Introgression of aflatoxin and fumonisin contamination resistance genes in maize hybrids

By

Nyashadzashe Chiuraise

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School of Agricultural, Earth and Environmental Sciences
College of Agriculture, Engineering and Science
University of KwaZulu-Natal
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DISSERTATION ABSTRACT

Maize is the principal crop in Africa, particularly in southern Africa. However, food security in the region is constantly threatened by the contamination of maize grain through mycotoxins, such as aflatoxins and fumonisins caused by *Aspergillus flavus* and *Fusarium verticillioides,* respectively. Food security is defined as the capacity of a nation to ensure that all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life. Aflatoxins and fumonisins are carcinogenic, teratogenic, mutagenic and immunosuppressive to both humans and livestock. Presently, breeding for reduced mycotoxin contamination is one of the best strategies to reduce aflatoxin and fumonisin contamination in maize grain. Although mycotoxin resistant maize inbred lines have been identified, currently, there are no aflatoxin and fumonisin resistant commercial maize hybrids available to farmers in southern Africa. Decades of research have resulted in the identification of maize inbred lines that are resistant to either aflatoxin or fumonisin accumulation but not to both. Therefore the current study aimed at stacking resistance genes to the two toxins in one germplasm line or hybrid.

The first objective of this study was to determine the current picture of mycotoxin contamination in southern African maize germplasm. Thus, a survey on South African and regional experimental hybrids was carried out during 2012/13 and 2013/14 seasons to determine the natural incidences of different types of ear rots and to identify the associated fungi. The second objective was to stack the resistance genes in a single product through introgression of aflatoxin and fumonisin resistance genes from tropical inbred lines into adapted inbred lines used in the subtropical and temperate conditions of southern Africa. Consequently, the resultant 72 single cross hybrids were evaluated for fumonisin contamination and 44 three-way cross hybrids and their progenies (146 $S_{2:3}$ families) were evaluated for both aflatoxin and fumonisin contamination under artificial inoculation, in South Africa.

Survey results showed that *F. verticillioides* was the most prevalent ear rot causing fungi followed by *Stenocarpella maydis*, *Fusarium graminearum* and *A. flavus.* These pathogens have potential to cause fumonisins, dipliotoxins, vomitoxins and aflatoxins.

Assessment of experimental hybrids indicated a significant variation (P <0.001) among hybrids for ear rot incidence, and contamination by mycotoxins. Five single cross hybrids accumulated consistently low fumonisin levels (<4 ppm) both in the greenhouse and field trials. Three 3-way cross hybrids displayed a combined low contamination level for both aflatoxins (<5ppb) and fumonisins demonstrating potential for stacking resistance genes in the end product. Four $S_{2:3}$ families also accumulated low levels of both aflatoxins and fumonisins below the legal limits of 5 ppb and 4 ppm, respectively, further demonstrating that new maize inbred lines can be developed by stacking mycotoxin genes. Therefore the study indicated a significant progress towards breeding mycotoxin resistant hybrids. Recommendations for upscaling this achievement are discussed.

DECLARATION

I, Nyashadzashe Chiuraise declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:
   a. Their words have been re-written but the general information attributed to them has been referenced;
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

4. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the reference section.

Signed:

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Nyashadzashe Chiuraise (Candidate)

As the candidate's supervisors we agree to the submission of this dissertation.

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Professor John Derera (Supervisor)

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Dr Kwasi S. Yobo (Co-supervisor)

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Drs Benice Sivparsad, Tafadzwa Mabhaudhi and Iona Basdew are also acknowledged for providing moral support during the course of my study.

DEDICATION

This dissertation is dedicated to all those who have helped me and guided me along the way.

Mostly, to my mother, Molly Chiuraise, and sister Vongai, who had confidence in me when I didn’t.

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List of Abbreviations

AER	Aspergillus ear rot
CGIAR	Consultative Group on International Agricultural Research
CIMMYT	International Maize and Wheat Improvement Centre
Codex	Codex Alimentarius Commission
CRP	CGIAR Research Programme
ELISA	Enzyme-linked immunosorbent assay
FER	Fusarium ear rot
GXE	Genotype by environment interaction
IITA	International Institute of Tropical Agriculture
SSA	Sub-Saharan Africa
FAOSTAT	Food and Agriculture Organisation Statistics
WHO	World Health Organisation

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

This introductory chapter provides information on the importance of maize in southern Africa. This section highlights the negative impact of maize ear rot diseases and mycotoxin contamination on food security, health and safety of consumers. It presents the problem statement, aim and objectives of the study, research questions, significance of the study and structure of the dissertation.

1.2 Importance of maize

Maize (*Zea mays* L.) is one of the three most important cereal crops in the world, after rice and wheat (Shiferaw et al., 2011). It is the most important crop in southern Africa. Maize grain contributes between 70-98% of energy requirements in southern Africa, which has the highest *per capita* maize grain consumption in the world (Smale et al., 2011). It is consumed as a major component (> 50%) in three daily meals (breakfast, lunch and supper) including weaning of babies and feeding the sick (Krivanek et al., 2007). Maize is the regional staple crop and any reduction in production has serious consequences on the economy, health and political stability. In Zimbabwe, for example, low maize grain production is associated with low gross domestic product (Richardson, 2005), while household food insecurity is synonymous with reduced maize production in the whole region. Adequate maize grain production, among other factors, has the potential to secure southern Africa's economies by providing sufficient food to the poor households and opportunities for growth in other economic sectors (Hommann-Kee Tui et al., 2013). Therefore maize is the crop in southern Africa which requires breeding attention.

Maize is the preferred crop in southern Africa because it is high yielding, easy to process, readily digested, and cheaper than other cereals. It is also a versatile crop. It can grow across a range of agro-ecological zones (McCann, 2009). Every part of the

maize plant has economic value which makes it appealing to the poor. The grain, leaves, stalk, tassel, and cob can all be used to produce a large variety of food and non-food products. Maize is the most appropriate energy crop for resource-poor and subsistence farmers in southern Africa. However, there are challenges that hamper production of sufficient maize to match requirements in southern Africa. The population growth outstrips maize production. Consequently, the gap between maize production and demand is filled by imports, mainly from the USA, Brazil and Argentina (Krivanek et al., 2007). It is against this background that there is a need for sufficient maize grain of suitable quality for human and livestock health to be produced in southern Africa.

1.3 Problem statement

Food security is defined as the capacity of a nation to ensure that “all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life” (FAO, 2006). Maize is the major staple crop in southern Africa; hence it should be of the right nutrition, quality and safe to consumers. However, maize grain being consumed in the region is oftenly contaminated with fungi, such as *Aspergillus flavus*, *Fusarium verticillioides*, *Stenocarpella maydis* and *Fusarium graminearum*. These fungi produce mycotoxins, such as aflatoxin, fumonisin, dipliotoxin, deoxynivalenol and zearalenone, respectively. These toxins are carcinogenic, mutagenic, teratogenic and immunosuppressive to both humans and livestock. Against this background, the purpose of this study was to stack aflatoxin and fumonisin resistance genes into a single maize germplasm line or hybrid. This would provide quality and safety insurance to small scale farmers, who do not have the capacity to check mycotoxin content in maize grain. Unfortunately such maize has not been developed, which negatively impacts on food security in the region.

1.4 Research questions

The following research questions were pivotal to the study:

1. To what extend are the natural incidence of ear rot diseases associated with mycotoxin contamination in southern African maize hybrids?

2. Is stacking of mycotoxin resistance genes in maize hybrids effective?
3. Is greenhouse screening capable of predicting mycotoxin contamination resistance in maize hybrids in the field?
4. Can the introgression of both aflatoxin and fumonisin resistance genes in adapted maize germplasm lines effective in breeding new productive lines for use as parents of new hybrids?

1.5 Significance of the study

Maize yields remain low and highly variable between years across southern Africa. With the exception of South Africa, the average yield hardly exceed 0.9 t ha^{-1}, South Africa has an average yield of 3.8 t ha^{-1}, (FAOSTAT, 2013). Among the contributing factors to yield reduction are the ear rot diseases. Ear rots not only contribute to yield losses but they are also associated with toxigenic fungi that contaminate grain with mycotoxins. The Food and Agricultural Organization (FAO) has estimated that up to 25% of the world's food crops are affected by mycotoxins each year, with annual losses of around one billion metric tons of food and food products (WHO, 2006). African countries lose an estimated $1.4 billion in potential trade revenue on cereals and nuts (Wild and Gong, 2010). Mycotoxin contamination prevents commodities from meeting international, regional and local regulation standards governing agricultural trade and food safety (Gnonlonfin et al., 2013).

The ear rot causing fungi, such as *Aspergillus flavus* (Aspergillus ear rot), *Fusarium verticillioides* (Fusarium ear rot), *Stenocarpella maydis* (Diplodia ear rot) and *Fusarium graminearum* (Gibberella ear rot) with incidences of 15-33% have been reported (Bigirwa et al., 2007). These fungi produce mycotoxins such as aflatoxin, fumonisin, dipliotoxin, deoxynivalenol and zearalenone, respectively. These toxins are associated with liver cancer, hepatitis and neural tube defects in humans (Balazs and Schepers, 2007; Mukanga et al., 2010). In addition, the depressed immune systems of people under long term, high level exposure of aflatoxins may increase the severity of epidemics of acquired immunodeficiency syndrome (AIDS), malaria, tuberculosis, and other diseases as well as impaired child development (Warburton and Williams, 2014).

In livestock, mycotoxins cause diseases and neural defects, such as ataxia, paresis and paralysis (Wu and Munkvold, 2008).

Several management practices have been employed to control mycotoxin contamination. They include cultural, chemical, physical, biological and breeding methods (Wagacha and Muthomi, 2008). However, no single control strategy is completely effective when environmental conditions are extremely favourable for the growth of the fungi. The most desirable and sustainable method for managing mycotoxins contamination in maize grain would be the use of resistant maize varieties. Past experience has demonstrated that the use of new varieties alongside improved management options can offset yield losses by up to 40% (Thornton et al., 2009). Unfortunately, no such commercial varieties are currently available in eastern and southern Africa. Although several maize genotypes with resistance to aflatoxin and fumonisin contamination have been identified (Menkir et al., 2008,Small et al., 2012), these sources of resistance lack desirable agronomic backgrounds. Furthermore, adaptation and their level of resistance are not adequate to satisfy commercial needs. Previous studies have not shown resistance to aflatoxin and fumonisin in a single hybrid, which is desirable. This information has led to the need to undertake this study. The results of this study will help to identify maize hybrid varieties resistant to ear rots and mycotoxin accumulation for use by smallholder farmers. The study will also identify families with improved resistance, which may serve as more appropriate materials for developing new maize inbred lines with ear rot and mycotoxin contamination resistance.

1.6 Research Objectives

The main objective of this study was to enhance maize grain quality by improving resistance of maize hybrids to contamination by mycotoxins, which are caused by ear rot fungal diseases in maize. This was achieved through introgression of aflatoxin and fumonisin contamination resistance genes into adapted maize inbred lines.

The specific objectives were:

a) To determine the natural incidences of ear rot diseases, which are associated with mycotoxin contamination in southern African maize hybrids.
b) To introgress aflatoxin and fumonisin contamination resistance genes from tropical inbred lines into South African adapted maize inbred lines.
c) To evaluate the efficacy of stacking ear rot, aflatoxin and fumonisin resistance genes in single and three-way cross hybrids.
d) To determine progress in introgressing the ear rots, aflatoxin and fumonisin resistance genes in $S_{2:3}$ maize families.
e) To determine the correlation between greenhouse and field disease screening.
f) To determine the correlation between ear rots severity and mycotoxin contamination in maize hybrids.
g) To determine the correlation between aflatoxin and fumonisin contamination
h) To determine the correlation between mycotoxin contamination and agronomic traits.
i) To quantify genetic gains and heritability estimates of aflatoxin and fumonisin contamination resistance.

1.7 Research hypotheses

Five research hypotheses were tested in the study:

1. There is a high prevalence of natural incidence of ear rot diseases associated with mycotoxin contamination in southern African maize hybrids.
2. Resistance to ear rots and mycotoxin contamination of maize hybrids in the field can be predicted using greenhouse data
3. Stacking of mycotoxin resistance genes is an effective way of improving hybrids for mycotoxin resistance
4. The introgression of both aflatoxin and fumonisin resistance genes in South African adapted maize germplasm lines is effective for breeding new productive maize inbred lines with mycotoxin resistance. These lines will be valuable for use as parents of new hybrids with mycotoxin resistance.

5. Ear rot, aflatoxin and fumonisin contamination resistance are highly heritable traits therefore high genetic gains can be obtained in breeding maize hybrids and inbred lines for mycotoxin and ear rot resistance.

1. 8 Dissertation outline

The layout of the dissertation is as follows:

Chapter One: General introduction

- Provides the study background and outlines the scope, aim and objectives, problem statement, significance of the study and structure of the dissertation.

Chapter Two: Literature review

- Presents the theoretical framework of the study by reviewing literature pertaining to the importance of maize in African diets, the effect of aflatoxin and fumonisin contamination on health and trade. Breeding for host resistance is explored as the best control strategy.

Chapter Three: Research design and methodology

- Presents the layout and management of greenhouse and field experiments. Field data collection methods, isolation and inoculation of pathogens techniques, aflatoxin and fumonisin quantification, and data analysis are presented.

Chapter Four: Results

- Findings on the natural incidence of ear rots in southern African maize are presented. Results on the evaluation of fumonisin contamination resistance in single crosses as well as aflatoxin and fumonisin resistance in three-way crosses and their progenies are also presented.

Chapter Five: General discussion

- It provides a general discussion of the findings in relation to the findings of existing research that informs the study.

Chapter Six: Conclusions, implications and recommendations

- Summarizes the key findings of the research chapters and presents the overall conclusions and recommendations for future breeding programs.

1.9 Conclusion and summary of research focus

Contamination of maize grain by mycotoxins significantly reduces its economic value and exposes consumers to health risks. Substantial economic losses have been reported along the value chain from maize growers, grain handlers to livestock and poultry producers. It is estimated that 5.5 billion people worldwide, with a greater proportion of them in sub-Saharan Africa, are chronically exposed to mycotoxin contamination of maize grain. Therefore it is prudent to emphasize breeding for mycotoxin resistance in the maize research programme. This study is a response to the call by the Consultative Group on International Agricultural Research (CGIAR) maize research programme (CRP). Breeding resistant varieties would be a viable cost-effective and environmentally friendly option to combat the problem of mycotoxin contamination in maize grain. Although mycotoxin resistant maize inbred lines have been identified, currently there are no aflatoxin and fumonisin resistant commercial maize hybrids, which are available to small-scale farmers. There is therefore a need to use the identified resistant maize inbred lines to breed for mycotoxin resistance in maize hybrids, which will be ultimately delivered to the small-scale farmers in sub-Saharan Africa and South Africa. The following chapter provides a review of the relevant literature on mycotoxin contamination on maize and discusses the breeding progress and strategies implemented to date.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

This review examines the importance of maize and the problem of mycotoxin contamination, its implications to food or feed safety. It highlights the danger caused by aflatoxins and fumonisins in sub-Saharan Africa (SSA) and explores breeding for host resistance in maize as a possible management intervention. Lastly conclusions are drawn in relation to objectives of the research, and the identified knowledge gaps are highlighted.

Maize is an important staple food for more than 1.2 billion people in SSA and Latin America (FAOSTAT, 2013). Worldwide consumption of maize is more than 116 million tons per year, with SSA consuming 30% (FAOSTAT, 2010). Eastern and southern Africa use 85% of their production as food, while Africa as a whole uses 95%, compared to other world regions that use most of their maize as animal feed (Shephard, 2008). Lesotho has the largest consumption *per capita* with 174 kg per year. Ninety percent of white maize consumption is in Africa and Central America. It fetches premium prices in southern Africa, where it represents the main staple food. Yellow maize grain is preferred in most parts of South America and the Caribbean. It is also the preferred animal feed in many regions as it gives a yellow colour to poultry, egg yolks and animal fat (Wu and Munkvold, 2008). In South Africa it is produced for livestock feed.

Maize accounts for 30−50% of low-income household expenditures in eastern and southern Africa. In southern Africa, where maize has the highest *per capita* consumption in the world, maize grain contributes 70-98% of energy requirements (van der Westhuizen et al., 2010). Maize is processed and prepared in various forms depending on the country. Ground maize is prepared into porridge in eastern and southern Africa, while maize flour is prepared into porridge in west Africa (Fapohunda, 2010). Ground maize is also fried or baked in many countries. In all parts of Africa,

green (fresh) maize is boiled or roasted on its cob and served as a snack. The grains are rich in vitamins A, C and E, carbohydrates, and essential minerals, and contain 9% protein (Tang et al., 2013). However this varies according to variety. The yellow hybrids have higher protein and contain vitamin A which is lacking in white maize. They are also rich in dietary fibre and calories which are a good source of energy (Shephard, 2008).

In South Africa (SA), maize is the most important grain crop, serving as both the major feed grain and the staple food for the majority of the population. It plays an important role in the South African economy. Approximately 12.8 million tons of maize (7.8 million tons of white and 4.98 million tons of yellow) are produced annually (South African Grain Information Services (SAGIS), 2011) of which approximately 600 000 tons are produced by small scale farmers. The surplus maize is usually exported to other African countries. Yellow maize is mostly used for animal feed production, while the white maize is primarily used for human consumption (Department of Agriculture, 2011). Maize also serves as a raw material for manufactured products, such as paper, paint, textiles, medicine and food.

Maize is produced throughout SA with the Free State, Mpumalanga and North West provinces being the largest producers, accounting for approximately 83% of total production. It is produced mostly under rain-fed conditions and less than 10% is produced under irrigation. Commercial maize farmers are estimated at 9000 and they cultivate nearly 3 million hectares of land and employ about 150 000 farm workers (South African Grain Information Services (SAGIS), 2011). Currently, the maize milling industry employs approximately 5 300 workers, while the formal animal feed industry employs an estimated 2 500 people. The total processing industry employs between 4 000 and 5 000 people. It is prudent to employ considerable resources on maize research and development that emphasise both quality and yield. Therefore, the current study focuses on improving maize for mycotoxin contamination resistance.

2.2 The problem of mycotoxins

Mycotoxins are defined as toxic secondary metabolites synthesized by different fungal species and contaminate various agricultural commodities either during production, harvest, storage or food processing (Gnonlonfin et al., 2013). Once the crop becomes infected under field conditions, the fungal growth continues with increasing vigour at post-harvest and storage conditions. Genotypes, drought, soil types, and insect activity are important in determining the likelihood of pre-harvest contamination (Guo et al., 2008). Humidity, temperature, and aeration during drying and storage are also important factors.

Although mycotoxins have impacted mankind since the beginning of organized crop cultivation, their effects have largely been ignored until the past 50 years (Wild and Gong, 2010). The scientific study of mycotoxins began in 1960 when a large number of turkey birds died in England due to consumption of contaminated groundnut meal imported from Brazil (Blout, 1961). With advanced technology very small quantities of many of the important mycotoxins can now be detected and accurately measured in foods and feeds.

Among the thousands of species of fungi, only about 100 belonging to genera *Aspergillus*, *Penicillium* and *Fusarium* are known to produce mycotoxins (Miller, 2008). Out of the 300–400 mycotoxins known, the most important are aflatoxins, ochratoxins, deoxynivalenol (DON), zearalenone, fumonisin, T-2 toxin and T-2 like toxins (trichothecenes) (Silvia et al., 2012). Deoxynivalenol, zearalenone, T-2 toxin and fumonisin are all produced by fungi of the genera *Fusarium*. *Aspergillus* section *Flavi*, especially *A. flavus* and *A. parasiticus*, produce aflatoxins and ochratoxins. Food borne mycotoxins of greatest significance in tropical developing countries are the fumonisins and aflatoxins (Schjoth et al., 2008). While aflatoxins occur mostly in maize and groundnuts, the prevalence of fumonisins in maize is 100% or close to it in all surveillance data that have been reported on maize from different parts of Africa (Mukanga et al., 2010,Wagacha and Muthomi, 2008). In SA, fumonisins in maize have been extensively researched (Shephard, 2008). Reports are also available on the

presence of fumonisins in other African countries including Kenya, Benin, Ghana and Zimbabwe (Kimanya et al., 2008).

Unlike the bacterial toxins that are macro-molecular proteins that produce symptoms in a few hours, because the body recognizes them as antigens and produces antibody-mediated reaction, mycotoxins are low molecular weight toxic metabolites of fungal origin (Gnonlonfin et al., 2013). When ingested, inhaled or absorbed through the skin they cause lowered performance, sickness or death in humans and animals (Bankole et al., 2006). Owing to their potent toxic nature and fairly common occurrence under natural conditions, mycotoxins have attracted worldwide attention in recent years. Some acute diseases with evidence of association with mycotoxins include aflatoxic hepatitis in Kenya (2004-2005), enteric ergotism in India, varcular ergotism in Ethiopia and deoxynivalenol mycotoxicosis in India and China (Probst et al., 2007). According to Miller (2008), among food contaminants, mycotoxins have greater consequences in terms of both human and animal health as well as economics. The current study focuses on aflatoxins and fumonisins which are discussed in the next section.

2.3 Aflatoxins

Aflatoxins are mycotoxins produced by many strains of *Aspergillus flavus, A. parasiticus, A. nominus* and *A. niger* molds. *Aspergillus flavus* and *A. parasiticus* are the most commonly implicated causal agents of aflatoxin contamination, with *A. flavus* by far the most common (Silvia et al., 2012). They belong to a family of compounds called difuranocoumarins produced by a polyketide pathway. Aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are the four major aflatoxins based on their blue (B) or green (G) fluorescence under ultraviolet light and their relative mobility by thin-layer chromatography on silica gel (Do and Choi, 2007). Besides these four toxins, aflatoxin M_1 and M_2 are derivatives of AFB_1 and AFB_2, and are found in milk and meat of AFB_1 and AFB_2 consuming animals (Balazs and Schepers, 2007). These were first isolated from milk of lactating animals fed with feeds contaminated with aflatoxins; hence, the M designation. *Aspergillus flavus* produces mainly AFB_1 and AFB_2, whereas *A. parasiticus* produces AFG_1 and AFG_2 in addition to aflatoxins AFB_1 and AFB_2 (Wu,

2010). These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds (Figure 2.1).

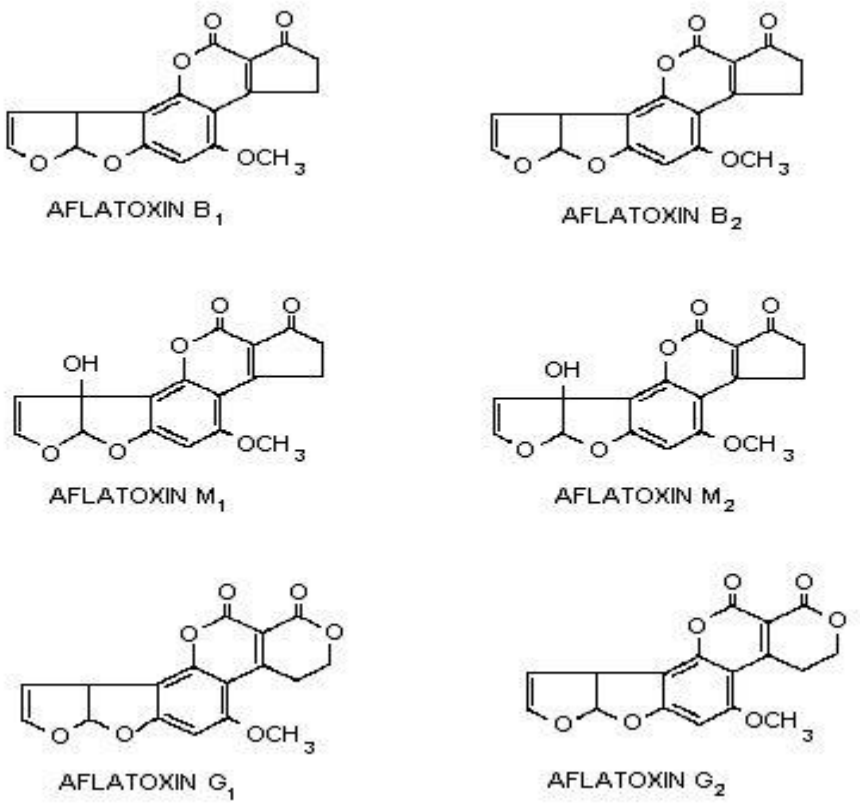

Figure 2.1 Chemical structure of aflatoxins compounds: AFB_1, AFB_2, AFM_1, AFM_2, AFG_1 and AFG_2 (Wu, 2010).

Aflatoxin B_1 is the most potent naturally formed carcinogen, and generally, if the term aflatoxin is used in the singular, the author is referring to AFB_1 (Gnonlonfin et al., 2013).

