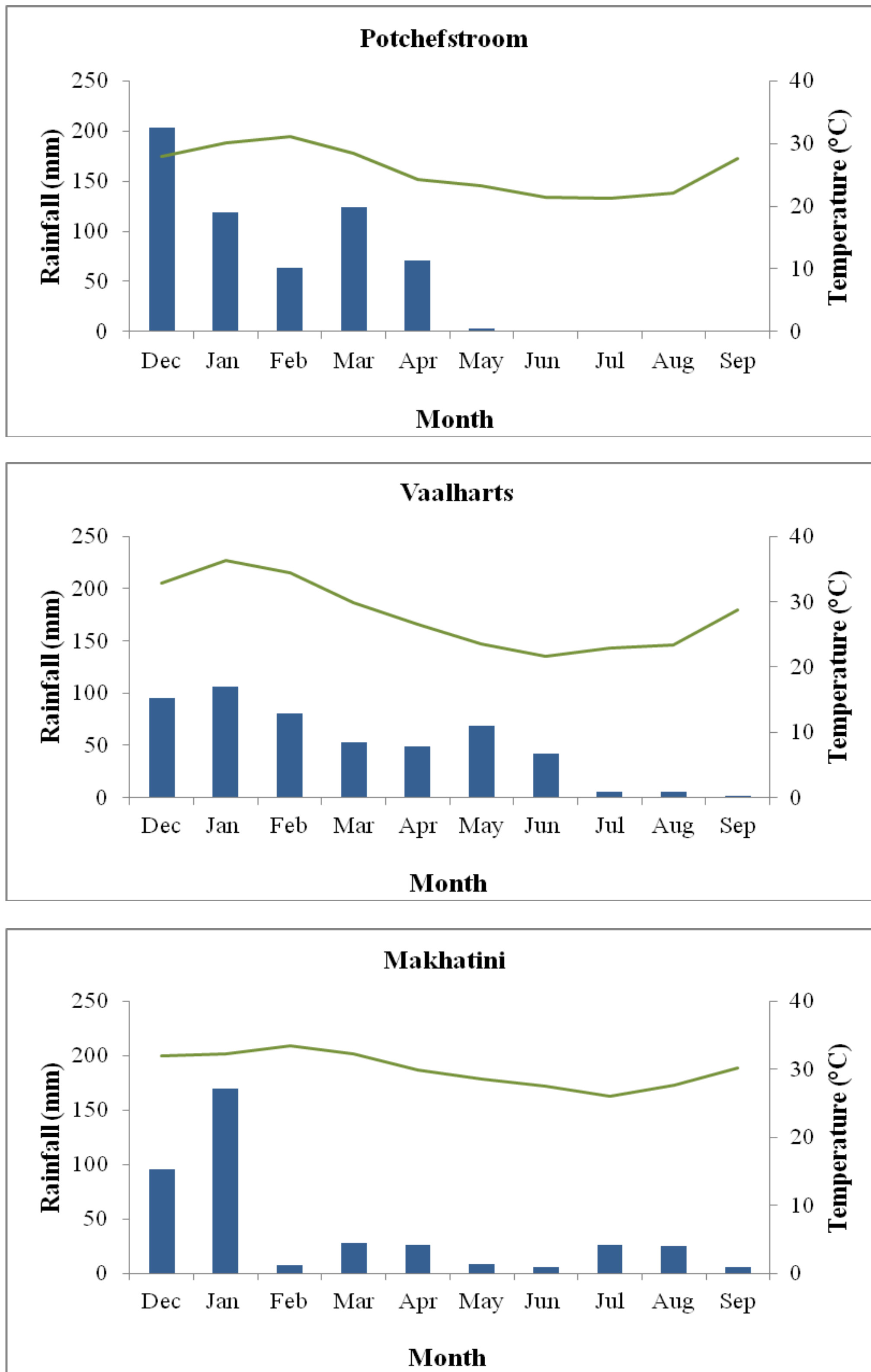


Fig. 6. Weather data recorded from Agricultural Research Council's weather stations for the 2012/2013 season at the respective field locations. Bar and line series indicate total monthly rainfall and mean monthly temperature, respectively, for the planting season.

Stellenbosch University http://scholar.sun.ac.za