Aflatoxins are well recognized as potent toxic carcinogenic, mutagenic, immunosuppressive, and teratogenic agents and are common contaminants of foods, particularly in the staple diets of many developing countries posing a serious health risk to consumers (Balazs and Schepers, 2007). Their quantity in food and feed is therefore closely monitored and regulated in most countries. The United Nations food

standards body Codex Alimentarius Commission has set maximum levels of total aflatoxin (Aflatoxin B_1+B_2+G_1+G_2) in food as 15 parts per billion (ppb/µg/kg) and 5 ppb for aflatoxin B_1 (Codex, 2014). The European Union has a maximum level of 2 ppb for aflatoxin B_1 and 4 ppb for total aflatoxin in crops (EC, 2007). The United States of America: Food and Drug Administration (FDA) sets the limit for total aflatoxin at 20 ppb, 5 ppb for aflatoxin B_1 (FDA, 2001). In South Africa, the maximum levels of total aflatoxins is 10 ppb and 5 ppb for aflatoxin B_1.

Aflatoxin poisoning has been associated with eating home grown maize and storing it under damp conditions (Mboya et al., 2011). Acute aflatoxin poisoning has often occurred in Eastern and Central provinces of Kenya. The 2004 aflatoxin-poisoning outbreak in Kenya is the most severe case documented worldwide. The outbreak covered more than seven districts and resulted in 317 case-patients and 125 deaths from eating maize with aflatoxin B_1 concentrations as high as 4,400 ppb - 220 times the Kenyan limit for foods (Probst et al., 2007). In SA, several school children under the feeding scheme in the Eastern Cape Province fell ill in 2001 after consuming contaminated peanut butter with aflatoxin levels of about 30 times higher than the legal limit of 10 ppb prompted authorities to replace peanut butter by alternative products (Shephard, 2003). Aflatoxin contamination is most prevalent in countries that are situated between 40ºN and 40ºS of the equator thereby potentially exposing up to 5 billion people in the developing world (Figure 2.2).

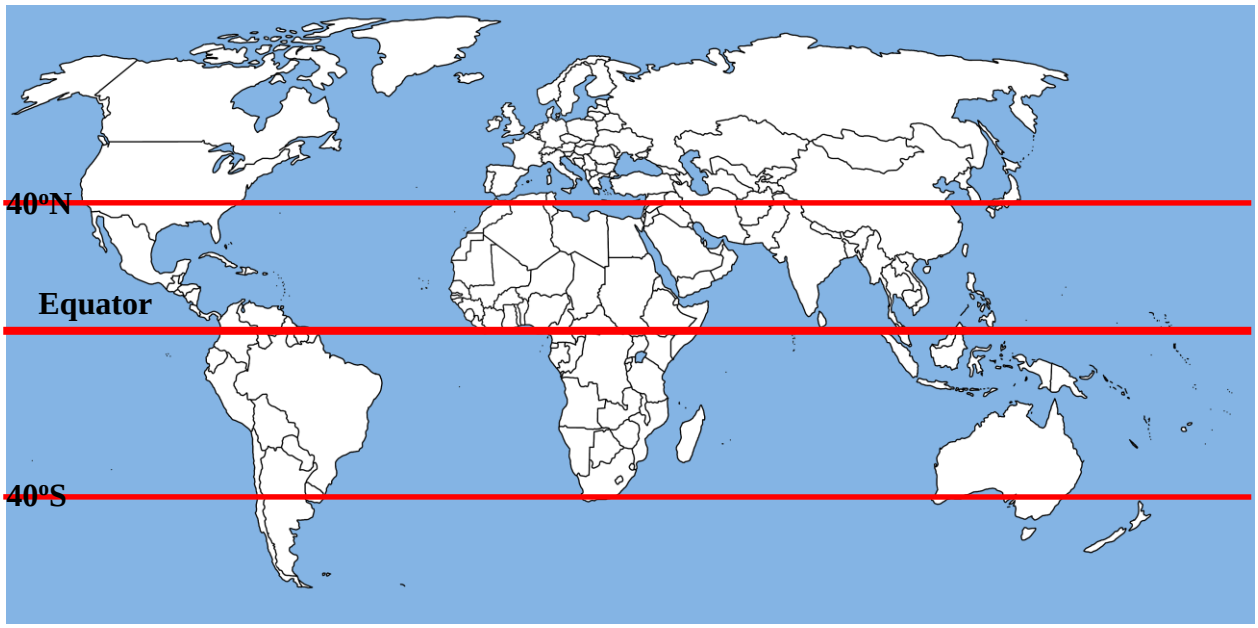

Figure 2.2 World map showing zone of perennial contamination risk by aflatoxins, 40ºS and 40ºN of the equator. Adapted from Gnonlonfin et al. (2013).

2.3.1 Aspergillus flavus Link

2.3.1.1 Classification

Kingdom: Fungi
Phylum: Ascomycota
Order: Eurotiales
Class: Eurotiomycetes
Family: Trichocomaceae
Genus: *Aspergillus*
Species: *flavus*

Aspergillus flavus may be divided into two distinct morphotypes, the S and L strains (Do and Choi, 2007). Each morphotype is composed of many clonal lineages (called vegetative compatibility groups or VCGs) defined by a vegetative compatibility system that limits gene flow between dissimilar individuals (Cary et al., 2011). Both morphotypes and VCGs differ in many characteristics. The most frequently studied characteristic is the aflatoxin-producing ability. The S strain, on average, produces

much higher concentrations of aflatoxins than does the L strain (Probst et al., 2007). Consequently, the S strain is the primary target for management of aflatoxin contamination in vulnerable crops (Williams, 2006). Species in the genus *Aspergillus* are characterized by a distinctive spore-bearing structure; the aspergillum (Figure 2.3).

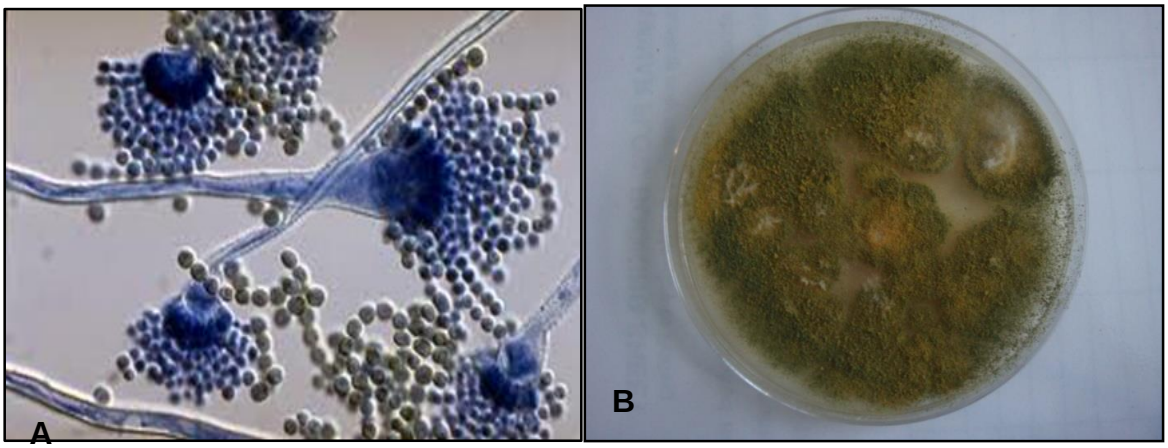

Figure 2.3 **A**. Characteristic conidiophores of *Aspergillus flavus* (Frisvad et al., 2005); **B**. Characteristic yellow-green moulds of *Aspergillus flavus* on PDA media (photo: N. Chiuraise, 2014)

2.3.1.2 Host range

Aspergillus flavus has a broad host range as an opportunistic pathogen or saprobe. It is an extremely common soil fungus. In the field, *A. flavus* is predominantly a problem in the oilseed crops; maize, groundnuts, cotton seed and tree nuts (Cary et al., 2011). Under improper storage conditions, *A. flavus* is capable of growing and forming aflatoxin in almost any crop seed (Mukanga et al., 2010). It is a pathogen of animals and insects. In humans it is predominantly an opportunistic pathogen of immuno-suppressed patients. Typical symptoms on maize include green moulds covering grain (Figure 2.4).

Figure 2.4 *Aspergillus flavus* infected maize at harvest stage (photo: N. Chiuraise, 2014)

2.3.1.3 Life cycle

The fungus forms millions of sclerotia (spores) in insect-damaged kernels before harvest. These sclerotia are dispersed into the soil during harvest and can be spread by wind, water or agricultural practices (Arora et al., 1992). The sclerotia survive in soil and produce conidiophores and conidia during the following season. Invasion of maize by *A. flavus* occurs via silks (Frisvad et al., 2005). Once *A. flavus* is present in plant tissue, it can continue to grow. Senescencing silk is a suitable media for microbial growth and provide entry for fungi into the ear (Dawlal et al., 2012). The fungal mycelium spreads superficially among the kernels and penetrates the kernels mainly through the pericarp. Insects, such as maize stalk borer (*Busseola fusca* Fuller), that feed on maize ears in the field and stored maize predispose kernels to fungal infection through physical damage, while storage insect pests open the kernels to fungal invasion (Mboya et al., 2011).

Insect damage of maize is a good predictor of mycotoxin contamination, and can serve as an early warning. Insects carry the spores from plant surfaces to the interior of the stalk or kernels or create infection wounds due to the feeding of the larvae on stalks or kernels (Henry, 2013). Insect-damaged kernels provide an opportunity for the fungus

to circumvent the natural protection of the integument and establish infection sites in vulnerable interior (Miller, 2008). Wounding by insects may provide infection courts and allow kernels to dry down to moisture content more favorable for growth of *A. flavus* and aflatoxin production. Figure 2.5 below shows a summarized life cycle of *A. flavus*.

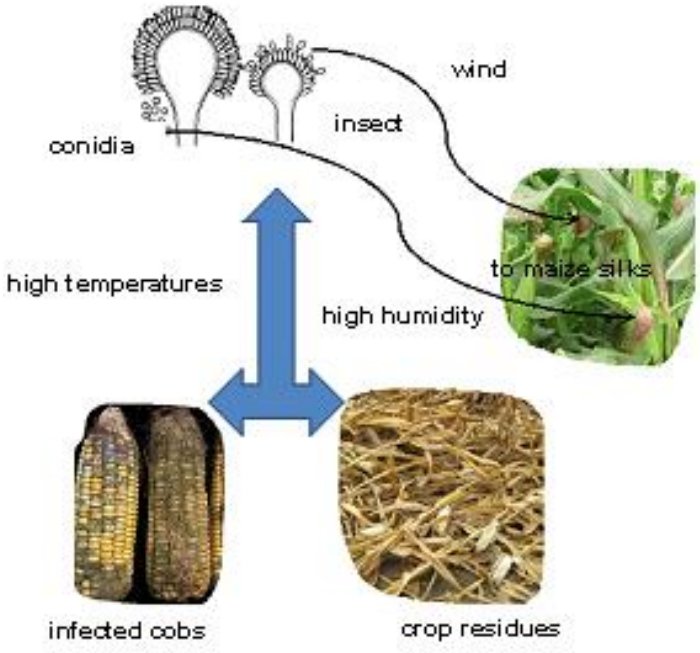

Figure 2.5 A summarized life cycle of *A. flavus,* adapted from Miller (2008)

2.4 Fumonisins

Fumonisins are secondary toxic metabolites produced by fungi of the genus *Fusarium* and are common contaminants of maize-based foods and feeds worldwide. They were first isolated and identified in South Africa in 1988 (Mangia, 2009). Fumonisins are produced by several *Fusarium* species (van der Westhuizen et al., 2010) including:

- *F. verticillioides* (Sacc.) Nirenberg,
- *F. proliferatum* (Matsushina) Nirenberg,
- *F. nygamai* Burgess &Trimboli,
- *F. anthophilum* (A. Braun) Wollenweber,
- *F. dlamini* Marasas, Nelson &Toussoun,

• *F. napiforme* Marasas, Nelson & Rabie,

• *F. thapsinum* Klittich, Leslie, Nelson & Marasas,

• *F. globosum* Rheeder, Marasas & Nelson.

Amongst these, *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers (Duncan and Howard, 2010).

There are at least 28 different forms of fumonisins, most designated as A-series, B-series, C-series, and P-series (Funnell-Harris and Pedersen, 2011). Fumonisin B_1 (Figure 2.6) is the most common and economically important form, followed by FB_2 and FB_3. Maize is the most commonly contaminated crop, although these toxins can occur in a few other crops as well.

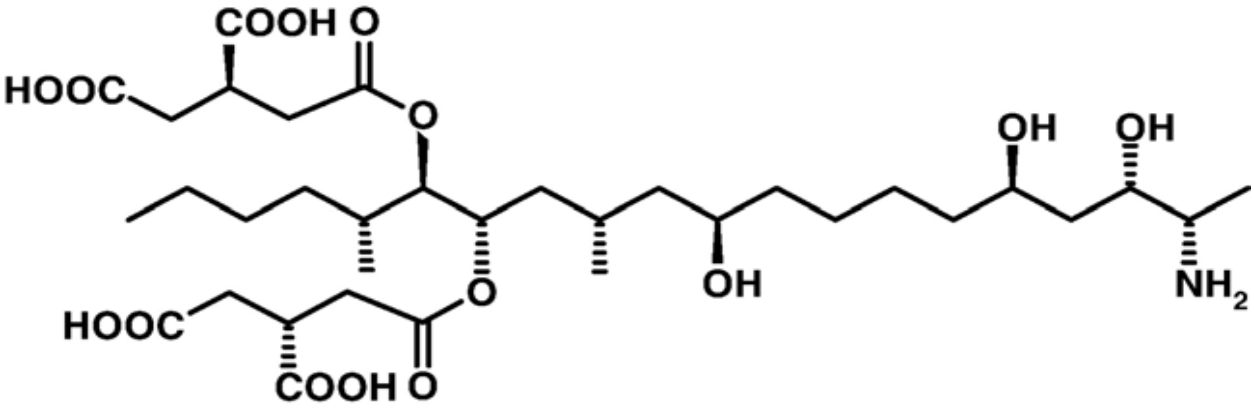

Figure 2.6 Chemical structure of Fumonisin B_1 produced by *Fusarium verticillioides* (Ncube et al., 2011)

Fumonisins have been implicated as a possible cause of oesophageal and liver cancer as well as neural tube defects in humans, blind staggers, a serious disease in horses, and porcine edema, a disease in swine (Sun et al., 2007). These toxins are poorly absorbed in the digestive tract and are quickly removed from the body of experimental animals. However, they mainly remain in liver and kidney (Serrano et al., 2012). Due to these inherent risks to human and animal health through the consumption of contaminated grains, guidelines for fumonisin content in maize products intended for food and animal feed have been implemented in a number of countries (Van Egmond et al., 2007). The United Nations food standards body Codex Alimentarius Commission has set maximum levels for total fumonisin (Fumonisin B_1+B_2+B_3) at 4 ppm (mg/kg) in raw maize grain and 2 ppm in maize flour and maize meal (Codex,

2014). The European Union has more strict regulation with maximum levels for total fumonisin set at 2 ppm (EC, 2007). The United States of America: Food and Drug Administration (FDA) sets 4 ppm for total fumonisins (FDA, 2001). In South Africa, a limit of 4 ppm for total fumonisins is set (Marasas et al., 2012). However, in some southern African countries tolerable limits generally does not exist or is not enforced (Wagacha and Muthomi, 2008). Therefore, a substantial portion of the maize crop in many parts of southern Africa could be affected when environmental conditions favour fumonisin accumulation in grain. The maize which is produced in the subsistence farming sector is consumed without checking the level of mycotoxins.

2.4.1 *Fusarium verticillioides* (Sacc.) Nirenberg

2.4.1.1 Classification

Kingdom: Fungi
Phylum: Ascomycota
Order: Eurotiales
Class: Eurotiomycetes
Family: Trichocomaceae
Genus: *Fusarium*
Species: *verticillioides*

Fusarium verticillioides (teleomorph *Gibberella moniliformis* (Sacc.) Nirenberg) is a widely distributed pathogen able to cause maize seedling blight, root rot, stalk rot and kernel or ear rot (Duncan and Howard, 2010) but also can infect vegetative and reproductive tissues without any symptom development (Hefny, 2012). Insect herbivory pressure and physiological stress facilitate disease development. Conidia are critical in the infection process of this fungus and might be necessary for systemic colonization of the maize plant (Funnell-Harris and Pedersen, 2011). Both symptomatic and asymptomatic kernel infections by *F. verticillioides* can result in decreased quality of maize and economic losses due to contamination by fumonisins. Reducing fumonisin contamination in maize will require greater understanding of how

F. verticillioides infects and systemically colonizes maize tissues and an assessment of what role conidia might play in plant-fungal interactions.

The small, hyaline, mostly single-celled microconidia of *F. verticillioides* are produced in long catenate chains arising from morphologically simple phialides (Figure 2.7). The chains of conidia are well adapted for wind, rain and vector dispersal (Dorn et al., 2011). This efficient dispersal of microconidia undoubtedly facilitates dominance in maize field environments. Sexual reproduction, although not common in nature, would be facilitated by microconidia serving as spermatia for fertilization of protoperithecia (Saleh et al., 2012). Thus, microconidia are clearly important for survival, reproduction and dispersal of *F. verticillioides.*

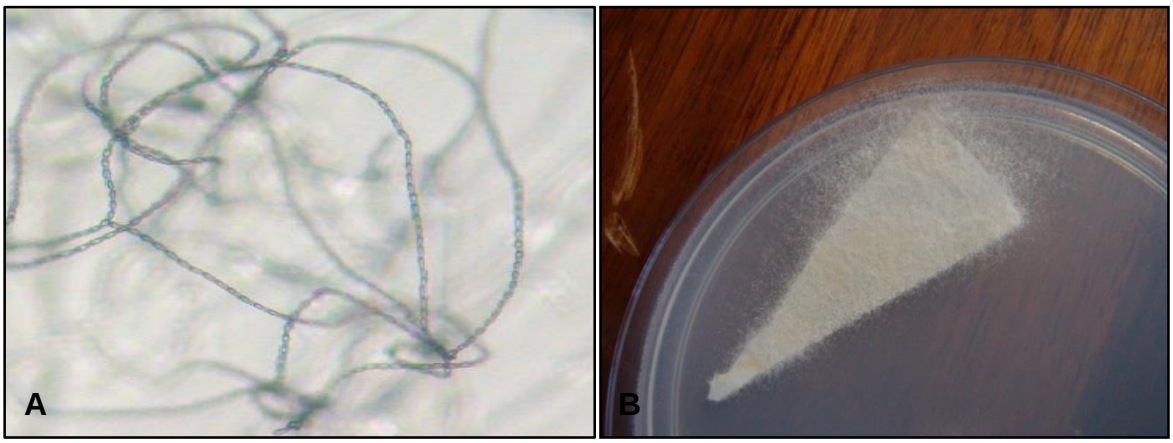

Figure 2.7 A. Microconidia chains of *Fusarium verticillioides* (photo: N. Chiuraise, 2014); B. Sporulation of *Fusarium verticillioides* on SNA media with filter paper under UV light (photo: N. Chiuraise, 2014).

2.4.1.2 Host range

Fusarium verticillioides infects a wide range of cultivated crops including maize, sorghum, sugarcane, wheat, cotton, banana, pineapple and tomato (Waśkiewicz et al., 2012). *Fusarium* ear rot caused by *Fusarium verticillioides* is a major economic concern to maize producers and the processing industry worldwide due to the losses in grain yield and quality (Adejumo et al., 2007). It is favored by warm and dry conditions. However, warm and wet conditions following inoculation at the silking stage have been reported to be conducive for disease development (Ncube et al., 2011).

Intact or split kernels covered with white or pinkish white mold are typical symptoms of ear rot infection by *F*. *verticillioides* (Figure 2.8).

Figure 2.8 *Fusarium verticillioides* infection of kernels scattered on the ear (photo: N. Chiuraise, 2014).

2.4.1.3 Life cycle

Fusarium verticillioides grows as a maize endophyte in both vegetative and reproductive tissues, often without causing disease symptoms in the plant (Menkir et al., 2006). Together with other *Fusarium* species that cause *Fusarium* ear rot, *F. verticillioides* overwinters on maize and grass residue and is more often seen in no-till, minimal-till, continuous maize fields and infected crop debris (Waśkiewicz et al., 2012). Mycelium in infected crop debris produce macroconidia and microconidia that are wind and rain splash disseminated, infecting ears through silks and colonizing kernels (Figure 2.9). *Fusarium verticillioides* can also infect maize plants systemically in which case ears may be infected through the ear shank. Insects, such as the *Lepidoptera* and *Coleoptera* spp., have been reported to act as vectors and transfer *F. verticillioides* spores between plants or cause plant injury that enable the fungi to infect the ears (Wild and Gong, 2010).

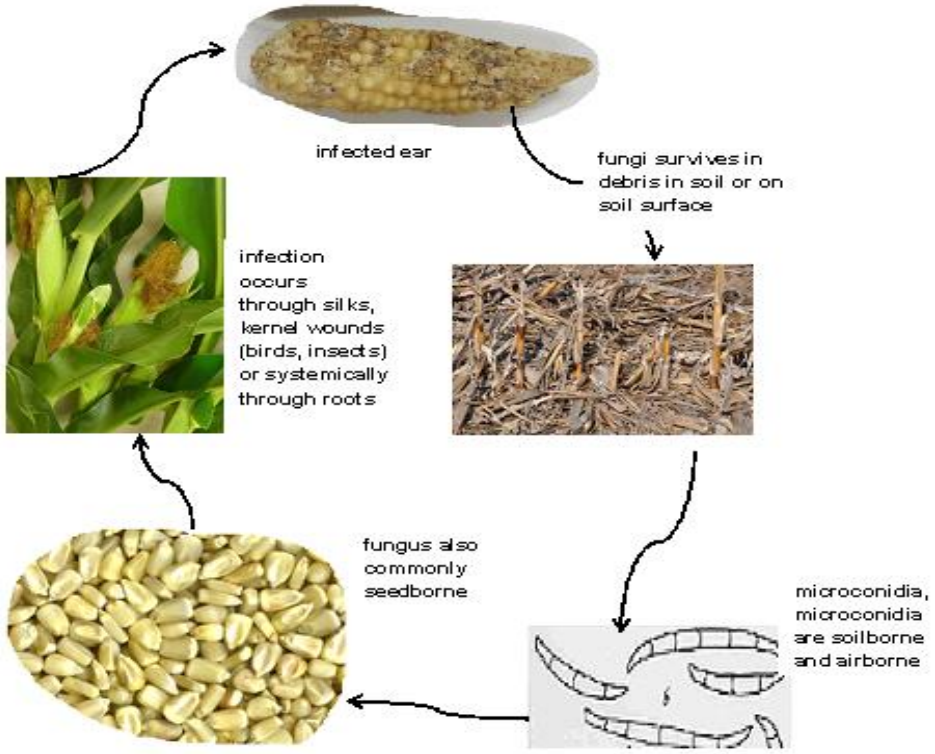

Figure 2. 9 Life cycle of *Fusarium verticillioides* adapted from Schjoth et al. (2008).

Often only kernels that are damaged due to insect feeding or silk cutting are prone to a *Fusarium* infestation, but under ideal conditions undamaged kernels can be infected. Ears that remain upright during heavy rainfall events and/ or have poor husk cover tend to be more susceptible to extensive rotting (Williams and Windham, 2009). This suggests that plant breeders should select for the drooping ear that shed moisture to improve resistance to infection.

2.5 Other ear rots

2.5.1 Diplodia ear rot

Stenocarpella is recognized as the most important ear rot pathogen in nearly all countries where maize is produced (Rossouw et al., 2009). While *S. maydis* is reported from humid zones wherever corn is grown, *S. macrospora* is most prevalent in humid subtropical and tropical zones, where plants exhibiting dry-ear rot and stalk rot may also display symptoms of leaf striping (Donald et al., 2010). Severe crop infestation can result in significant yield losses of more than 50% from the presence of

light-weight, rotted ears with discolored, shriveled, and non-viable seeds (Figure 3.1C). Infection by *Stenocarpella* spp. spread by wind of insect dispersal of conidia. Conidia in *S. maydis* are straight, curved or irregular, septate, smooth-walled and pale-brown with rounded or truncated ends (Figure 2.10)

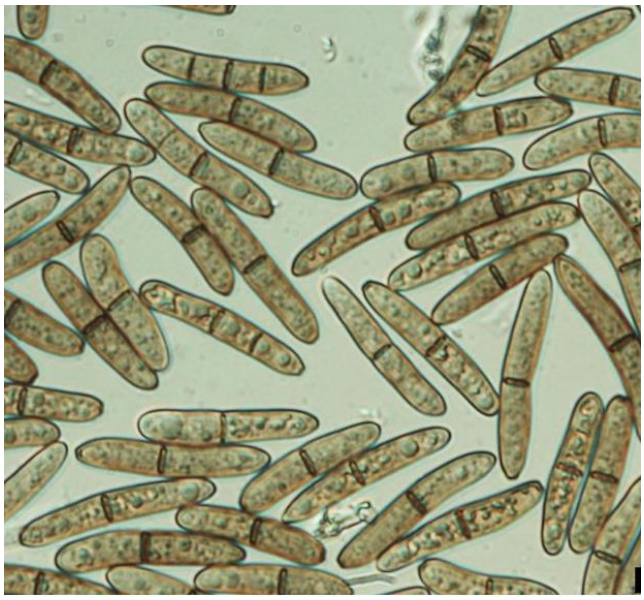

Figure 2.10 Conidia of *Stenocarpella maydis* (Adapted from (Rossouw et al., 2009)

Stenocarpella maydis ear rot is of further importance because of its association with authenticated field outbreaks of diplodiosis, a common nervous disorder (neuromycotoxicosis) of cattle and sheep grazing on infected maize crop residue in southern Africa (Moremoholo et al., 2010) and in Argentina (Odriozola et al., 2005). Studies on the characterization of secondary metabolites, diplodiotoxins are still ongoing to understand its extend of toxicity to humans and livestock. Previous studies have reported that flint cultivars are more resistant than dent (Czembor and Ochodzki, 2009), and resistance breeding offers promise for control, although no maize lines appear immune.

2.5.2 Gibberella ear rot

Fusarium graminearum Schwebe. (teleomorph: *Gibberella zeae* (Schwebe.) which causes *Gibberella* ear rot (GER) of maize continue to be a significant problem in tropical and subtropical areas in SSA. It is mostly of significant concern to pork

producers. The production of the secondary metabolite, zearalenone causes infertility in male swine and hyperestrogenism in female swine. Deoxynivalenol (DON, vomitoxin) causes feed refusal and vomiting in swine which leads to a reduction in weight gain and feed conversion (Sampietro et al., 2011). The fungus gains entry into the maize ear by two routes. One infection route is via the silk. Conidia or ascospores land and germinate on the silk, entering the ear by way of mycelium growing down the silk (Picot et al., 2012). The second route is through wounds in the husk created by insects, birds or hail.

The biotic causes of husk wounds probably result in the greater frequency of *Fusarium* spp. infection because insects and birds can be vectors of the fungus. Cool, wet weather within three weeks after silking favours the disease. Previous work has demonstrated some maize hybrids and inbreds that vary in their response to GER (Kazan et al., 2012). Symptoms include pink to reddish mould usually starting at the tip of the ear; mould growth development between husks and ears; superficial black specks develop on the husk and ear shanks (Ma et al., 2013) (Figure 3.1D). Septate sickle shaped macroconidia are distinguishing characteristics of *F. graminearum* (Figure 2.11).

Figure 2.11 Macroconidia of *Fusarium graminearum* (Adapted from Leslie and Summerell (2006).

2.6 Breeding for resistance

Several methods for reducing severity of *Aspergillus*, *Fusarium* ear rot and mycotoxin contamination in maize grain have been suggested, but the most effective and economical method is the development of resistant hybrids.

2.6.1 Genetic variation

Genetic variation describes naturally occurring genetic differences among individuals of the same species (Acquaah, 2009). Genetic variation is known to exist for ear rots and mycotoxin contamination resistance in maize, and stably resistant breeding lines have been developed. However, transfer of resistance to elite cultivars has been hampered by low heritability, caused by high genotype × environment interaction, a highly quantitative nature, and difficulty in accurate phenotyping (Warburton et al., 2013). In order to screen genotypes across seasons and environments with uniform levels of infection, maize breeders have resorted to the use of artificial inoculation. Natural infection is highly variable across seasons and environments making it difficult to differentiate between susceptible and resistant genotypes. Previous studies have observed significant differences between inbred lines using the silk channel and kennel wounding inoculation methods (Afolabi et al., 2007,Brown et al., 2013,Robertson-hoyt et al., 2007)

While stable resistance is a positive advancement in the efforts to breed resistant maize hybrids, all resistant breeding lines identified to date contain tropical germplasm in their backgrounds (Menkir et al., 2008). For example Williams et al. (2014) reported that resistance to mycotoxin producing *Fusarium* and *Aspergillus* species in existing inbreds have been derived from tropical sources, such as NC300, Mp313E, and Mp715. NC300 was derived from a three-way cross of tropical hybrids, and Mp313E and Mp715 were derived from Tuxpeño, a tropical landrace from México. Warburton and Williams (2014) described the tropical Mexican landrace Tuxpeño as the progenitor or one main contributor for most of the resistant lines and likely the source of resistance. Tropical germplasm tend to be tall, late, and prone to lodging, in addition to lower yielding than commercial hybrid checks when grown in subtropical and temperate conditions in South Africa. Because of the highly quantitative nature of host

plant genetic resistance to *A. flavus* and *F. verticillioides* infection and aflatoxin and fumonisin contamination in maize, it has been very difficult to transfer the resistance from these older breeding lines into a more agronomically acceptable idiotype using only phenotypic selection (Donald et al., 2010).

2.6.2 Heritability

Heritability is defined as the proportion of total variance that is genetic (Hallauer et al., 2010). Heritability is important because it aids the breeder in determining the amount of progress or genetic gain that can be made through certain breeding strategies. It is a measure of the degree (0 to 100%) to which offspring resemble their parents for a specific trait. A high heritability means the offspring have a greater chance of exhibiting an expression of the trait that is similar to that of the parents; whereas a low heritability shows a lower level of resemblance (Odriozola et al., 2005). There are two types of heritability, broad and narrow sense heritability. Broad sense heritability (H^2) is the proportion of phenotypic variation (σ^2_P) that is the result of the total genetic variation (σ^2_G) including both dominance and epitasis effects ($H^2 = \sigma^2_G/\sigma^2_P$). Narrow sense heritability (h^2) is the proportion of phenotypic variance (σ^2_P) that is the result of additive genetic variation, σ^2_A, ($h2 = \sigma^2_A/\sigma^2_P$) (Nyquist, 1991). The heritability for any trait can be calculated.

In a study by Menkir et al. (2006), broad-sense heritability estimates were found to be moderate to high for *Aspergillus* ear rot (AER) and aflatoxin contamination resistance suggesting that selection for resistance should be feasible. In a similar study Robertson-Hoyt et al. (2006) reported relatively high heritabilities for *Fusarium* ear rots ($H^2 = 0.47$) and fumonisin contamination resistance ($H^2 = 0.86$). This implies that breeding for resistance to fumonisin contamination can be achieved by selection in multiple environments for resistance to *Fusarium* ear rot (FER).

2.6.3 Gene action

Gene action refers to the behavior or mode of expression of genes in a genetic population (Acquaah, 2009). There are four types of gene action: additive, dominance, epistatic and overdominance (Acquaah, 2009). Disease resistance is a complex phenomenon and different genes play different roles in helping out the plant against the pathogens. Different kinds of protein-protein interactions are involved at different growth stages (Eller et al., 2008). The interaction of different genes and their pathways must be revealed, to confirm the exact role of genes. Plant pathologists and breeders recognize two general types of resistance: qualitative and quantitative (Guo et al., 2001). Qualitative resistance typically confers a high level of resistance, and is usually race-specific, and based on single dominant or recessive genes. In contrast, quantitative resistance in plants is typically partial and race-nonspecific in phenotype, oligogenic or polygenic in inheritance and is conditioned by additive or partially dominant genes (Parlevliet, 2002). Although it is easier to work with qualitative resistance in crop genetic studies and in breeding, quantitative resistance is often the more useful in an agronomic context, due to its generally higher durability and broader specificity (Lindhout, 2002). In maize, the majority of disease resistance deployed in elite varieties in the field is quantitative in nature (White et al., 1990).

Results from previous studies strongly suggests that resistance to ear rots, aflatoxin and fumonisin contamination is quantitatively inherited with both simple (additive and dominance) and digenic (dominance x dominance) effects playing a major role in conditioning the inheritance of resistance (Leslie and Summerell, 2006,Mesterházy et al., 2012).

2.6.4 Correlations

A correlation is a measure that determines the degree to which two traits are associated (Manly, 2004). Previous studies have reported a number of correlations between ear rots, mycotoxin contamination and some agronomic traits in maize. For example the results of (Picot et al., 2012) clearly showed that a significant correlation between pericarp thickness and resistance exists and identified various properties of

the pericarp and its wax layer as resistance factors to *F. verticillioides*. These traits were consistent over two seasons under very differing ecological conditions. When the wax was removed, infection severity increased significantly. Ma et al. (2013) detected different thrips species on ears that increased the severity of FER infection. Husk looseness correlated with FER at the brown silk stage and also with the size of the thrips population. It was concluded that husk tightness plays an important part in epidemiology and disease development. Eller et al. (2008) reported that the kernel moisture content influences the degree of ear rot. This concurs with studies on stalk rot causing pathological drydown and thus influencing Gibberella ear rot (GER) (Mesterházy et al., 2012) as well as FER.

Results from an earlier study showed inbred lines developed with selection for GER also exhibited high levels of resistance to FER and common smut (*Ustilago zeae*) in inoculated trials (Sampietro et al., 2011), indicating that it may be possible to develop hybrids with resistance to multiple *Fusarium* spp. (Mesterházy et al., 2012). Tang et al., (2013) found that the heritabilities for mycotoxin values were similar or higher than those found for ear rot data (both *F. graminearum* and *F. verticillioides*). This is in agreement with studies done by (Henry et al., 2009) which identified genotypes with good resistance to both *F. verticillioides* and *A. flavus*. Correlations between ear rot severities of the two pathogens (r = 0.72) and between aflatoxin and fumonisin concentrations (r = 0.61) led to the conclusion that good resistance to both species in the same genotype is attainable. (Robertson-hoyt et al., 2007) also came to the same conclusion.

2.6.5 Breeding progress and gains through selection

Breeding progress is the increase in the level of a quantitative trait that results from selection (Poehlman, 1994). Resistance to ear rot infection and mycotoxin contamination is highly quantitative, and maize breeders have difficulty incorporating polygenic resistance alleles from unadapted donor sources into elite breeding populations without having a negative impact on agronomic performance. Therefore, progress in preventing aflatoxins and fumonisins in maize has been slow. Some of the

hoped for technologies and advances, such as short cuts for evaluating resistance to aflatoxins and fumonisins contamination in the laboratory and more efficient procedures for evaluating resistance in the field, are still in development (Warburton and Williams, 2014). More work is needed to understand the reasons and conditions under which these mycotoxins are produced. Studies by Small et al. (2012) identified inbred lines, CML 444 and CML390 with potential resistance to FER and fumonisin contamination. They are adapted to the medium altitude environments (800-1600 masl) in eastern and southern Africa.

Williams and Windham (2009) analysed fumonisin accumulation in a diallel analysis using *A. flavus*-resistant and *A. flavus*-susceptible inbreds inoculated with *F. verticillioides* and *A. flavus*. The inbreds Mp715 and MP 717 revealed high aflatoxin and fumonisin resistance. Inbred Mp313E revealed resistance only against fumonisins, not aflatoxin. Therefore inbred lines MP715 and MP717 could be valuable sources of resistance to both *A. flavus* and *F. verticillioides* infection, and aflatoxin and fumonisin contamination. Studies by Robertson et al. (2006) and Li et al. (2011) are in agreement that four QTLs on chromosome 3,4,5 and 6 accounted for 2.5-10.2% of the phenotypic variation of Fusarium ear rot and fumonisin contamination. Among the four QTLs, the QTL on chromosome 4 (bin 4.06) flanked by markers bnlg1621-bnlg1137, has the largest effect on resistance to Fusarium ear rot and fumonisin contamination. This locus can be utilised in facilitating marker assisted selection in maize breeding programs.

Menkir et al. (2008) also identified six maize inbred lines, TZAR 101-6 at IITA, Nigeria with resistance to aflatoxin contamination. The lines combine both temperate and tropical genomes. These resistant sources are a valuable contribution to the aflatoxin resistance breeding efforts in SSA. Four resistant inbred lines: Mp715, Mp717, Mp718, and Mp719 that were developed at Mississippi State University have been released as sources of resistance (Williams and Windham, 2001,Williams and Windham, 2006,Williams and Windham, 2012). These lines were selected primarily from southern US germplasm. An additional three resistant lines: GT601, GT602, and GT603 were developed from GT-MAS:gk population and released as sources of resistance to accumulation of aflatoxins (Guo et al., 2011,Guo et al., 2007).

Studies by Kelley et al. (2012) identified maize genes associated with host plant resistance or susceptibility to *A. flavus* infection and aflatoxin contamination using a combination of microarray analysis, qRT-PCR analysis, and QTL mapping methods. A gene encoding glycine-rich RNA binding protein 2 was found to be associated with the host hypersensitivity and susceptibility in inbred line Va35. A nuclear pore complex protein YUP85-like gene was found to be involved in the host resistance in inbred line Mp313E. These findings will be important in identification of DNA markers for breeding maize lines resistant to aflatoxin accumulation.

2.6.6 QTL analysis for ear rot and mycotoxin contamination

Genomic regions (or loci) responsible for quantitative effects known as quantitative trait loci (QTL) can be utilised in molecular breeding by the use of molecular markers to transfer specific QTLs from resistant breeding lines (donor parents) to current elite (but susceptible) inbreds (Robertson-hoyt et al., 2007). This could greatly speed up resistance breeding efforts. For example, markers could be used to introgress identified QTL to elite parents via marker-assisted backcrossing by using markers that can be either cheaply run in a small laboratory, or automated and run very quickly in a larger one. Thus all plant breeding programs (large or small) would be able to use the markers to efficiently pyramid and transfer resistant QTLs from one or more donor lines into elite maize inbreds. Alternatively, markers could be used to estimate the breeding value of lines based on the magnitude of effects associated with marker alleles for mycotoxin contamination resistance and other traits of interest. (Moremoholo et al., 2010).

Quantitative trait loci mapping studies have identified many potential QTLs for aflatoxin, *A. flavus*, and ear rot resistance (Robertson-hoyt et al., 2007); (Kazan et al., 2012,Leslie and Summerell, 2006,Poehlman, 1994)) and some of these have been identified in multiple studies or from more than one donor line. Unfortunately, no single QTL has been identified that can explain a majority of the phenotypic variation within a study (major gene resistance) or a significant amount of the variation in every study. However, at least one QTL with a moderately large, repeatable, additive effect has

been found from each resistance source mapped to date (Poehlman, 1994). Therefore, the development of markers from within the largest QTL from multiple different resistance sources will allow the possibility of pyramiding these genes with large additive effects into elite breeding lines. This will allow this trait to be more easily manipulated in practical breeding programs.

(Robertson-hoyt et al., 2007) found that QTLs for *F. verticillioides* resistance were also effective against *A. flavus*. The genotypic correlations between ear rot data of the two pathogens (r = 0.99) were very close. On chromosome 5, a large effect QTL was identified. The resistance QTLs against *A. flavus* and *F. verticillioides* were occasionally clustered on the same chromosomes. Another attempt was the meta-analysis of QTLs associated with ear rot resistance (Hallauer et al., 2010). The analysed data of 14 studies, representing *F. graminearum, F. verticillioides* and *A. flavus*, showed that QTLs against the three fungi were clustered on the same chromosomes (chromosome 4 and 5) (Mesterházy et al., 2012). These data seem to support the idea of common resistance on QTL level. At present, it is not clear whether the QTLs in a cluster are individually effective to all three fungal pathogens or whether they are specialized to different fungal species and the cluster effect secures the broad sense resistance. The former is more likely on the basis of wheat studies (Mesterházy et al., 2012) .

2.6.7 Genotype by environment interaction

FAO (2006) defined genotype x environment (GxE) interaction as the failure of genotypes to achieve the same relative performance in different environments. Donald et al. (2010) reported that the main difficulty in detecting QTLs that express resistance in multiple environments is the high GxE interaction variance that *A. flavus* and aflatoxin resistance displays. Afolabi et al. (2007) and Small et al. (2012) previously reported a significant GxE interaction for FER and fumonisin contamination in Nigeria and South Africa, respectively. Due to the nature of plant diseases, successful infection of the host and subsequent production of mycotoxins is dependent on prevailing environmental conditions, susceptibility of the plant host to the pathogen, as

well as insect vector activity under some circumstances (Mesterházy et al., 2012,Warburton and Williams, 2014). Further, it has been reported that maize genotypes grown outside of their normal production zones are likely to be more susceptible to ear rots and mycotoxin contamination (Menkir et al., 2008). For these reasons, it is important that locally adapted material be used in breeding programs. Potentially resistant lines are evaluated over an extended range of conditions, representative of the localities where they will eventually be grown, to further confirm their resistant status.

2.6.8 Conclusion

The review of literature has shown that plant breeding has the potential to reduce ear rot severity and mycotoxin contamination in maize grain. Indeed previous studies have shown significant and positive correlations between ear rot severity and mycotoxin contamination, aflatoxin and fumonisin contamination resistance, and mycotoxin contamination and selected agronomic traits. Previous studies have also shown moderate to high heritability estimates for aflatoxin and fumonisin contamination resistance. A single study reported the simultaneous screening of maize inbred lines in the greenhouse and field experiments with a significant positive correlation. However, the following knowledge gaps were identified; consumers of maize grain in southern Africa are unaware of their level mycotoxin contamination exposure due to ear rot diseases. Studies on the efficacy of stacking ear rot, aflatoxin and fumonisin contamination resistance genes in maize hybrids have not yet been undertaken. It is also noted that commercial hybrids with combined resistance to both aflatoxin and fumonisin contamination are non-existent.

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CHAPTER THREE

RESEARCH DESIGN AND METHODOLOGY

3.1 Introduction

The chapter presents the research design and methodology employed to address the study's aims and objectives. It describes how the survey on the incidence of ear rots on southern African maize hybrids was carried out. It also explains the design and management of greenhouse and field trials. Further it reveals the morphological and molecular characterisation of the pathogens surveyed and the ones used for artificial inoculation. The chapter also presents the methods for quantifying mycotoxin content and collection of data on agronomic traits.

3.2 Research design

Two approaches were taken to answer research objectives described in Chapter one. Firstly a survey of natural incidence of the ear rots which cause toxins in maize grain was conducted using a sample of regional experimental hybrids. Secondly, new experimental hybrids were designed by stacking aflatoxin and fumonisin resistance genes in three-way cross hybrids. The three-ways were advanced to the $S_{2:3}$ families. These experimental materials were artificially inoculated with *A*. *flavus* and *F. verticillioides*. At harvest the grain was evaluated for ear rot infection and mycotoxin contamination. The details of germplasm, experimental design and management are described in the following sections.

3.3 Screening for ear rot incidences in southern African maize hybrids

3.3.1 Germplasm

Experimental hybrids with different levels of physiological maturities (early, medium and late) from breeding programs in four countries in southern Africa namely; Zimbabwe, Zambia, Malawi and South Africa were observed in the regional trials, organised by the International Maize and Wheat Improvement Center (CIMMYT). A set

of 50 early, 63 medium and 54 late maturing hybrids were surveyed in 2012/13. In 2013/14, a set of 60 early, 60 medium and 40 late maturing hybrids were surveyed (Table 3.1). The hybrids 11C1579 and 11C1774 were used as local checks in the survey. All hybrids had white grain which is preferred by consumers in the region.

Table 3.1 List of hybrids screened for natural incidences of ear rot diseases at Cedara Research Station, South Africa.

Maturity group	Number of hybrids		
	2012/13	**2013/14**	**Total**
Early	50	60	**110**
Medium	63	60	**123**
Late	54	40	**94**
Total	**167**	**160**	**327**

3.3.2 Experimental design

The seeds of the hybrids were planted at Cedara Research Station (29°54`S, 30°26`E, altitude 1066 m), during the 2012/13 and 2013/2014 season. Cedara Research Station was chosen as a suitable site to conduct the study because it has a warm, misty and humid climate providing perfect conditions for ear rots disease development. It is also known as a disease hotspot in South Africa. The experiments for both seasons were planted in three replications arranged in an alpha-lattice design with five plots per incomplete block. Plots consisted of two rows per hybrid of 5 m with spacing of 0.9 m between the rows and 0.3 m within the rows making a plot size of 9 m². The trial was planted by hand with two seeds per station and thinned down to one plant at three weeks after seedling emergence. Each row had a maximum potential of 17 plants resulting in 34 plants per plot.

3.3.3 Management

The experiment was conducted under natural conditions, that is, no irrigation was applied or artificial inoculation of disease. Basal fertilizer (NPK) was applied (75 kg N,

50 kg P, 25 kg K per hectare) before planting. The top dressing of 120 kg per hectare in the form of Limestone Ammonium Nitrate, LAN (28% N) was applied four weeks after crop emergence. Hand weeding and other cultural practices were conducted as and when they were required. The cultural practices, which are recommended for maize in South Africa, were followed. This included application of insecticide granules to control stalk-borer, and application of herbicides, such as atrazine and gramoxone, to control the weeds. The trials were manually harvested during May 2013 and May 2014, respectively.

3.3.4 Data collection

The rainfall, temperature and relative humidity data for 2012/13 and 2013/14 seasons were recorded. The experimental data was collected on a whole plot basis for each hybrid. Flowering dates were recorded as number of days from planting to 50% of the plants showing silks for silking date or shedding pollen for anthesis date. Anthesis silking interval was calculated by subtracting silking date from anthesis date. At harvest diseased ears per plot were counted and categorized based on the visual symptoms of the ear rots. The symptoms were classified as *Aspergillus*, *Fusarium*, *Diplodia* or *Gibberella* ear rot (Figure 2.3). The characteristic symptoms of *Aspergillus* ear rot are yellow-green mycelia growth on the kennels (Figure 2.3A). *Fusarium* ear rot is characterized by cottony, whitish-pink growth typically occurring individually or in groups scattered randomly on ear (Figure 2.3B). *Diplodia* ear rot is dense whitish fungal growth matted between the kernels and between the ear and the husk, beginning at the base of ear progressing towards the tip (Figure 2.3C). Pink to reddish mould usually starting at the tip of the ear characterize *Gibberella* ear rot (Figure 2.3D). Grain yield was measured as plot weight and transformed to t/ha. Data on insect damage was taken in 2013/14 season due to the observed high incidence. Insect damage ratings were recorded from 1=no damage or tunnelling to 9=heavy damage or tunnelling (Badu-Apraku et al., 2012). Grain texture was recorded using a rating scale of from 1=hard, completely rounded flint kernel to 5=soft, distinct dent (CIMMYT, 1985). The grain moisture content was determined using the grain moisture meter, MC-7825G (ONMI instruments, UK).

3.4 Isolation and morphological identification of cultures

Kernels infected with suspected *Fusarium* spp. were surface sterilised with 2% jik (sodium hypochlorite) for one minute before being washed three times with distilled water. The kernels were then cultured on selective media Synthetic Nutrient Deficient Agar (SNA- glucose 0.2 g, sucrose 0.2 g, KH_2PO_4 1 g, KNO_3 1 g, $MgSO_4$ 0.25 g, KCL 0.5 g, agar 14 g/L). Pieces of sterile filter paper were placed on the SNA media to enable quick sporulation. Plates were incubated at 25°C under UV light for 14 days. Formation of microconidia chains (Leslie and Summerell, 2006) were used as a morphological tool to confirm the *F. verticillioides* isolate in the laboratory at University of KwaZulu-Natal (UKZN). The chains of microconidia were observed under a light microscope (Figure 2.7). Deep purple colouration and sickle shaped macroconidia under a light microscope were used as characteristic tools for confirmation of *F. graminearum* (Figure 2.11)

Kernels infected with suspected *A. flavus* were surface sterilised with 2% jik for one minute (sodium hypochlorite) before being washed three times with distilled water. The *Aspergillus flavus* Selective Media (AFPA – dichloran 0.002 g, ferric ammonium citrate 0.5 g, peptone 10 g, K_2HPO_4, $MgSO_4.7H_20$ 0.5 g, chloramphenicol 0.2 g, agar 15 g/L) was used to culture the infected kernels. Plates were incubated at 28°C for seven days. Yellow-green moulds on media and the observation of a spore-bearing structure (the aspergillum) were used as morphological characteristic tools to confirm *A. flavus* in the laboratory (Figure 2.3).

Suspected *Diplodia* ear rot kernels were also surface sterilised before being plated on potato dextrose agar (PDA). The plates were incubated at 28°C for seven days. White mycelium covering the surface of the media and black spherical pycnidia on the kernels are the morphological characteristics of *S. maydis*. Microscopic observation revealed conidia as straight, curved or irregular, septate, smooth-walled and pale-brown with rounded or truncated ends (Figure 2.10).

3.5 Molecular characterization of cultures

Molecular techniques which include deoxyribonucleic acid (DNA) extraction, amplification and sequencing were employed. DNA was isolated using the DNA mini

plant extraction kit (Qiagen, Valencia, CA) by following the manufacturer's protocol after the mycelium was placed in Eppendorf tubes and ground with ca. 10 µg sterile, chemically treated sand. Extracted DNA was used as template in polymerase chain (PCR) reactions. Part of the transcript elongation factor (TEF) was amplified using the primer set elongation factor 1 (EF1) [5'- CGAATCTTTGAACGCACATTG -3'] and EF2 (5'- CCGTGTTTCAAGACGGG -3') (O'Donnell K et al., 1998) . For *A. flavus* and *S. maydis* internal transcribed spacer (ITS) region primers, ITS1 (5'- TCC GTA GGT GAA CCT GCG G- 3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') were used for the amplifications of the target genomic regions of the fungal isolates (White et al., 1990).

The polymerase chain reaction (PCR) reaction consisted of 1x DreamTaq reaction buffer with $MgCl_2$, dNTPs (250 µM each), primers (0.2 µM each), template DNA (25 ng) and DreamTaq polymerase (0.5 U) (Thermo Fisher Scientific, Waltham, MA). The PCR reaction conditions, for the TEF and ITS gene region were amplified by initial denaturation at 94°C for 2 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and elongation at 72°C for 1 min, with a final elongation step at 72°C for 5 min. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (Qiagen, Valencia, CA).

DNA sequences were determined from PCR amplicons using the ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, (Applied Biosystems, Warrington, UK) using the primers EF1 and EF2. Consensus sequences were compiled in BioEdit and BLAST comparisons done on *Fusarium spp., Aspergillus* spp. and *Stenocarpella spp.* parts of MycoBank database (http://www.mycobank.org). A 99% match confirmed the species as *F. verticillioides* (Accession no. KF562131.1), *F graminearum* (JX118859.1), *A. flavus* (KF309063.1) and *S. maydis* (KC311732.1).

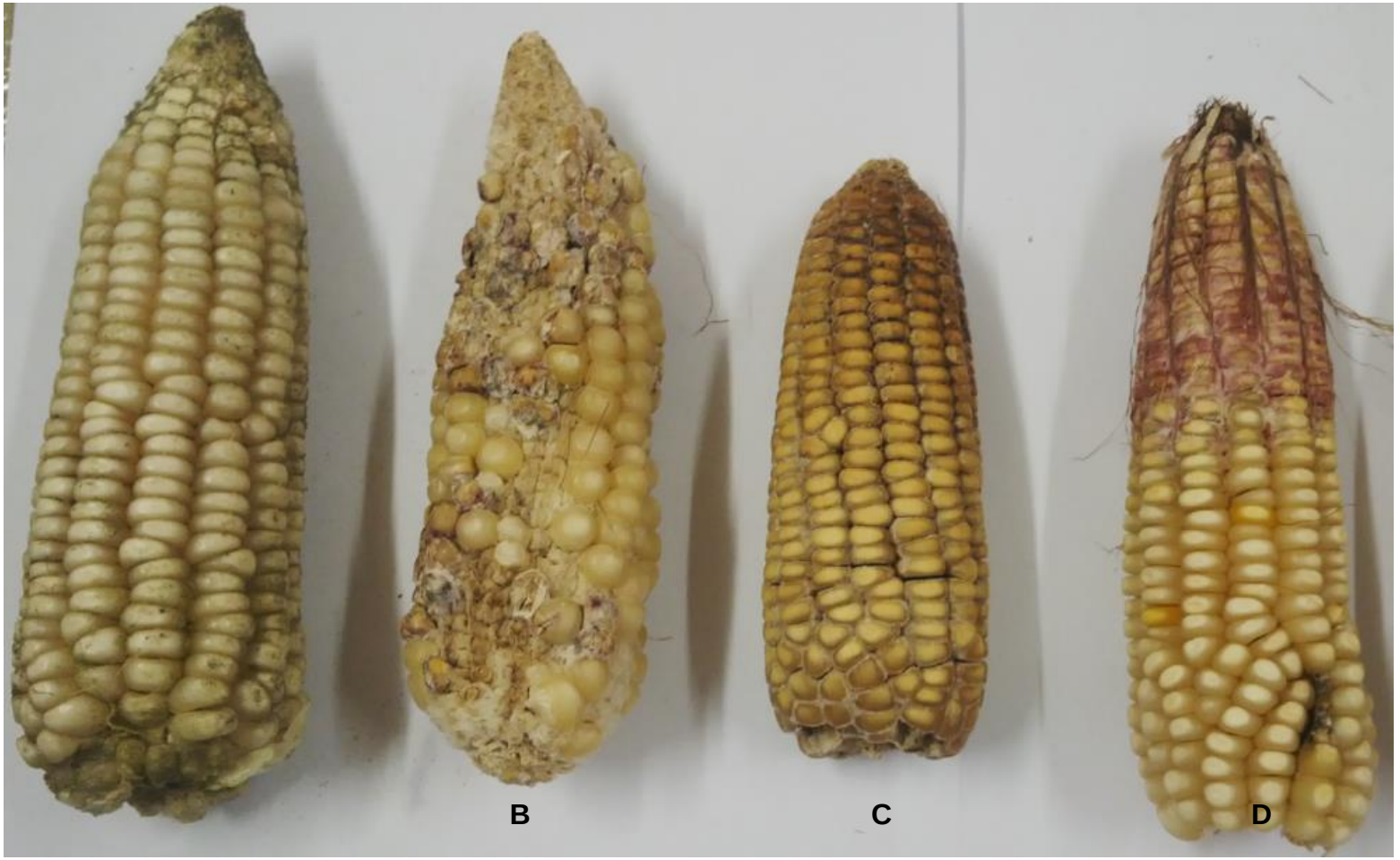

Figure 3.1 Characteristic symptoms of *Aspergillus* ear rot (A), *Fusarium* ear rot (B), *Diplodia* ear rot (C) and *Gibberella* ear rot (D).

3.6 Introgression of aflatoxin and fumonisin contamination resistance genes in maize hybrids

3.6.1 Germplasm

The maize inbred lines, TZAR102 and TZAR103, with resistance to aflatoxin contamination were obtained from the International Institute of Tropical Agriculture (IITA) maize program at Ibadan, Nigeria. These maize inbred lines combine temperate and tropical genomes in their background, which makes them suitable sources for breeding aflatoxin resistance in South African germplasm lines. In addition they contain sufficient genes of resistance to major foliar diseases, which compromise grain yield in the southern Africa region. These materials are sufficiently described by Menkir et al. (2008).

The maize inbred lines, CML444 and CML390, with resistance to fumonisin contamination were obtained from the International Maize and Wheat Improvement Centre (CIMMYT) regional research station in Harare, Zimbabwe. These lines were found to accumulate low levels of fumonisin, < 5ppm (Small et al., 2012), qualifying them as suitable sources for use in breeding for fumonisin resistance, in South African maize inbred lines. Additionally, these lines have been shown to be resistant to *Fusarium* ear rot infection which is associated with contamination of grain with fumonisins and other mycotoxins. They have white grain and high yield potential in hybrids. They are adapted to the medium altitude environments (800-1600 masl.), in eastern and southern Africa.

The following adapted inbred lines from the maize program at the University of KwaZulu-Natal (UKZN) were used as recipients of genes for resistance to contamination by aflatoxin and fumonisin: PA1, PB1, 08CED6-7, DTA; DTAB103, 10MAK9-32B, 10MAK10-27, 10MAK9-34, DTAB-112, DTAB-84, DTAB-83, 10MAK10-1 and 12UK5-1. An additional 28 experimental lines were included.

Table 3.2. List of recipient germplasm of genes for resistance to contamination by aflatoxin and fumonisin.

Entry	Inbred line	Grain colour	Entry	Inbred line	Grain colour
1	PA1	White	29	12MAKCB4-105	White
2	PB1	White	30	12MAKCB4-106	White
3	08CED6-7	White	31	12MAKCB4-111	White
4	DTA	White	32	12MAKCB4-120	White
5	DTAB103	White	33	12MAKCB4-123	White
6	10MAK9-32B	White	34	12MAK9-1	White
7	10MAK10-27	White	35	12MAK9-136	White
8	10MAK9-34	White	36	12MAK9-112	White
9	DTAB-112	White	37	CML395	White
10	DTAB-84	White	38	N3	White
11	DTAB-83	White	39	PL720	White
12	10MAK10-1	White	40	B17	White
13	12UK5-1	White	41	09MAK24-1	White
14	12MAKCB3-1	White			
15	12MAKCB3-2	White			
16	12MAKCB4-3	White			
17	12MAKCB4-6	White			
18	12MAKCB4-7	White			
19	12MAKCB4-79	White			
20	12MAKCB4-104	White			
21	12MAKCB4-110	White			
22	12MAKCB4-123	White			
23	PAN6227F2-8	White			
24	09UK16-37B	White			
25	PAN53	White			
26	PAN6611	White			
27	08CED-7-2	White			
28	12MAKCB4-104	White			

3.6.2 Stacking of resistant genes into adapted germplasm to be improved

The genes for resistance to aflatoxins and fumonisins were stacked into South African adapted maize inbred lines by crossing them with the resistant inbred lines. A total of 41 recipient lines were crossed to two fumonisin resistant inbred lines (donors): CML444 and CML390, to generate 82 F_1 single crosses, during the 2012 winter season, at Makhathini Research Station (27°39`S; 32°10`E; altitude 72 m). These 82 single crosses were crossed with two aflatoxin resistant inbred lines: TZAR102 and TZAR103, in the greenhouse during the 2012/13 summer season. This resulted in 44 three-way cross hybrids stacked with aflatoxin and fumonisin resistant genes. The three-way cross is equivalent to the S_1 generation seed. These three-way crosses were self-pollinated to produce the $S_{1:2}$ generation seed at Makhathini Research Station, during the 2013 winter season. The resultant 146 $S_{1:2}$ families were then advanced to $S_{2:3}$ at Cedara Research Station (29°54`S, 30°26`E, altitude 1066 m), during the 2013/14 summer season. Figure 4.1 shows the schematic diagram of the introgression of resistant genes into adapted lines over four seasons.

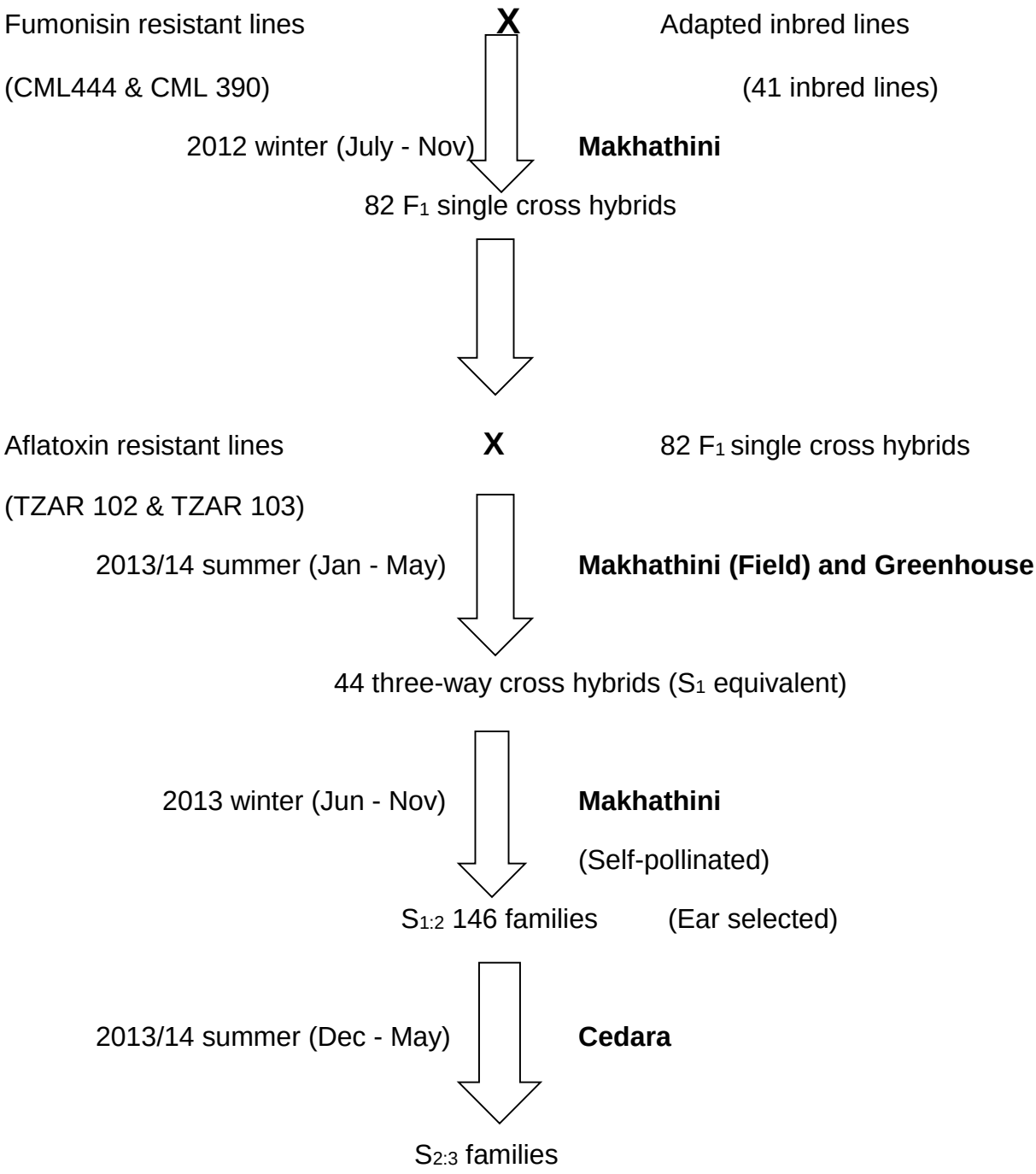

Figure 3.2 Schematic diagram of introgression of aflatoxin and fumonisin contamination resistance in South African maize germplasm lines.

3.6.3 Experimental design

3.6.3.1 Single crosses - greenhouse evaluation

The 82 single crosses were planted in a randomised complete block design (RCBD) with four replications in the greenhouse during the 2012/13 summer season. The trial was set up in 30cm plastic pots filled with composted pine bark growth media. Two replications were crossed with the aflatoxin resistant inbred lines to generate three-way crosses. The other two replications were artificially inoculated with *F. verticillioides* for *Fusarium* ear rots and fumonisin contamination evaluation.

3.6.3.2 Single crosses – field evaluation

The 82 single crosses were also planted simultaneously with the greenhouse trial in the field at Makhathini Research Station, during the 2012/13 summer season. The trial was planted in single row plots with 17 plants per row. Plots were spaced 0.9 m between rows and 0.3 m between stations arranged in a RCBD with two replications. Both replications were artificially inoculated using the silk channel inoculation method with *F. verticillioides* for FER and fumonisin contamination evaluation.

3.6.3.3 Three-way crosses evaluation

The 44 three-way crosses from the greenhouse were planted at Makhathini during the 2013 winter season (Jun-Oct). The trial was planted in single row plots with 17 plants per row. Plots were spaced 0.9 m between rows and 0.3 m between stations arranged in an RCBD with two replications. Both replications were artificially inoculated with both *F. verticillioides* and *A. flavus*. In each replicate both pathogens were inoculated onto ears by partitioning a plot with 17 plants such that eight plants were inoculated with *F*. *verticillioides* and another eight plants with *A. flavus* leaving one plant in-between.

3.6.3.4 $S_{2:3}$ families evaluation

The 146 families were planted at Cedara Research Station, during the 2013/14 summer season (Dec-May). The inbred lines CML390 and CML444 were used as positive controls for fumonisin contamination resistance. TZAR102 and TZAR103 were used as positive

controls for aflatoxin contamination resistance. Three $S_{1:2}$ family bulk of the TZAR102 x TZAR103 cross was also included as a positive control, which was at the same level of inbreeding with the test entries. Sixteen drought tolerant $S_{1:2}$ families (MTX-144-153, MTX-155-7), which were not introgressed with aflatoxin and fumonisin resistant genes were used as negative controls or susceptible controls for both aflatoxin and fumonisin contamination. The experiment was planted in single row plots by hand with two seeds per station and thinned down to one plant at three weeks after seedling emergence. Each row plot had a maximum potential of 17 plants. Plots were 5 m long with spacing of 0.9 m between rows and 0.3 m between stations arranged in a RCBD. In each plot, five plants were self-pollinated to advance to the $S_{3:4}$ generation, and the remaining 12 plants were partitioned such that six plants were artificially inoculated with *F. verticillioides* and another six plants with *A. flavus*. The nursery was manually harvested on the 28th of May 2014.

3.6.4 Management

The field trial on three-way crosses was conducted under irrigation. The nursery of $S_{2:3}$ families was conducted under rain-fed conditions with no supplementary irrigation. All field experiments on single, three-way crosses and $S_{2:3}$ families were managed as described in Section 3.3.3.

3.6.5 Isolation, inoculum preparation and inoculation of pathogens

3.6.5.1 Isolation and morphological identification of cultures

Isolates of *F. verticillioides* and *A. flavus* for inoculation in the greenhouse and in the field were obtained from naturally infected maize ears at Cedara Research Station near Pietermaritzburg. The fungal isolates were isolated and identified as described in Section 3.4 and 3.5.

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3.6.5.2 Inoculum preparation

Fungal isolates were routinely maintained in 15% glycerol at -80ºC. Prior to inoculation *F. verticillioides* and *A. flavus* were cultured on SNA and AFPA, respectively. Conidia were washed from the surface of the agar media with sterile distilled water and filtered through two layers of sterile cheesecloth. The concentration was adjusted to $1x10^6$ conidia/ml using a haemocytometer, 0.1% Tween 20 (Fisher Biotech, Fairlawn, NJ) per litre was added as surfactant. Inoculum suspension was used within 24 hours of preparation

3.6.5.3 Inoculation

At flowering the maize ears were inoculated using a 10 ml syringe 7-10 days after mid-silking. Conidia suspension (5 ml) was injected down the silk channel of primary ears of all plants at the blister (R2) growth stage (Chungu et al., 1997). Ears were covered after inoculation with plastic shoot bags for two days to maintain high humidity and to protect the inoculum from being drained by rain or dried by excessive heat.

3.6.6 Disease assessment

Ears were manually harvested at maturity. At each harvest date, ears in each plot were hand-picked, dehusked and evaluated for severity of ear rot symptoms. Disease severity was assessed by determining the percentage of each ear covered by symptoms using a 7- class rating scale, in which 1 = no infection, 2 = 1 to 3%, 3 = 4 to 10%, 4 = 11 to 25%, 5 = 26 to 50%, 6 = 51 to 75%, and 7 =76 to 100%, as described by Afolabi et al. (2007). The characteristic symptoms of infection are pinkish or white mycelial growth for *Fusarium* ear rot (FER) and yellow-green mycelia growth for *Aspergillus* ear rot (AER). These are shown in Figure 2.3 A and B in Chapter 2. Ears were oven-dried after disease assessment to approximately 14% grain moisture content. Grain was hand-shelled and bulked by plots. Two hundred grains from bulked samples of each plot were separated into symptomless and symptomatic fractions (discoloured kennels). The latter fraction contained kernels that were visibly mouldy, darkened, streaked, or chalky in appearance. The incidence of discoloured kernels was determined by expressing the number of visibly discoloured kernels as a proportion of 200 grains from bulked samples of each plot multiplied by 100.

3.6.7 Data collection on agronomic traits

Grain texture was recorded using a rating scale of 1 = hard, completely rounded kernel, flint to 5 = soft, distinct dent, in accordance with the protocols used at CIMMYT (CIMMYT, 1985). Husk cover was rated using a scale from 1 = long husk covering the entire length of the ear to 9 = short husk with ear protruding and kernels exposed. Insect damage ratings were recorded from 1 = no damage or tunneling to 9 = heavy damage or channeling (Badu-Apraku et al., 2012). Moisture content was determined using the grain moisture meter MC-7825G (ONMI instruments, UK).

3.6.8 Aflatoxin analysis

Bulked grain from each plot was ground using a Cyclotech sample mill to pass through a 1-mm mesh and stored in a cold room at 4ºC. Two replicates of 5 g samples were used for both aflatoxin and fumonisin extraction. Ground samples were extracted with 25 ml of 70% methanol using a multi-tube shaker for 20 minutes. The samples were then centrifuged for 10 minutes at 4 000rpm and 1 ml of the obtained supernatant was diluted with 1 ml of distilled water. Contamination of grain samples by aflatoxin B_1 was quantified using a direct competitive enzyme-linked immunosorbent assay (ELISA) technique following the methods described in the manufacturer's instructions (BIOO Scientific Corporation, 2014) as described below.

A 50 µl aliquot of each aflatoxin B_1 standards (0 ppb, 0.05 ppb, 0.1 ppb, 0.2 ppb 0.4 ppb and 0.8 ppb) were dispensed in duplicates separately into twelve mixing wells, and 50µl of each sample extract were also added in duplicates to all remaining mixing wells. To each mixing well, 100 µl of enzyme conjugate (horseradish peroxidase) was dispensed in the well, and after mixing by gently rocking the plate manually for 1 minute, the plate was covered with a foil paper and incubated for 30 minutes at room temperature (20-25ºC). The contents of the mixing wells were removed, and after a 3 x wash step, 150 µl of aflatoxin B_1 antibody were added and the plate was incubated for another 30 minutes. Another 3x wash was done and 100 µl of substrate 3-3-5-5 tetra methyl benzidine (TMB) was added and incubated for 15 minutes at room temperature. Reactions were stopped by adding 100 µl of Stop Buffer to each well and optical densities were measured with

Fluostar Optimal plate reader (BMG Labtech, Gauteng, SA) with 450 nm wavelength. Samples with aflatoxin concentrations higher than the highest standards were diluted with the extraction solvent, and results obtained were multiplied by the dilution factor. The minimum detection limit was 0.05 ppb or µg kg^{-1} and the recovery rate was >80%.

3.6.9 Fumonisin analysis

The total fumonisin contamination of grain samples was quantified using a direct competitive ELISA technique following the methods described in the manufacturer's instructions (BIOO Scientific Corporation, 2014) as described below.

A 50 µl aliquot of each fumonisin B_1 standards (0 ppm, 0.05 ppm, 0.1 ppm, 0.2 ppm, 0.4 ppm and 0.8 ppm) were dispensed in duplicates separately into twelve mixing wells, and 50µl of each sample extract were also added in duplicates to all remaining mixing wells. To each mixing well, 50 µl of 1X Antibody 2 was added and then 50 µl of 1X Antibody 1 to each well. The wells were mixed by gently rocking the plate manually for 1 minute. The plate was incubated for 30 minutes at room temperature covered with a foil paper.

The contents of the mixing wells were removed, and after a 3 x wash step with 300 µl of 1X Wash Solution, 100 µl of TMB was added and incubated for 15 minutes at room temperature. Reactions were stopped by adding 100 µl of Stop Buffer to each well and optical densities were measured with Fluostar Optimal plate reader (BMG Labtech, Gauteng, SA) with 450 nm wavelength. Samples with fumonisin concentrations higher than the highest standards were diluted with the extraction solvent, and results obtained were multiplied by the dilution factor. The minimum detection limit was 0.05 ppm or mg $kg^{-1}$and the recovery rate was >80%.

3.7 Statistical analysis

3.7.1 Analysis of Variance

All statistical analysis of all data of hybrids and $S_{2:3}$ families were performed in GenStat (version 14, VSN International). Differences between hybrids were determined with Fisher's unprotected least significant differences (LSD) test. For the survey on ear rot

incidence, linear Pearson's correlation coefficients were determined for the relationship between ear rot incidences, yield, days to mid-silking, grain moisture content, insect damage and grain texture. Data on ear rot incidence of hybrids was transformed to arcsine of the percentage of ear rots incidence. Although the experiment was designed as an alpha-lattice, the trial was analysed as RCBD without violating any of the assumption for the statistical model.

For introgressed hybrids, linear correlation coefficients were determined for the relationship among ear rots severity, percentage of discoloured kernels, aflatoxin and fumonisin concentration. Data on discoloured kernels of hybrids was transformed to arcsine of the percentage of ear rots incidence to normalize residuals. For $S_{2:3}$ families, linear correlation coefficients were determined for the relationship between aflatoxin and fumonisin concentration with AER, FER, husk cover, grain texture and grain moisture content using Pearson correlation coefficients based on untransformed means.

The following model was used for general analysis of variance (ANOVA):
Response =population mean + replication effects + hybrids effects + random error effects.

$$Y_{ij} = \mu + b_i + t_j + e_{ij}$$

where, Y_{ij} = response in the *i*th replication of the *j*th hybrid

μ = population mean

b_i = *i*th replication

t_j = *j*th hybrid effect

e_{ij} = random error

The replications were considered as random effects and hybrid effects were treated as fixed.

3.7.2 Estimation of heritability

The REML tool was used to estimate the variance components. The replications were considered as fixed effects and hybrids effects were treated as random. Heritability was calculated on an entry mean basis using the following formula (Nyquist, 1991):

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_e^2}{r}}$$

Where H^2 is the broad sense heritability, σ_G^2 is the genotypic variance, σ_e^2 is the overall error variance and r is the number of replications for the experiment.

3.7.3 Estimation of genetic gain

The realized genetic gain was calculated as the difference between the population mean and the mean of the selected hybrids or families (Nyquist, 1991) :

$$\Delta G = \mu_2 - \mu_1$$

Where μ_2 = mean of selected hybrids or families, μ_1 = population mean.

The predicted genetic gain was estimated as described by (Nyquist, 1991):

$$\Delta G = R = i \sigma_p H^2$$

Where ΔG is the genetic gain, R is the response to selection, i is the selection intensity, H^2 is the broad sense heritability and σ_p is the phenotypic variance. 10% of the hybrids or families were selected for advancement in the breeding program therefore the selection intensity (i) of 1.76 was used to predict the genetic gain.

3.8 Conclusion

This chapter described the research design and experiment in detail. This includes field, greenhouse and laboratory experiments which were conducted to answer the research objectives. The findings from the study are presented in Chapter 4.

CHAPTER FOUR

RESULTS

4.1 Introduction

This chapter presents the outcomes of this study and highlights the patterns observed. The results are presented in two sections. The first section presents findings from the screening on natural incidence of ear rots in southern African maize hybrids. The second section presents results from the evaluation of ear rots and fumonisin contamination of experimental single cross hybrids. The section also presents results on the evaluation of ear rots and aflatoxin and fumonisin contamination of experimental three way cross hybrids and $S_{2:3}$ families. A conclusion on the chapter is drawn.

4.2 Screening of natural incidence of ear rots in southern African maize hybrids

4.2.1 Weather data

Temperature, rainfall and relative humidity data for the duration of the study are presented in Figure 4.1 and 4.2.

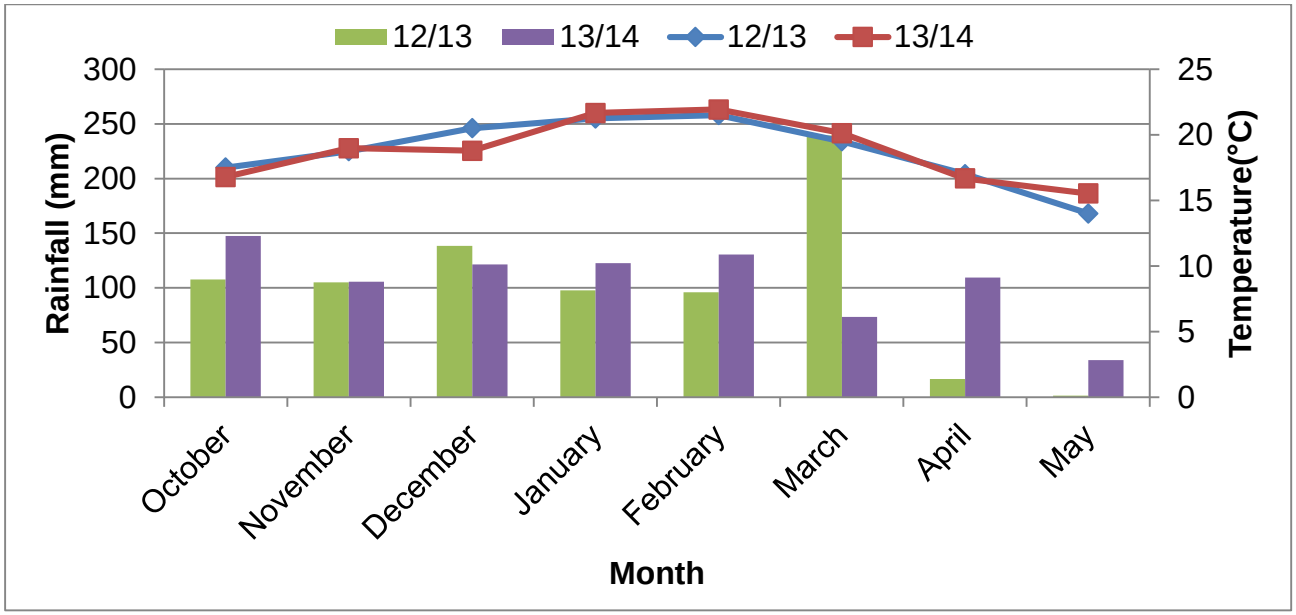

Figure 4.1 Monthly rainfall (in bars) and mean temperatures (line graph) during the experimental period (2012/13 and 2013/14) at Cedara Research Station, South Africa. (Source: Agricultural Research Council, 2014).

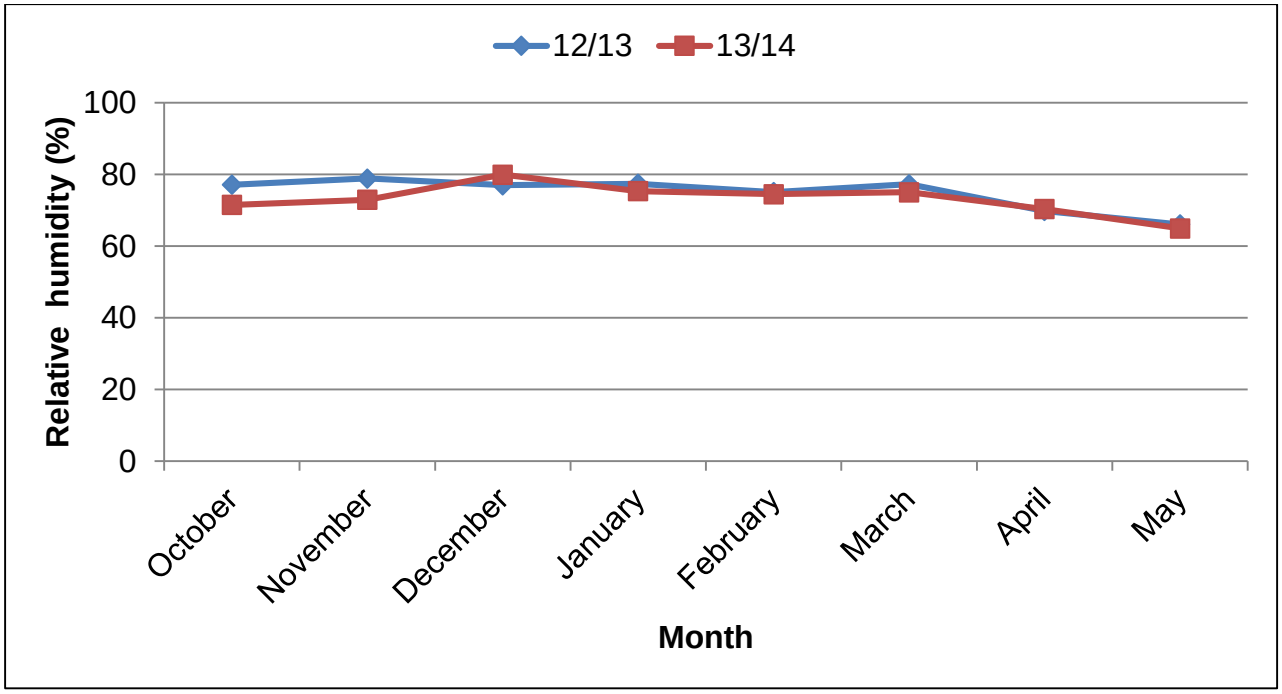

Figure 4.2 Monthly mean relative humidity during the experimental period (2012/13 and 2013/14) at Cedara Research Station, South Africa. (Source: Agricultural Research Council, 2014).

4.2.2 Incidences of ear rots causing fungi

In the 2012/13 summer season, *S. maydis* was the most prevalent (42.61%) ear rot causing fungi, followed by *F. verticillioides* (28.19%), *A. flavus* (15.06%) and *F. graminearum* (14.32%). In the 2013/14 summer season, the most prevalent fungi was *F. verticillioides* (48.7%) followed by *S. maydis* (22.13%), *F. graminearum* (16.62%) and *A. flavus* (12.52%). Over the two seasons, *F. verticillioides* recorded the highest mean (38.46%), *S. maydis* was second (32.37%), *F. graminearum* was third (15.45%) and *A. flavus* had the least incidence (13.79%) [Figure 4.3].

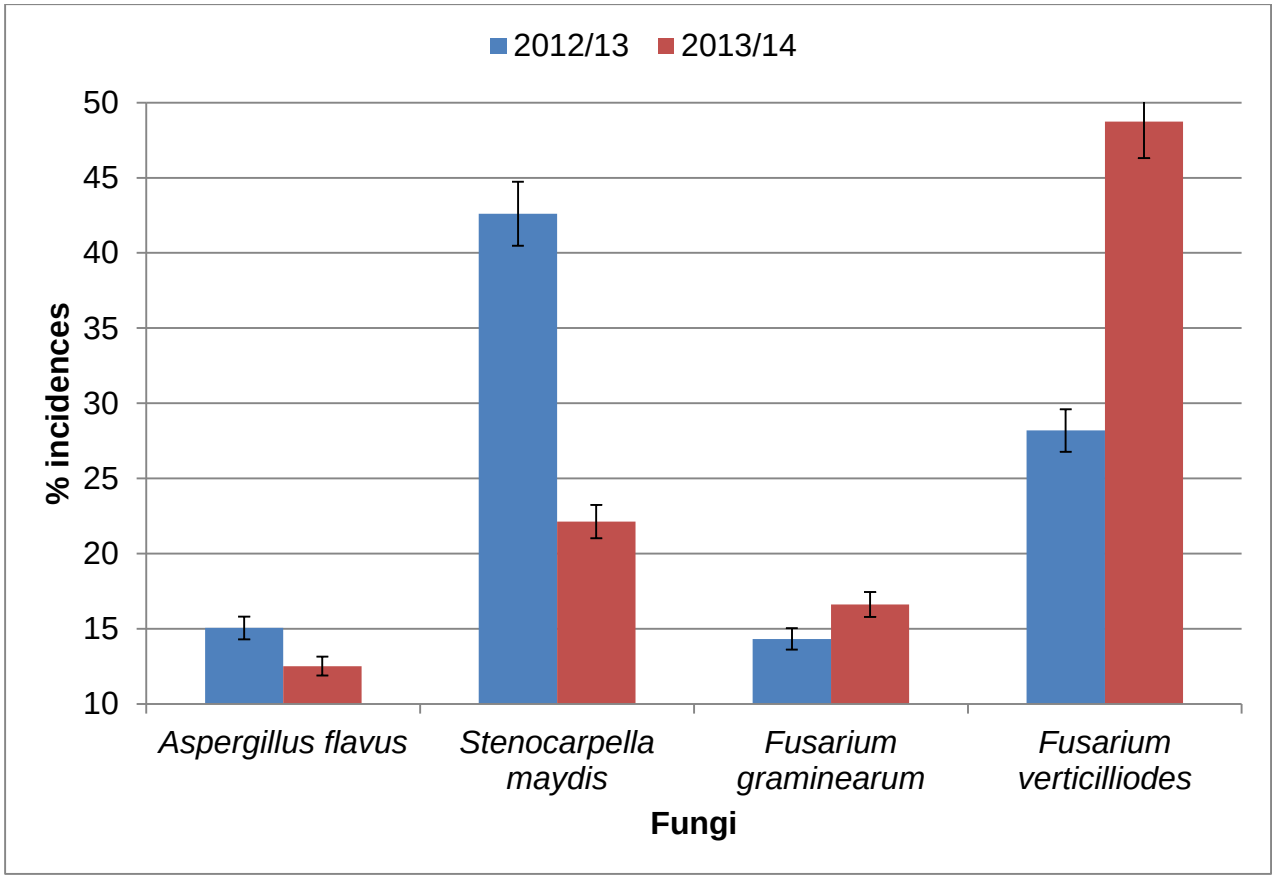

Figure 4.3 Prevalence of ear rots causing fungi in 327 maize hybrids, during the 2012/13 and 2013/14 summer seasons at Cedara Research Station, South Africa.

Ear rot incidences in maize hybrids were more prevalent during the 2013/14 summer season than the 2012/13 summer season. There was significant variation ($P < 0.001$) among hybrids for ear rots resistance over the two seasons. Analysis of variance for ear rots incidence is presented in Table 4.1 and 4.2. Mean squares of hybrids were highly significant ($P < 0.001$) for ear rots incidence in early maturing, medium maturing and late maturing hybrids in both seasons.

Table 4.1 Analysis of variance for ear rots incidence for early, medium and late maturity maize hybrids surveyed during 2012/13 season.

	Early maturity		Medium maturity		Late maturity	
Source	df	ms	df	ms	df	ms
Replication	2	4.33	2	0.29	2	2.33
Entry	49	1.69*	64	2.76**	54	3.82**
Error	98	1.00	128	1.63	108	1.99
Total	149		194		164	
Trial statistics						
l.s.d	1.62		2.06		2.28	
cv%	29.3		38.0		41.4	

*, **, *** indicates the term is significant at P < 0.05, P < 0.01, P < 0.001, respectively.

Table 4.2 Analysis of variance for ear rots incidence for early, medium and late maturity maize hybrids surveyed during 2013/14 season.

	Early maturity		Medium maturity		Late maturity	
Source	df	ms	df	ms	df	ms
Replication	2	10.26	2	8.94	2	0.24
Entry	59	2.23***	59	3.20***	39	3.72***
Error	118	1.13	118	1.13	78	1.30
Total	179		179		119	
Trial statistics						
l.s.d	1.72		1.72		1.85	
cv%	20.4		19.3		19.2	

*, **, *** indicates the term is significant at P < 0.05, P < 0.01, P < 0.001, respectively.

4.2.3 Reaction of hybrids to ear rots infection

Lowest ear rots incidences were observed in early maturing hybrids for both seasons (12.99% and 28.71%) with a combined mean of 20.85%. Medium maturing hybrids had an incidence of 13.27% and 32.13% for the 2012/13 summer season and 2013/14 summer season, respectively, with a combined mean of 22.7%. The highest incidences were observed in late maturing hybrids for both seasons (14.17% and 37.3%) with a combined mean of 25.73% (Figure 4.4).

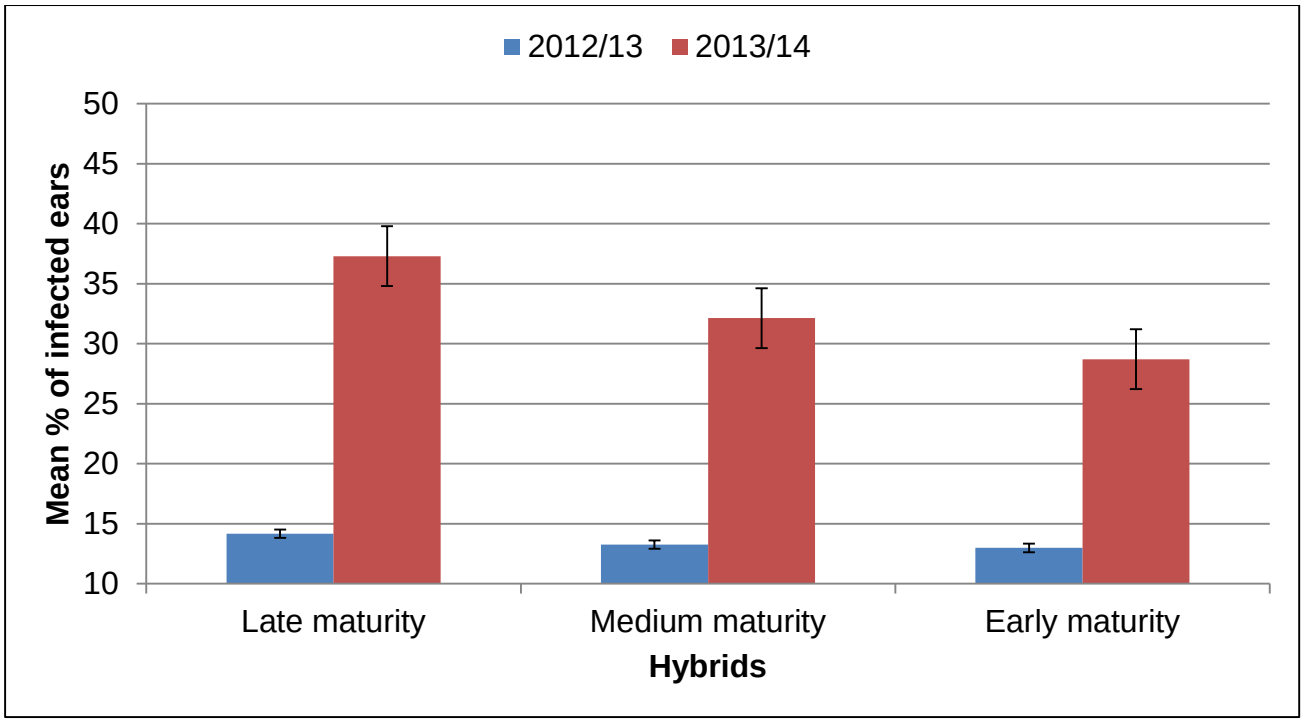

Figure 4.4 Mean incidences of percentage infected ears of early, medium and late maturing experimental hybrids, during 2012/13 and 2013/14 seasons, at Cedara Research Station.

4.2.4 Associations between ear rots incidence and selected agronomic traits

Yield per plot at harvest, days to mid silking, grain moisture content, insect damage and grain texture were tested to ascertain any correlation with incidences of ear rots. Significant ($P < 0.001$) and negative correlations were observed between yield and ear rots disease incidences in all hybrids over the two seasons. The highest correlation was

observed in early maturing hybrids in both 2012/13 and 2013/14 seasons, and the lowest in medium maturing hybrids in the 2012/13 season (Table 4.3). Late maturing hybrids had the lowest negative correlation in 2013/14 season. Significant ($P < 0.05$) positive associations were observed in 2013/14 between days to mid-silking and ear rots disease incidences in early and medium maturing hybrids. No significant ($P > 0.05$) correlations was observed in late maturing hybrids in both seasons. Insect damage and incidences of ear rots showed significant ($P < 0.001$) positive correlations for all the hybrids. Results also showed significant ($P < 0.001$) and positive correlations between ear rot incidences and grain texture for all types of hybrids (Table 4.3).

Table 4.3 Pearson's correlation coefficients of ear rot incidence with selected agronomic traits in 327 maize hybrids, at Cedara research station.

	Early maturity		**Medium maturity**		**Late maturity**	
	2012/13	**2013/14**	**2012/13**	**2013/14**	**2012/13**	**2013/14**
Trait			**Ear rot % incidence**			
Yield	-0.43***	-0.52***	-0.18**	-0.48***	-0.40***	-0.41***
Days to mid-silking	0.13ns	0.16*	0.03ns	0.22*	-0.06ns	0.11ns
Days to mid-pollen	0.12ns	0.17**	0.02ns	0.21**	-0.07ns	0.08ns
Anthesis to silking interval	0.14ns	-0.03ns	0.02	0.09ns	0.05ns	0.19**
Grain moisture content	-0.01ns	-0.04ns	0.03	-0.07ns	0.03	-0.13ns
Insect damage	-	0.70***	-	0.74***	-	0.70***
Grain texture	-	0.27***	-	0.30***	-	0.24**

*, **, ***, ns indicates the term is significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, not significant at $P \geq 0.05$ respectively.

4.3 Single cross hybrids evaluation for fumonisin contamination

Analysis of variance for *Fusarium* ear rot, fumonisin concentration and percentage discoloured kernels is presented in Table 4.4. Mean squares of single cross hybrids were highly significant ($P < 0.001$) for *Fusarium* ear rot, fumonisin concentration and percentage discoloured kernels in both the greenhouse and field trial at Makhathini.

Table 4.4 Analysis of variance for *Fusarium* ear rot, fumonisin concentration and percentage discoloured kernels for the greenhouse and field trials.

	Greenhouse					Field				
	***Fusarium* ear rot**		**Fumonisin concentration**	**Discoloured kernels**		***Fusarium* ear rot**		**Fumonisin concentration**	**Discoloured kernels**	
Source	df	ms	ms	df	ms	df	ms	Ms	ms	
Rep.	1	0.56	0.21	1	0.00	1	0.03	0.01	0.01	
Entry	71	4.63***	335.46***	58	0.44***	71	6.52***	34.07***	0.49***	
Error	71	0.46	0.13	53	0.00	71	0.61	0.00	0.01	
Total	143			112		143				
Trial statistics										
Lsd		28.6	1.8		0.13		23.1	0.24	0.19	
cv		1.36	0.71		7.8		1.55	0.6	13.0	

*, **, *** indicates the term is significant at P < 0.05, P < 0.01, P < 0.001, respectively.

4.3.1 Ear rot severity assessment

Continuous distribution curves were observed for the frequency distributions of *Fusarium* ear rot severity (Figure 4.5). The histograms for *Fusarium* ear rot severity showed negative skewness for both the greenhouse and field trial. *Fusarium* ear rot severity on the single cross hybrids ranged from 1.0 to 7.0 with mean of 2.9. Forty-nine percent of the hybrids showed low disease severity (≤3) in both the greenhouse and in the field. The incidence of discoloured kernels due to *Fusarium* ear rot ranged from 0 to 68% with mean of 14.44%. Forty-seven percent of the hybrids exhibited lower incidences of discoloured kernels (≤10%). The mean visual rating of *Fusarium* ear rot on maize ears in the greenhouse and the field trials was 2.4 and 3.4, respectively (Table 4.7).

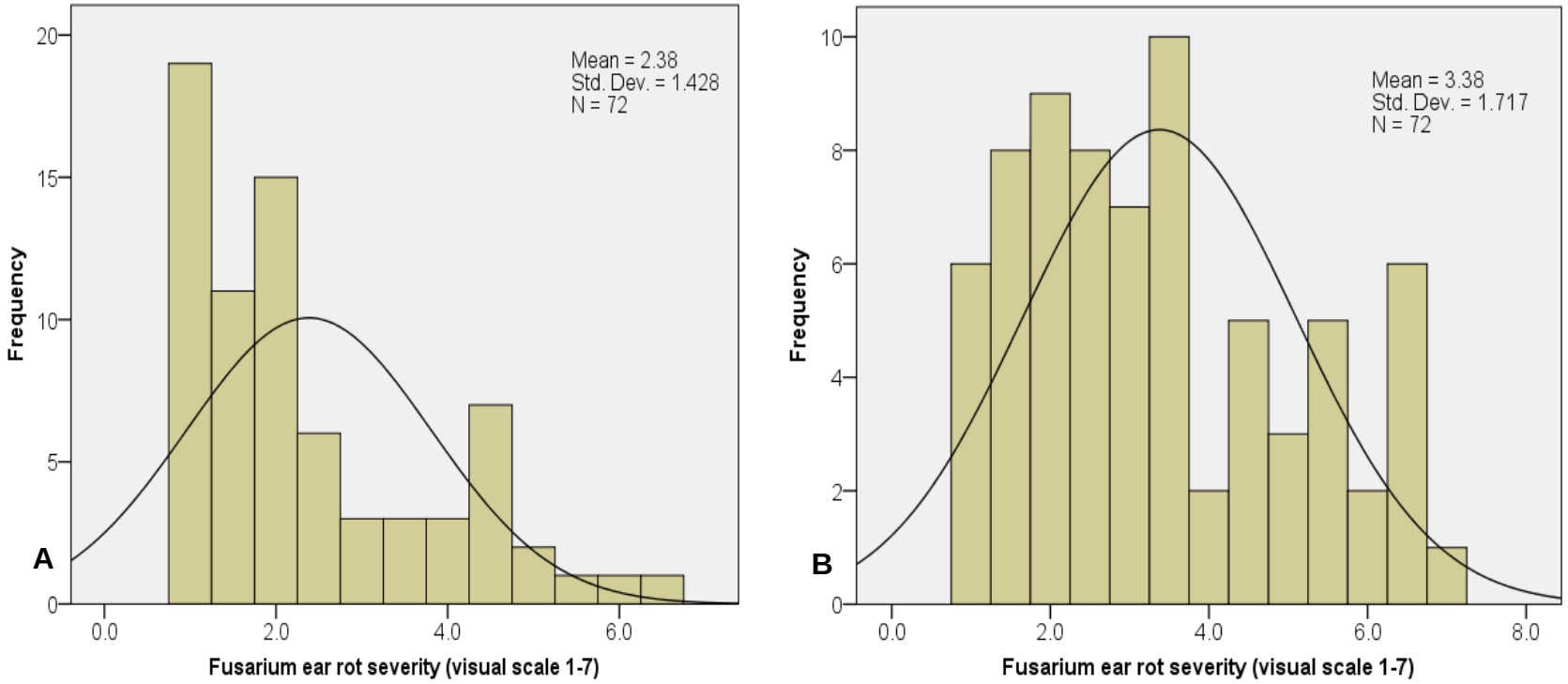

Figure 4.5 Frequency distributions of *Fusarium* ear rot severity of single cross maize hybrids in the greenhouse (A) and in the field (B).

4.3.2 Fumonisin contamination evaluation

Continuous variations were observed for the frequency distributions of fumonisin concentration (Figure 4.6). Bimodal histograms were observed for fumonisin concentrations in both the greenhouse and the field trial. At least five single cross hybrids consistently showed low fumonisin concentration levels of less than 4 ppm (mg kg^{-1}) in both the greenhouse and field which is the Codex Alimentarius Commission legal limit of total fumonisin in grain (Codex, 2014). The hybrids were: FUMH03, FUMH10, FUMH30, FUMH47, and, FUMH73 (Figure 4.7 and Table 4.5). Only FUMH47 exhibited lower accumulation levels than the resistant control, in both the greenhouse and the field (Figure 4.7 and Table 4.5). Four single cross hybrids (FUMH1, FUMH17, FUMH71 and FUMH74) showed moderate resistance (> 4-10 ppm) in both environments. Fourteen percent of the hybrids were susceptible in the two environments, accumulating between 10-20 ppm. Highly susceptible hybrids which consistently accumulated >30 ppm in both environments accounted for 17% of the single cross hybrids. Hybrid FUMH53 had the highest average in both environments (Figure 4.7 and Table 4.5).

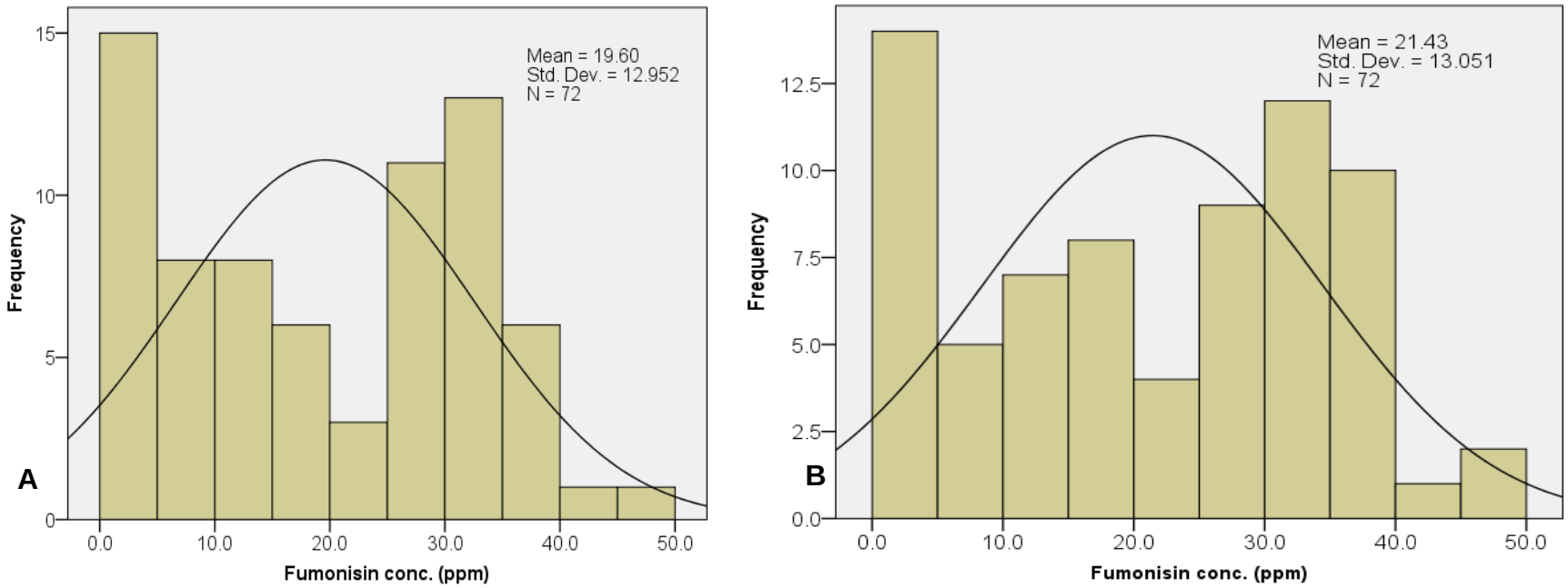

Figure 4.6 Frequency distributions of fumonisin concentration of single cross maize hybrids in the greenhouse (A) and in the field (B).

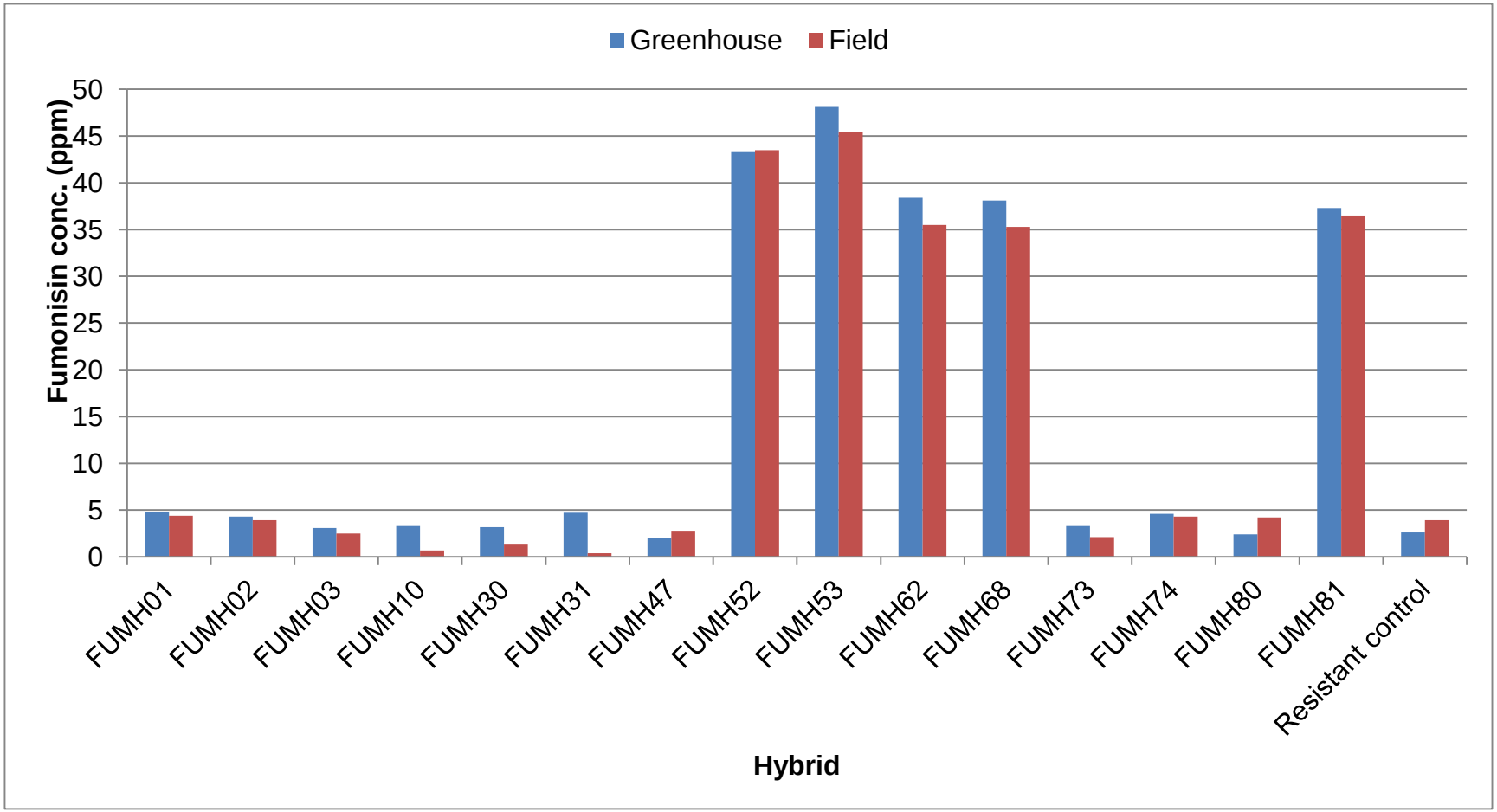

Figure 4.7 Mean fumonisin concentration of the top ten and bottom five single cross hybrids in the greenhouse and the field.

Table 4.5 Evaluation of 72 single cross maize hybrids for resistance to *Fusarium* ear rot, percentage of discoloured kernels and fumonisin contamination in the greenhouse and field.

		Greenhouse trial			Field trial		
Hybrid	**Grain texture**	**Fusarium ear rot (score)**	**Discoloured kernels (%)[x,y]**	**Fumonisin conc. (ppm)[z]**	**Fusarium ear rot (score)**	**Discoloured kernels (%)[x,y]**	**Fumonisin conc. (ppm)[x,z]**
Top 10							
FUMH10	Flint	1.0a	0.7b	3.3e	2.0a-c	1.4a-c	0.7b
FUMH30	Flint	1.0a	0.5ab	3.2e	1.0a	1.2ab	1.4c
FUMH47	Flint	1.0a	0.0a	2.0ab	1.5b-d	1.2ab	2.8f
FUMH31	Flint	2.0a-c	1.7cd	4.7f	1.5b-d	1.7c-f	0.4a
FUMH73	Flint	1.5ab	2.2d-f	3.3e	5.0l-n	2.2e-g	2.1d
FUMH03	Flint	2.0a-c	1.9c-e	3.1de	2.0b-d	1.9c-f	2.5e
FUMH80	Dent	1.0a	0.0a	2.4b-d	3.5f-h	1.4a-c	4.2hi
FUMH02	Flint	1.0a	0.5ab	4.3f	1.0ab	1.2ab	3.9h
FUMH74	Dent	1.0a	0.0a	4.6f	4.0e-g	1.0a	4.3i
FUMH01	Flint	1.0a	1.8c-e	4.8f	1.5ab	1.8c-f	4.4i
Bottom 5							
FUMH68	Flint	3.0c-e	4.9l-d	38.1M	5.5k-m	4.9no	35.3RS
FUMH81	Dent	2.0a-c	2.2d-f	37.3L	5.0l-n	2.2f-h	36.5U
FUMH62	Flint	4.0e-g	5.1m-o	38.4M	5.5k-m	5.1o	35.5S
FUMH52	Dent	1.0a	0.0a	43.3N	5.5kl	1.2ab	43.5Y
FUMH53	Dent	4.0e-g	5.8pq	48.1O	6.5l-n	5.8qr	45.4Z
Control							
Resistant control	Flint	1.0a	0.0a	2.6b-e	2.0d-f	1.0a	3.9h
Mean		2.4	2.48	19.8	3.4	2.76	21.4
l.s.d		1.4	0.64	0.7	1.5	0.51	0.3
cv (%)		28.6	13.0	1.9	22.9	9.3	0.6

[x]For each variable means followed by the same letter do not differ significantly according to Fischer's least significant difference test (P≤ 0.05).

[y]Data transformed to the $\log_{10}$ of the percentage of discoloured kernels.

[z]Fumonisin concentration = total of $FB_1 + FB_2 + FB_3$

4.3.3 Correlation between greenhouse and field results

The individual hybrids did not differ considerably in their fumonisin contamination between the greenhouse and field trial, as indicated by the high Pearson correlation coefficient (Figure 4.8)

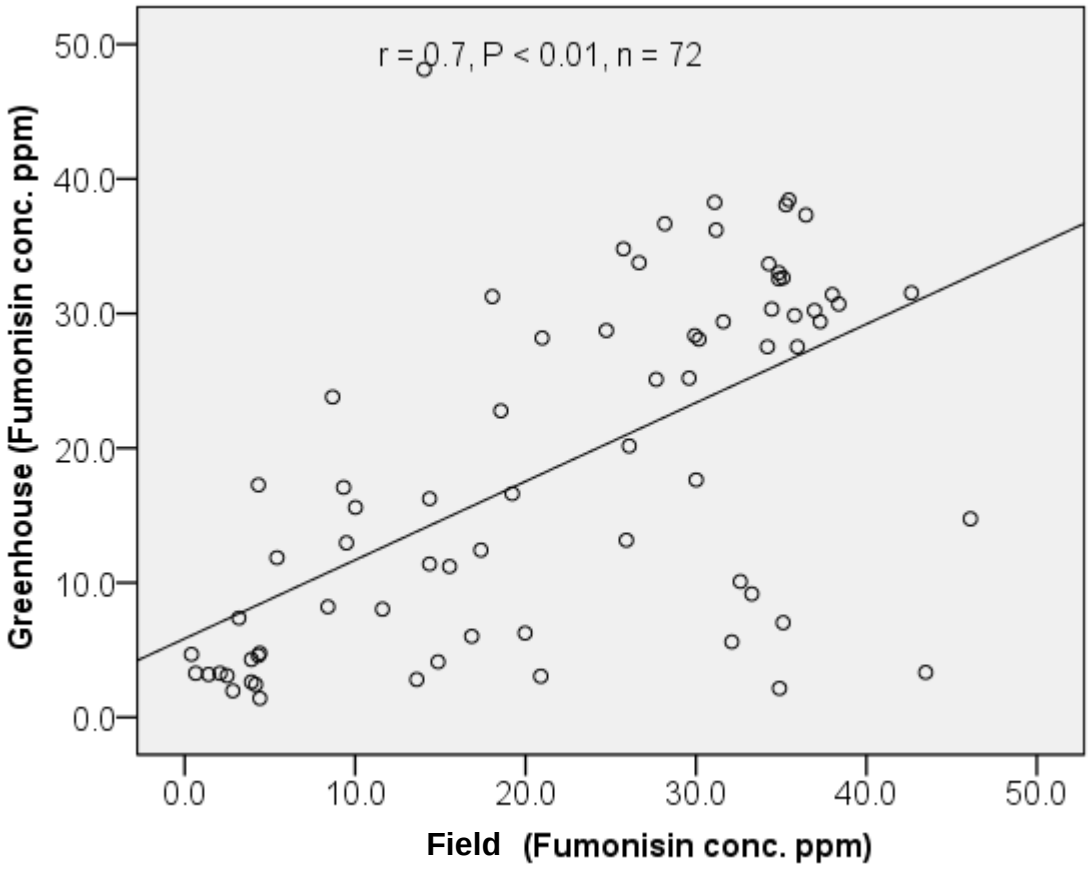

Figure 4.8 Linear correlations between fumonisin contamination in the greenhouse and field trial.

4.3.4 Correlation between ear rot severity and fumonisin contamination

A significant correlation was observed between FER and fumonisin concentration in the greenhouse and field trial (Figure 4.9). This finding is demonstrated, for example, by FUMH10, which had a mean disease severity rating of 1 (no infection) and

fumonisin contamination of 3.3 ppm in the greenhouse, or FUMH53, with a mean disease severity score of 6.5 and fumonisin contamination of 45.4 ppm in the field. The trend was not consistent because some hybrids, showed relatively high disease severity score and low fumonisin contamination. This is demonstrated by FUMH73 in the greenhouse, which had a mean visual score of 5 and fumonisin contamination level of 2.1 ppm. In sharp contrast, another set of hybrids exhibited a trend of low disease severity rating and high fumonisin contamination. The example is FUMH52 in the greenhouse, which had a small mean visual score of 1 while it accumulated high levels of 43.3 ppm of fumonisin (Table 4.5).

The data of the FER and fumonisins concentration in the greenhouse and field trial showed a significant correlation between the two environments (Figure 4.7). This correlation between the two environments is shown for example by FUMH01 which had a low ear rot severity score and accumulated low fumonisin content in both the greenhouse and the field trials. FUMH53 showed high ear rot severity score and accumulated high fumonisin content in both environments (Figure 4.7).

4.3.5 Correlation between percentage discoloured kernels and fumonisin contamination

Relatively weak but significant correlations were observed between fumonisin contamination and incidence of discoloured kernels in both the greenhouse and the field (Figure 4.10). This association was confirmed, for example, by FUMH38 which had a high percentage of discoloured kernels and a corresponding high fumonisin concentration in the two environments. However, inconsistencies were observed between the incidence of discoloured kernels and fumonisin concentration. Several hybrids showed low percentage of discoloured kernels but accumulated high fumonisins concentration and *vice versa*. The combined data of the two environments also showed a significant correlation (Figure 4.10).

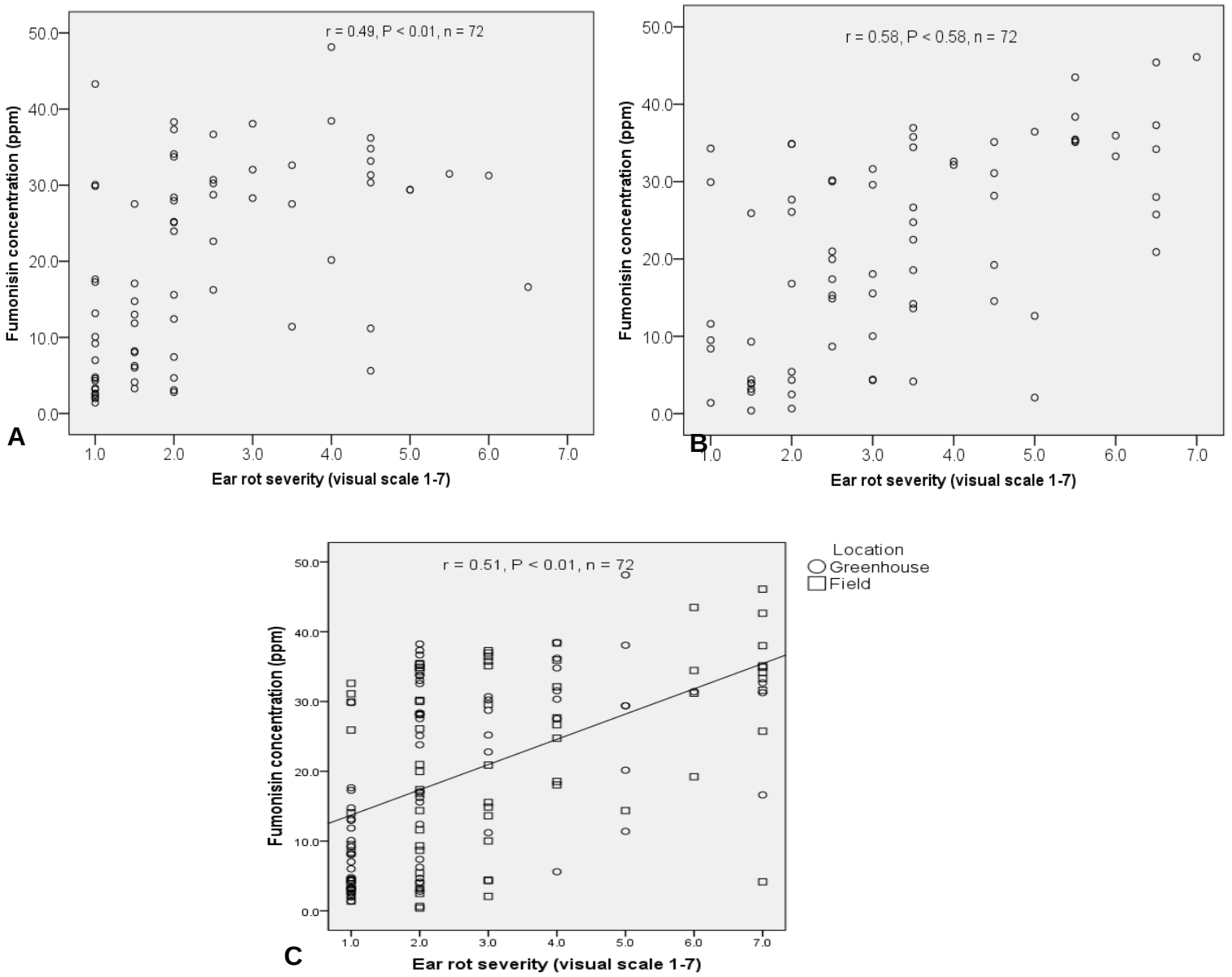

Figure 4. 9 Linear correlations of *Fusarium* ear rot severity and fumonisin contamination in the greenhouse (A), field (B) and (C) combined (greenhouse vs field).

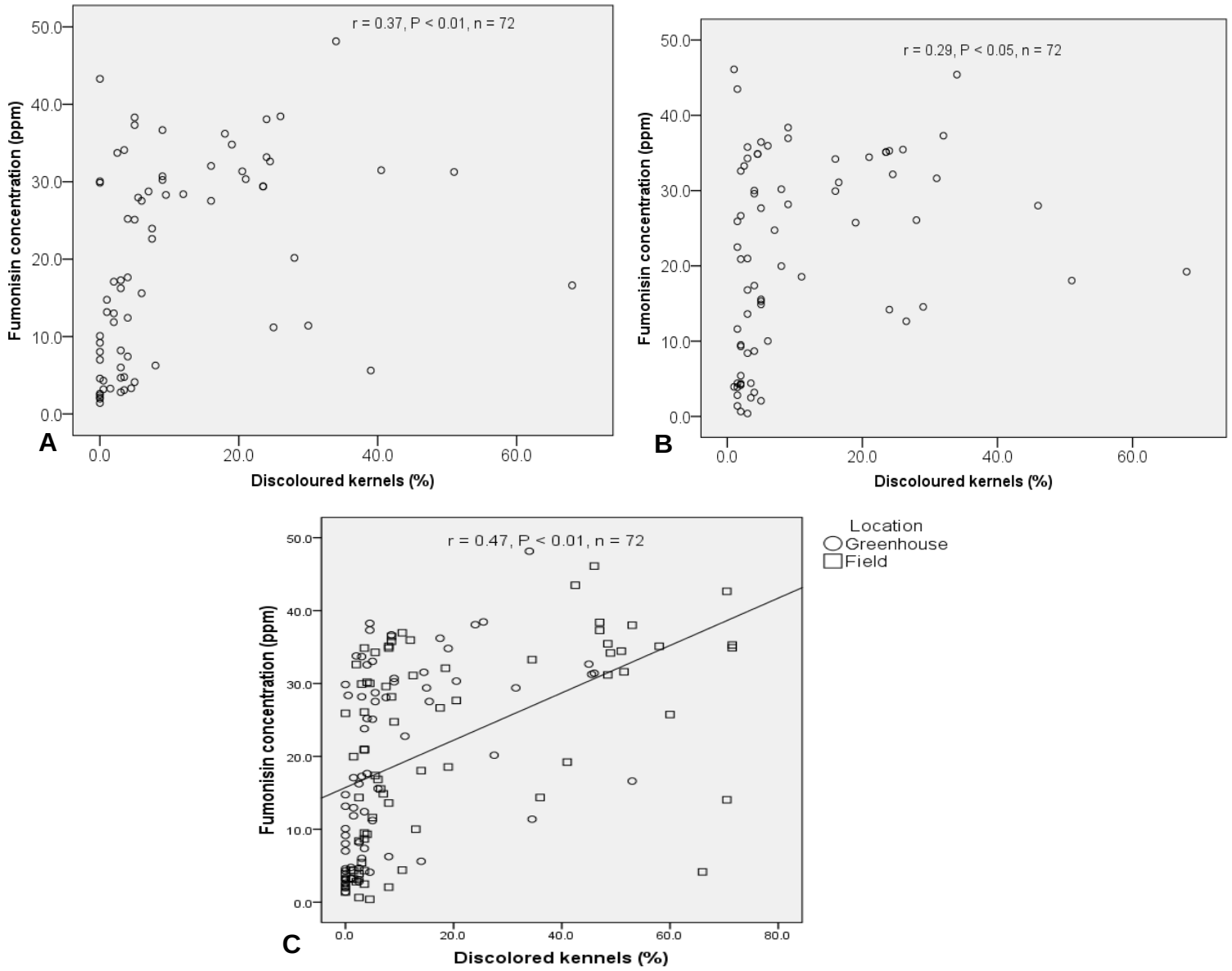

Figure 4.10 Linear correlations of percentage discoloured kernels and fumonisin contamination in the greenhouse (A), field (B) and (C) combined (greenhouse vs field).

4.3.6 Estimates of genetic gain and heritability

When the top 10% resistant single crosses were selected, high genetic gains were realised for fumonisin contamination resistance in both the greenhouse and field trials. The realised genetic gains for FER were moderate and low in the greenhouse and in the field, respectively. Low genetic gains were predicted in both environments for fumonisin contamination resistance and FER. Heritability estimates of fumonisin contamination resistance and FER were very high in both trials (Table 4.6 and 4.7).

Table 4. 6 Breeding gains and heritability in single cross maize hybrids in the greenhouse.

Trait	**Observed mean of selected single crosses ($\bar{x}$)**	**Population mean (μ)**	**Realized genetic gain ($\bar{x}$-μ)**		**Predicted genetic gain**		**Heritability (h^2)**
			(Mean)	**(%)**	**(Mean)**	**(%)**	
Fumonisins (ppm)	3.1	19.8	-16.7	-84	-2.9	-14.5	0.99
Fusarium ear rot score[x]	1.2	2.4	-1.2	-50	-2.4	-3.3	0.90

[x]1 = no infection, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75%, 7 = 76 -100%

Table 4.7 Breeding gains and heritability in single cross maize hybrids in the field at Makhathini.

Trait	**Observed mean of selected single crosses ($\bar{x}$)**	**Population mean (μ)**	**Realized genetic gain ($\bar{x}$-μ)**		**Predicted genetic gain**		**Heritability (h^2)**
			(Mean)	**(%)**	**(Mean)**	**(%)**	
Fumonisins (ppm)	2.5	21.4	-18.9	-88	-6.1	-32.2	0.99
Fusarium ear rot score[x]	2.4	3.4	-1.0	-29	-2.9	-4.0	0.91

[x]1 = no infection, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75%, 7 = 76 -100%

4. 4 Three-way cross hybrids evaluation for ear rot and mycotoxin contamination

Analysis of variance for *Fusarium* ear rot, fumonisin concentration, discoloured kernels for *Fusarium* ear rot, *Aspergillus* ear rot, aflatoxin concentration and discoloured kernels for *Aspergillus* ear rot is presented in Table 4.8. Mean squares of three way cross hybrids were highly significant ($P < 0.001$) for *Aspergillus* ear rot, aflatoxin concentration, percentage discoloured kernels for Aspergillus ear rot and fumonisin concentration. Mean squares for *Fusarium* ear rot and percentage discoloured kernels of *Fusarium* ear rot were highly significant ($P < 0.01$).

Table 4.8 Analysis of variance for ear rots, mycotoxin concentration and percentage discoloured kernels of three-way cross hybrids.

	***Fusarium* ear rot**		**Fumonisin concentration**		***Fusarium* discoloured kernels**		***Aspergillus* ear rot**		**Aflatoxin concentration**		***Aspergillus* discoloured kernels**	
Source	df	ms	df	ms	df	ms	df	ms	df	ms	df	ms
Rep.	1	4.55	3	33.06	1	0.69	1	1.14	3	270.47	1	0.00
Entry	43	2.93**	43	478.65***	43	0.27**	43	3.00***	43	221.91***	43	0.37***
Error	43	1.17	129	24.29	43	0.12	43	1.18	129	23.96	43	0.11
Total	87		175		87		87		175		87	
Trial statistics												
Lsd		2.19		6.9		0.7		2.19		6.85		0.68
cv		28.4		16.7		29.3		32.1		27.3		35.4

*, **, *** indicates the term is significant at P< 0.05, P< 0.01, P< 0.001, respectively

4.4.1 Ear rot severity assessment

Continuous variations were observed for the frequency distributions of FER and AER severity. The histograms for FER and AER severity, showed normal distribution curves (Figure 4.11). *Aspergillus* ear rot and FER severity on the three-way cross hybrids ranged from 1.0 to 6.5 with mean of 3.4 and 3.8, respectively (Table 4.9 and 4.10). Eighteen percent of the hybrids showed low disease severity (≤3) for both AER and FER. The incidence of discoloured kernels due to both FER and AER ranged from 1.0 - 57% and 1.0 - 54% with mean of 23.3% and 16.1%, respectively. Eighteen percent of the hybrids also showed lower incidences of discoloured kernels (≤10%).

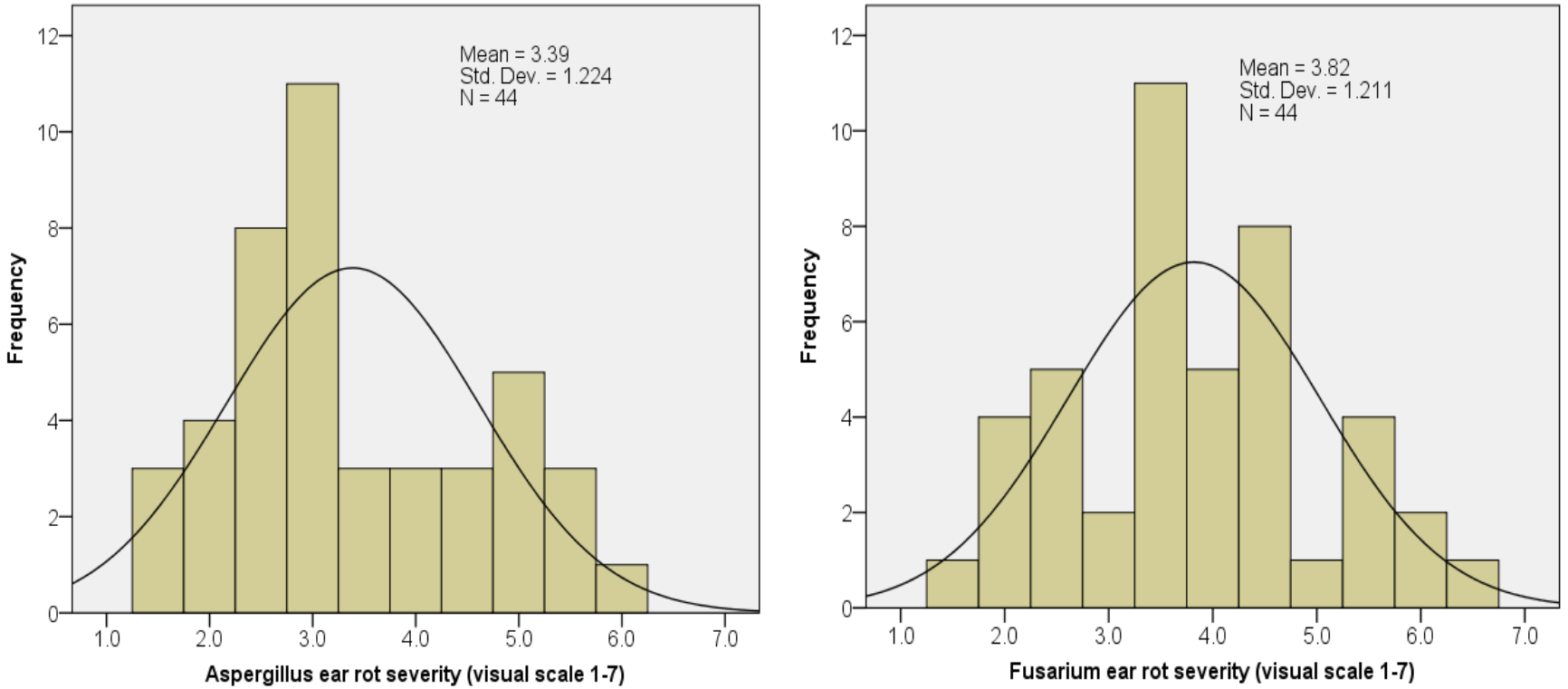

Figure 4.11 Frequency distributions of *Aspergillus* and *Fusarium* ear rot severity, of three-way cross maize hybrids.

4.4.2 Mycotoxin analysis

Continuous variations were also observed for the frequency distributions of aflatoxin and total fumonisin concentration (Figure 4.12). The histogram of aflatoxin concentration showed a normal distribution. A positively skewed histogram was observed for total fumonisin concentration. Mycotoxin analysis showed that at least three hybrids, such as FUMH/AFTX11, FUMH/AFTX12 and FUMH/AFTX18, have low concentration of aflatoxin (≤ 5 ppb) and fumonisin (≤ 4 ppm) contamination, respectively. However, there were some hybrids that had low aflatoxin concentration but high fumonisin concentration, for example FUMH/AFTX26 which had an aflatoxin content of 4.1 ppb and fumonisin content of 37.0 ppm. No hybrid showed high aflatoxin content but low fumonisin content (Figure 4.13; Table 4.9).

Four hybrids (FUMH/AFTX39, FUMH/AFTX22, FUMH/AFTX23, and FUMH/AFTX34) showed moderate resistance (> 5-10 ppb) to aflatoxin contamination, 32% were susceptible (> 10-20 ppb). Only FUMH/AFTX31 was highly susceptible to aflatoxin contamination, accumulating ≥ 30ppb. Four hybrids (FUMH/AFTX03, FUMH/AFTX2, FUMH/AFTX38, FUMH/AFTX16) showed moderate resistance (>4-10ppm) to fumonisin contamination. Five hybrids (FUMH/AFTX13, FUMH/AFTX19, FUMH/AFTX24, FUMH/AFTX17, FUMH/AFTX15) were susceptible (>10-20ppm). However, 66% of the hybrids showed high susceptibility to fumonisin contamination, accumulating ≥ 30 ppm.

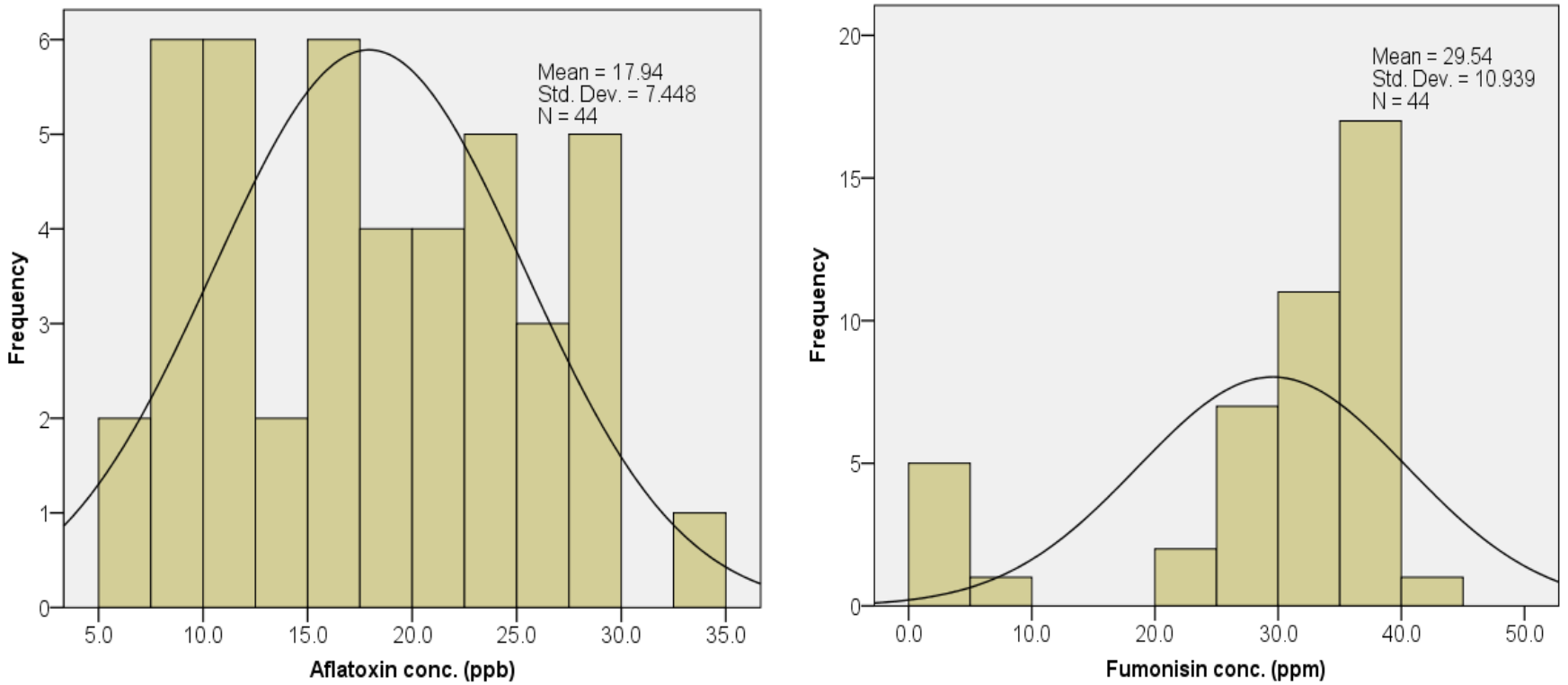

Figure 4.12 Frequency distributions of aflatoxin and fumonisin concentration of three-way cross maize hybrids.

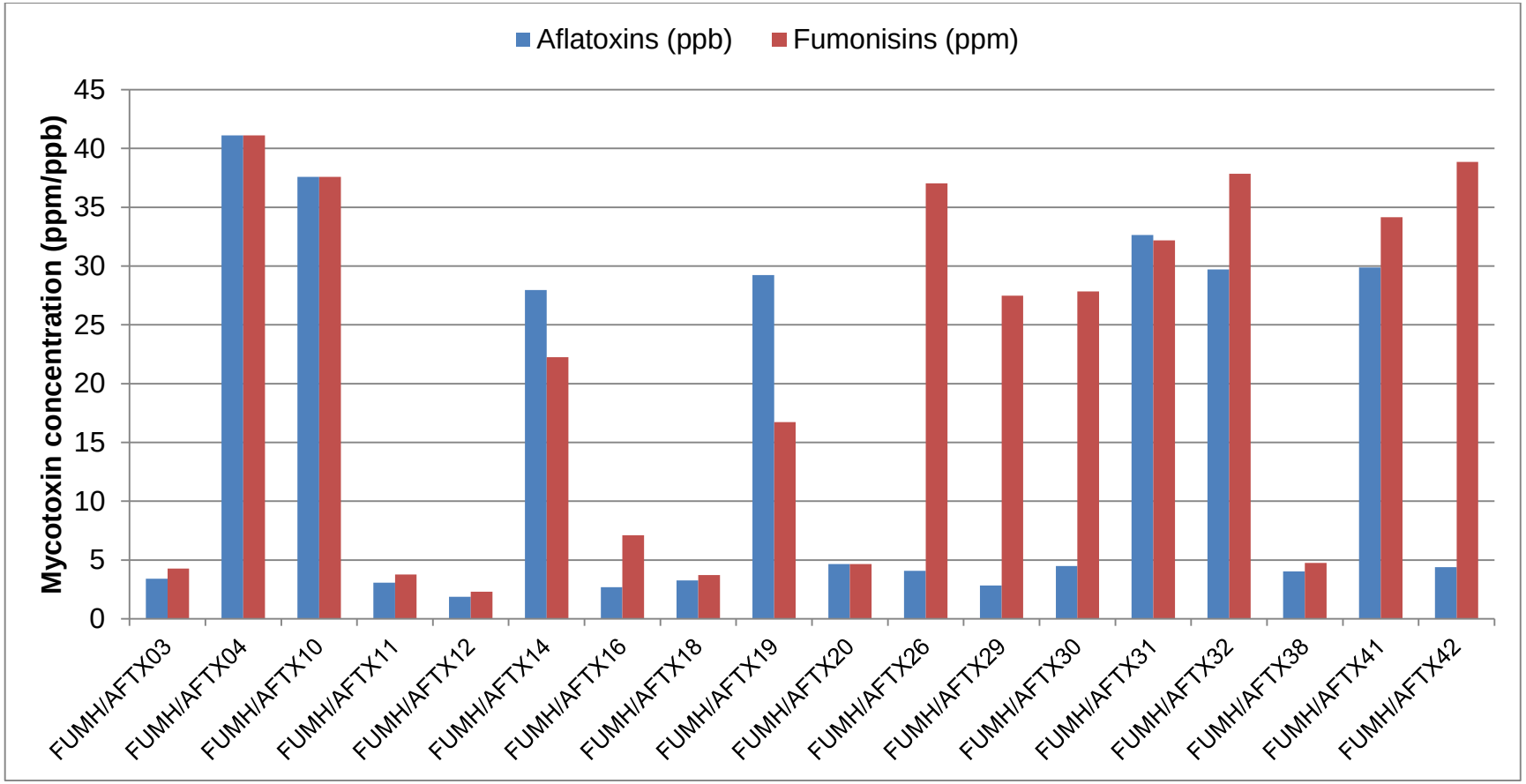

Figure 4.13 Mean fumonisin and aflatoxin contamination of few selected three-way cross hybrids in the field at Makhatini.

Table 4.9 *Aspergillus* ear rot and aflatoxin contamination resistance of selected three-way cross maize hybrids in the field at Makhathini.

Hybrid	Grain texture	*Aspergillus* ear rot[x]	Discoloured kernels (%)[x,y]	Aflatoxin B_1 conc. (ppb)[x]
Top 10				
FUMH/AFTX12	Flint	1.5a	1.5a	1.9a
FUMH/AFTX16	Flint	1.5a	1.6ab	2.7ab
FUMH/AFTX29	Flint	2.5a-c	2.5a-e	2.8ab
FUMH/AFTX11	Flint	2.5a-c	2.2a-e	3.1ab
FUMH/AFTX18	Flint	2.0ab	1.8ab	3.3ab
FUMH/AFTX03	Flint	2.5a-c	2.0a-c	3.4ab
FUMH/AFTX38	Dent	3.0a-d	1.8ab	4.0a-c
FUMH/AFTX26	Flint	3.0a-d	3.1a-g	4.1a-c
FUMH/AFTX42	Flint	2.0ab	1.6a	4.4a-c
FUMH/AFTX30	Flint	2.0ab	1.9ab	4.5a-c
Bottom 5				
FUMH/AFTX14	Flint	3.0a-d	3.1a-f	28.0j-l
FUMH/AFTX19	Flint	5.5ef	6.2i-j	29.2kl
FUMH/AFTX32	Flint	3.0a-d	2.2a-e	29.7kl
FUMH/AFTX41	Flint	5.5ef	6.0h-k	29.9kl
FUMH/AFTX31	Flint	5.0d.-f	6.2i-k	32.7l
Mean		3.4	3.52	15.2
l.s.d		2.2	2.75	9.6
cv(%)		32.1	38.8	31.2

[x]For each variable (*Aspergillus* ear rot severity, discoloured kernels, aflatoxin B_1 content), means followed by the same letter do not differ significantly according to Fischer's least significant difference test ($P \leq 0.05$).

[y]Data transformed to the arc sine square root of the percentage of discoloured kernels.

Table 4.10 *Fusarium* ear rot and fumonisin contamination of selected three-way cross hybrids in the field at Makhathini

Hybrid	Grain texture	*Fusarium* ear rot[x]	Discoloured kernels (%)[x,y]	Fumonisin (ppm)[x,z]
Top 5				
FUMH/AFTX12	Flint	1.5a	1.4a	2.0a
FUMH/AFTX18	Flint	2.5a-c	2.3a-e	3.7ab
FUMH/AFTX11	Flint	2.0ab	2.2a-d	3.8ab
FUMH/AFTX03	Flint	2.5a-c	1.8a-c	4.3ab
FUMH/AFTX20	Flint	2.0ab	3.9c-i	4.7a-c
FUMH/AFTX38	Dent	2.5a-c	2.3a-e	4.8a-c
FUMH/AFTX16	Flint	2.5a-c	2.3a-e	7.1c-f
FUMH/AFTX13	Dent	3.5a-e	3.4a-h	15.4b-e
FUMH/AFTX19	Flint	4.5c-g	5.4d-k	16.7c-f
FUMH/AFTX24	Flint	3.5a-c	1.6ab	17.2d-f
Bottom 5				
FUMH/AFTX10	Flint	5.0d-g	6.0g-k	37.6k-n
FUMH/AFTX32	Flint	4.0b-f	7.0i-k	37.8k-n
FUMH/AFTX42	Flint	6.5g	7.5k	38.9l-n
FUMH/AFTX36	Flint	4.5c-g	2.8a-g	39.1mn
FUMH/AFTX04	Flint	3.5a-e	3.8a-i	41.1n
Mean		3.8	4.42	26.1
l.s.d		2.2	3.26	12.1
cv(%)		28.0	36.5	22.8

[x]For each variable (*Fusarium* ear rot severity, discoloured kernels, fumonisin content), means followed by the same letter do not differ significantly according to Fischer's least significant difference test (P≤ 0.05).

[y]Data transformed to the arc sine square root of the percentage of discoloured kernels.

[z]Fumonisin concentration = total of FB_1 + FB_2 + FB_3

4.4.3 Correlation between ear rot and mycotoxin contamination

A significant and positive correlation was observed between FER and fumonisin contamination of maize hybrids (Figure 4.14). This result is shown for example by FUMH/AFTX03, which had a mean FER rating of 2.5 and fumonisin contamination of 4.3 ppm (Table 4.10). Not all hybrids followed a similar trend, for example FUMH/AFTX01 had a low mean FER score of 2.0 but accumulated a high fumonisin content of 29.42 ppm.

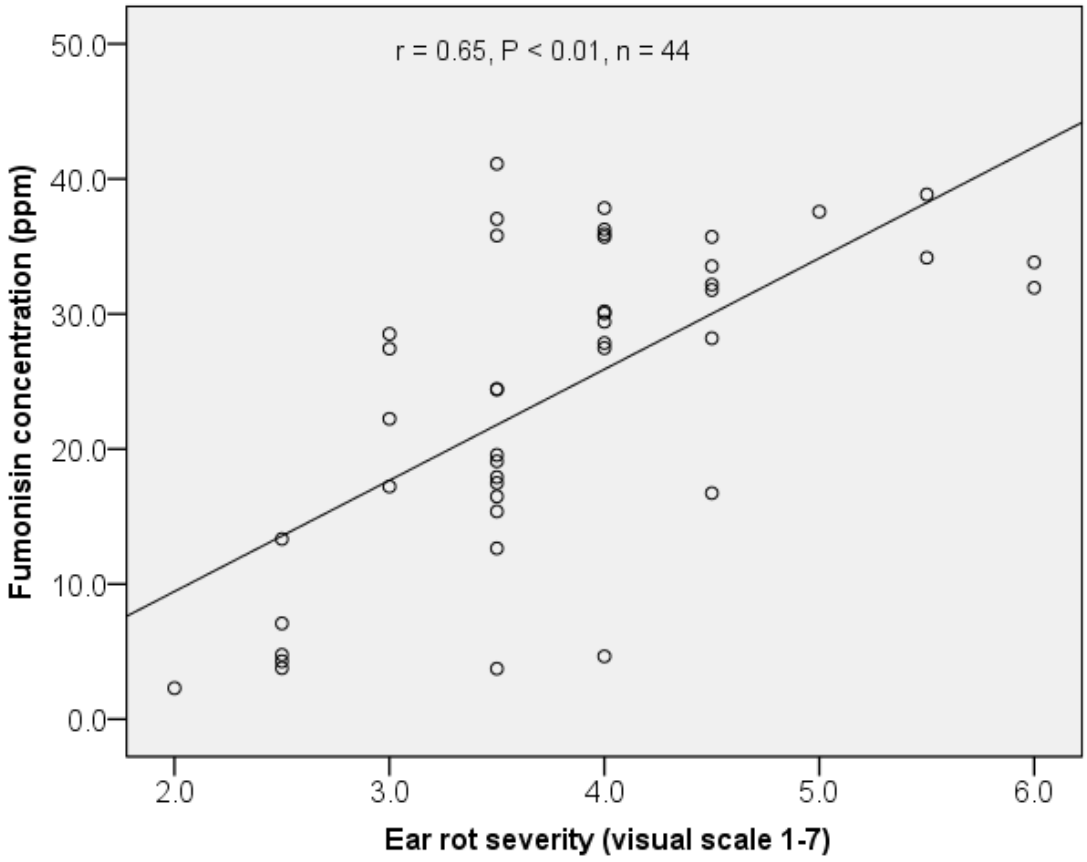

Figure 4.14 Linear correlations of *Fusarium* ear rot severity and fumonisin contamination of maize hybrids in the field at Makhathini.

The relationship between incidences of discoloured kernels and fumonisin contamination was also highly significant and positive (Figure 4.15.). This finding is shown by FUM/AFTX42, which had a high percentage of discoloured kernels (71%) and a resultant high fumonisin contamination level of 38.8 ppm (Table 4.10.). Outliers from this general trend were observed. For example, FUMH/AFTX36 had six percent discoloured kernels but accumulated a high fumonisin content of 39.11 ppm.

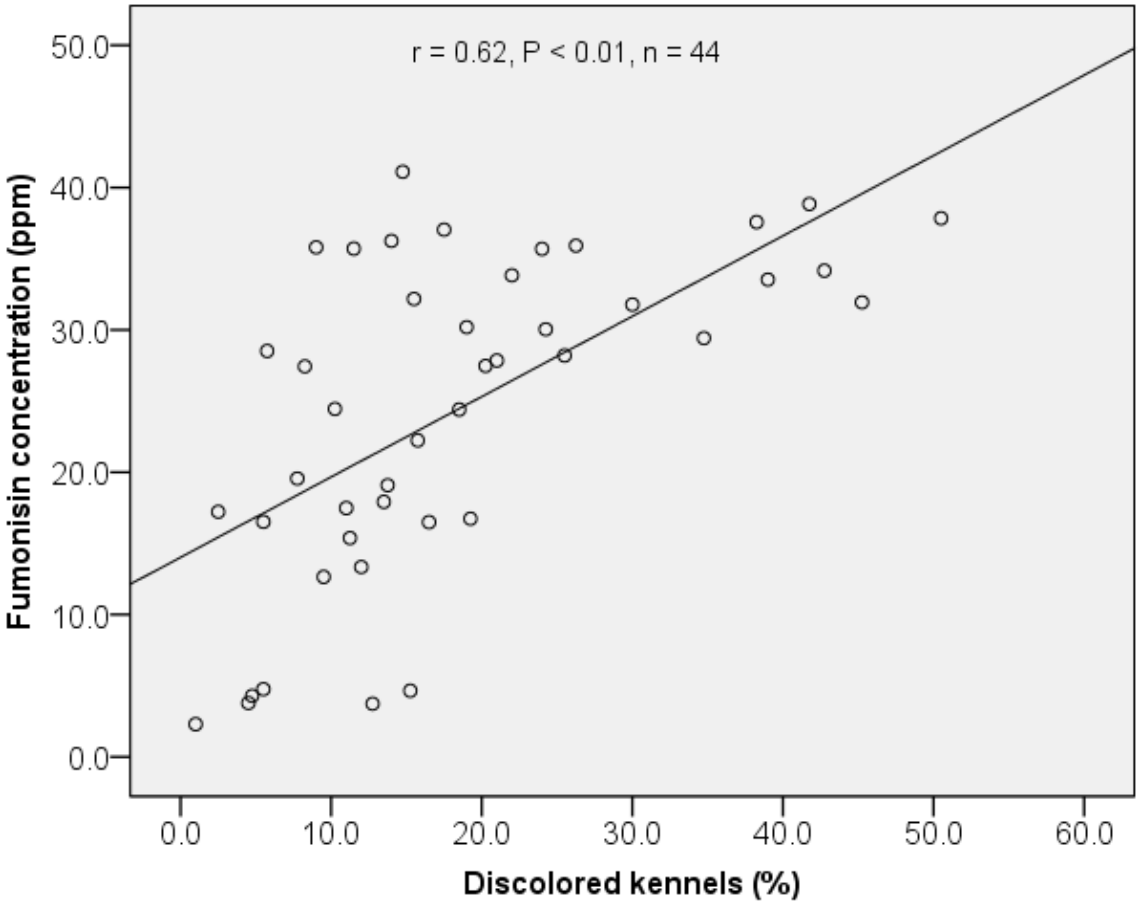

Figure 4.15 Linear correlations of percentage discoloured kernels and fumonisin contamination of maize hybrids in the field at Makhathini.

The linear correlation of AER and aflatoxin contamination was higher compared to the relationship between FER and fumonisin contamination (Figure 4.16). This result is shown by FUMH/AFTX12, which had a mean AER score of 1.5 and a low aflatoxin contamination of 1.9 ppb. Few hybrids did not follow this trend, for example FUM/AFTX 14 and FUM/AFTX32 had low ear rot severity scores, but accumulated high aflatoxin content.

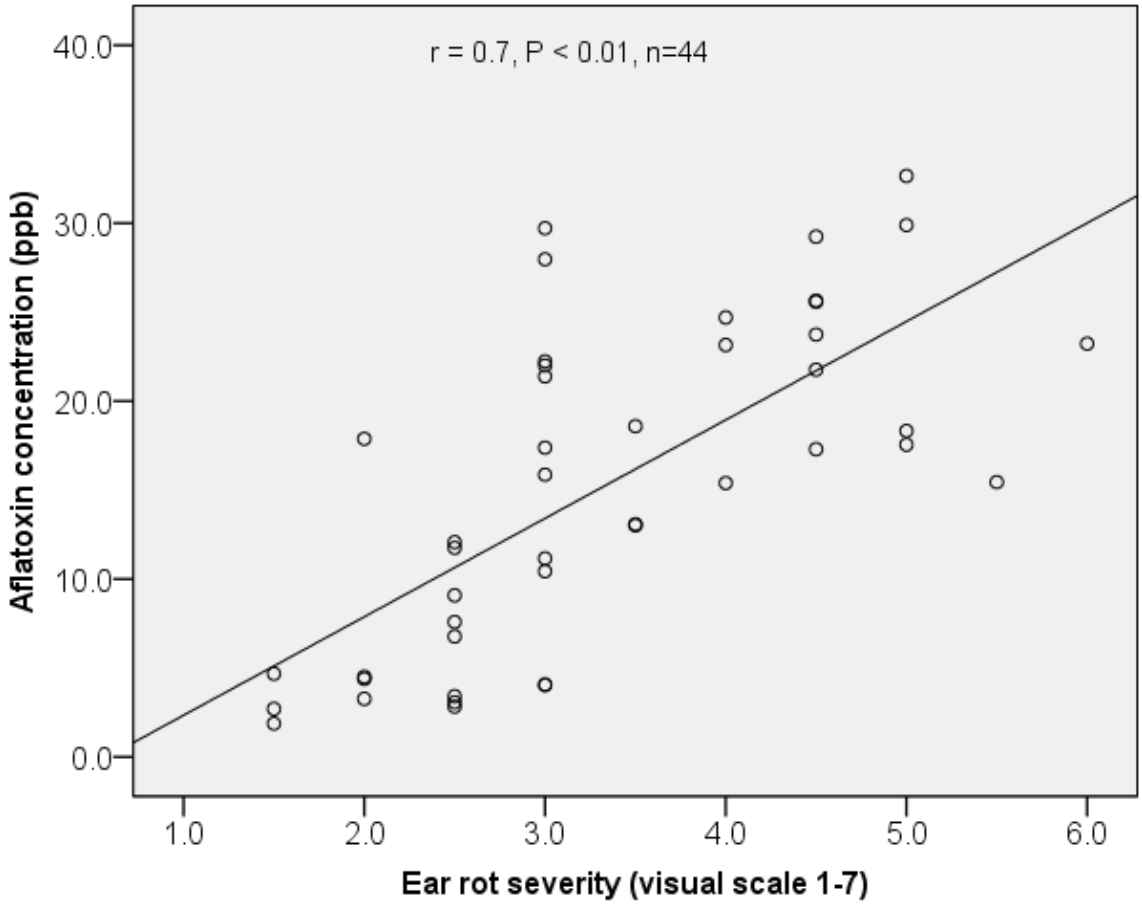

Figure 4.16 Linear correlations of *Aspergillus* ear rot severity and aflatoxin contamination of maize hybrids in the field at Makhathini.

A slightly lower but significant linear correlation was observed between the percentage incidence of discoloured kernels and aflatoxin contamination (Figure 4.17). Such a trend is shown by the hybrid FUM/AFTX39 which had a high percentage of discoloured kernels and corresponding high aflatoxin content. Such a trend was not obeyed by for example by FUM/AFTX32.

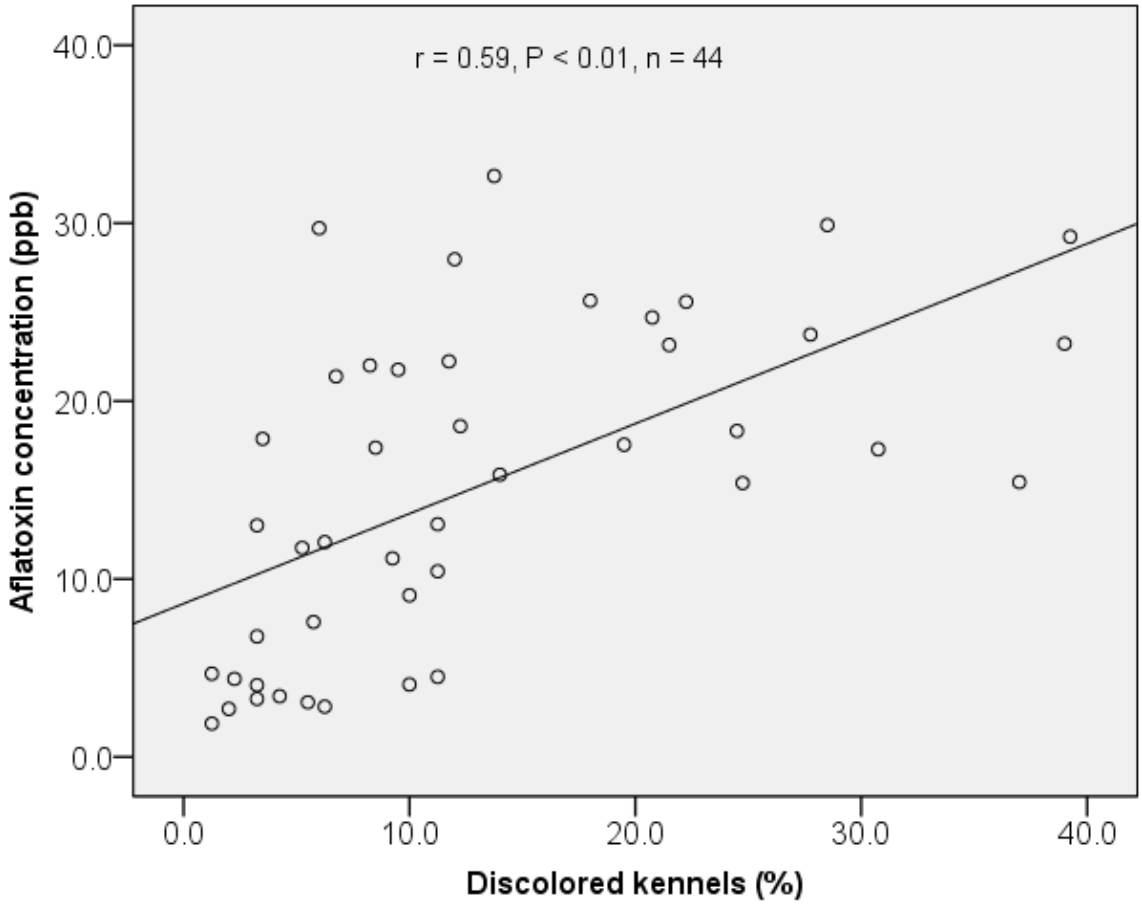

Figure 4.17 Linear correlation of percentage incidences of discoloured kernels and aflatoxin contamination of maize hybrids in the field at Makhathini.

A weaker but significant positive correlation was observed between fumonisin contamination and aflatoxin contamination among the hybrids (Figure 4.18). This finding is shown by FUMH/AFTX29, which had a high fumonisin contamination of 27.5 ppm and a low aflatoxin contamination level of 2.8 ppb (Table 4.10). Some hybrids, however, displayed a strong correlation in the accumulation of aflatoxins and fumonisins. An example is FUMH/AFTX12, which accumulated low concentration for both mycotoxins.

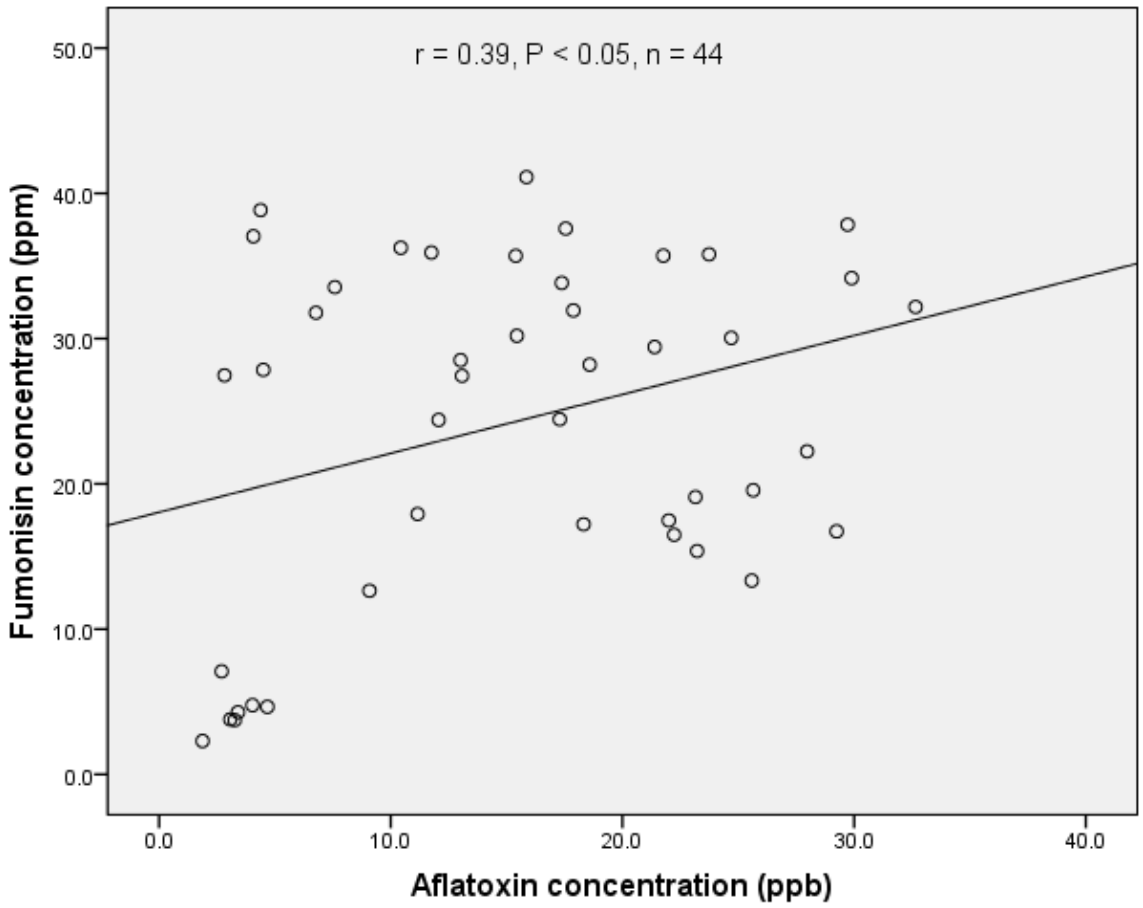

Figure 4.18 Linear correlations of fumonisin and aflatoxin contamination of maize hybrids in the field at Makhathini.

4.4.4 Estimates of genetic gain and heritability

When the top 10% three-way crosses were selected, high genetic gains were realised for aflatoxin and fumonisin accumulation. Moderately low genetic gains were predicted for both aflatoxin and fumonisin contamination resistance. Heritability estimates for aflatoxin and fumonisin contamination resistance were both high, whereas those for AER and FER were moderate. Moderately high genetic gains were realised for AER and FER. Low genetic gains were predicted for both AER and FER (Table 4.11).

Table 4.11 Breeding gains and heritability in three-way cross maize hybrids

Trait	Observed mean of selected three-way crosses($\bar{x}$)	Population mean (μ)	Realized genetic gain ($\bar{x}$-μ)		Predicted genetic gain		Heritability (h^2)
			(Mean)	(%)	(Mean)	(%)	
Aflatoxins (ppb)	2.6	15.2	-12.6	-83	-2.4	-16.1	0.89
Fumonisins (ppm)	3.5	26.1	-22.6	-87	-6.7	-25.5	0.95
Aspergillus ear rot score	2	3.4	-1.4	-44	-0.1	-1.7	0.61
Fusarium ear rot score	2.1	3.8	-1.7	-45	-0.1	-1.6	0.60

[x]1 = no infection, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75%, 7 = 76 -100%

Table 4.12 List of the 23 selected three-way cross maize hybrids.

Hybrid	Pedigree	Resistance
FUMH/AFTX03	CML390/12MAKCB4-6//TZAR102	Aflatoxin
FUMH/AFTX11	CML444/PL720//TZAR102	Aflatoxin
FUMH/AFTX12	CML444/DTA//TZAR102	Aflatoxin
FUMH/AFTX16	CML444X12MAK9-112//TZAR102	Aflatoxin
FUMH/AFTX18	CML390/12MAKCB3-1//TZAR103	Aflatoxin
FUMH/AFTX20	CML390/12MAKCB4-3//TZAR103	Aflatoxin
FUMH/AFTX26	CML390/12MAKCB4-48//TZAR103	Aflatoxin
FUMH/AFTX29	CML390/12MAKCB4-123//TZAR103	Aflatoxin
FUMH/AFTX30	CML390/PAN6227F2-8//TZAR103	Aflatoxin
FUMH/AFTX38	CML444/DTA//TZAR103	Aflatoxin
FUMH/AFTX42	CML444X12MAK9-131//TZAR103	Aflatoxin
FUMH/AFTX03	CML390/12MAKCB4-6//TZAR102	Fumonisin
FUMH/AFTX11	CML444/PL720//TZAR102	Fumonisin
FUMH/AFTX12	CML444/DTA//TZAR102	Fumonisin
FUMH/AFTX18	CML390/12MAKCB3-1//TZAR103	Fumonisin
FUMH/AFTX20	CML390/12MAKCB4-3//TZAR103	Fumonisin
FUMH/AFTX38	CML444/DTA//TZAR103	Fumonisin
FUMH/AFTX03	CML390/12MAKCB4-6//TZAR102	Aflatoxin + Fumonisin
FUMH/AFTX11	CML444/PL720//TZAR102	Aflatoxin + Fumonisin
FUMH/AFTX12	CML444/DTA//TZAR102	Aflatoxin + Fumonisin
FUMH/AFTX18	CML390/12MAKCB3-1//TZAR103	Aflatoxin + Fumonisin
FUMH/AFTX20	CML390/12MAKCB4-3//TZAR103	Aflatoxin + Fumonisin
FUMH/AFTX38	CML444/DTA//TZAR103	Aflatoxin + Fumonisin

4.5 Evaluation of $S_{2:3}$ families

4.5.1 Weather data

The weather data for the 2013/14 summer season is shown in Figures 4.19 and 4.20.

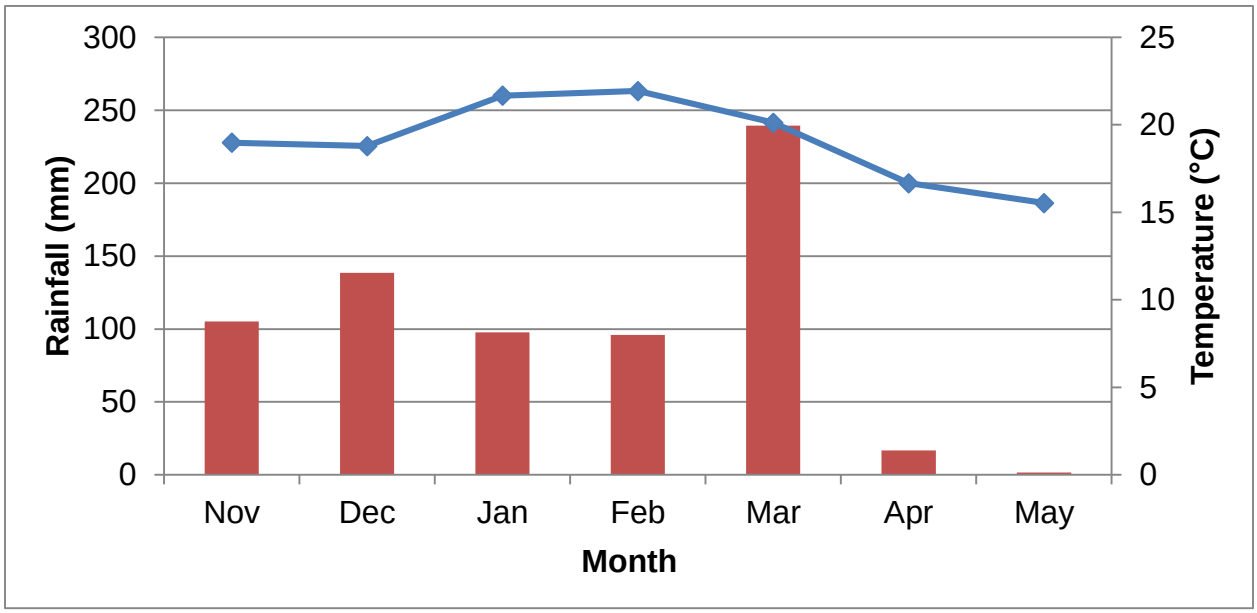

Figure 4.19 Monthly rainfall (in bars) and mean temperatures (line graph) for Cedara Research Station, during the experimental period, November 2013 to May 2014 (Source: Agriculture Research Council, 2014)

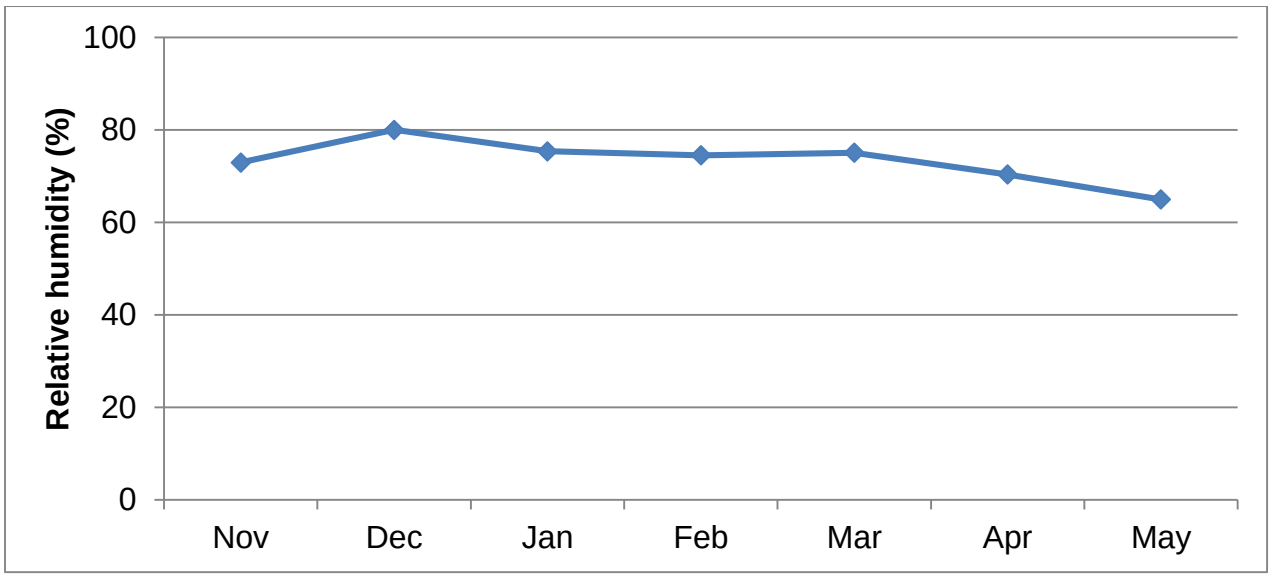

Figure 4.20 Monthly mean relative humidity for Cedara Research Station during the experimental period, November 2013 to May 2014 (Source: Agriculture Research Council, 2014)

4.5.2 Evaluation for ear rots and mycotoxin contamination

Analysis of variance for ear rots, mycotoxin content and selected agronomic traits is presented in Table 4.13. Mean squares of $S_{2:3}$ families were highly significant ($P < 0.001$) for FER, AER, aflatoxin and fumonisin content, husk cover, insect damage, grain texture and grain moisture content.

Table 4.13 Analysis of variance for ear rots, mycotoxin concentration and selected agronomic traits of $S_{2:3}$ maize families

	Fusarium ear rot		Fumonisin conc.		Aspergillus ear rot		Aflatoxin conc.		Husk cover		Insect damage		Grain texture		Grain moisture content	
Source	df	ms	df	ms	df	ms	df	ms	df	ms	df	ms	df	ms	ms	ms
Rep.	1	0.27	1	0.08	1	7.63	1	9.76	1	0.00	1	4.89	1	0.00	1	0.12
Entry	165	2.96***	165	566.30***	165	2.05***	165	250.92***	165	2.42***	165	5.12***	165	2.35***	165	3.30***
Error	165	0.39	165	0.34	165	0.48	165	0.21	165	0.03	165	1.02	165	0.00	165	0.14
Total	331		331		331		331		331		331		331		331	
lsd	1.23		1.15		1.36		0.91		0.34		2.0		0.11		0.73	
cv	19.6		2.8		26.5		2.3		6.6		16.4		2.3		2.7	

*, **, *** indicates the term is significant at P <0.05, P <0.01, P <0.001, respectively.

4.5.3 Ear rot severity assessment

Continuous variations were observed for the frequency distributions of FER and AER severity (Figure 4.21). The histograms of FER and AER severity showed normal distributions. *Aspergillus* ear rot severity ranged from a score of 1.0 to 5.3 with a mean of 2.6 (Table 4.14). Sixty-nine percent of the families had a low ear rot severity score (≤3) for AER. Fourteen percent of the families performed better than the resistant controls, which had a mean score of 1.5. Eighty-four percent of the families were more susceptible than the resistant control. MTX-55 had the highest score of 5.3.

FER ranged from 1.0 to 6.0 with a mean of 3.2 (Table 4.15). Fifty-six percent of the families had a low ear rot severity score (≤ 3) for FER. Sixteen percent of the families performed better than the resistant controls, which had a mean score of 2.0. Seventy-seven percent of the families were more susceptible than the resistant control. MTX-44 had the highest score of 6.0

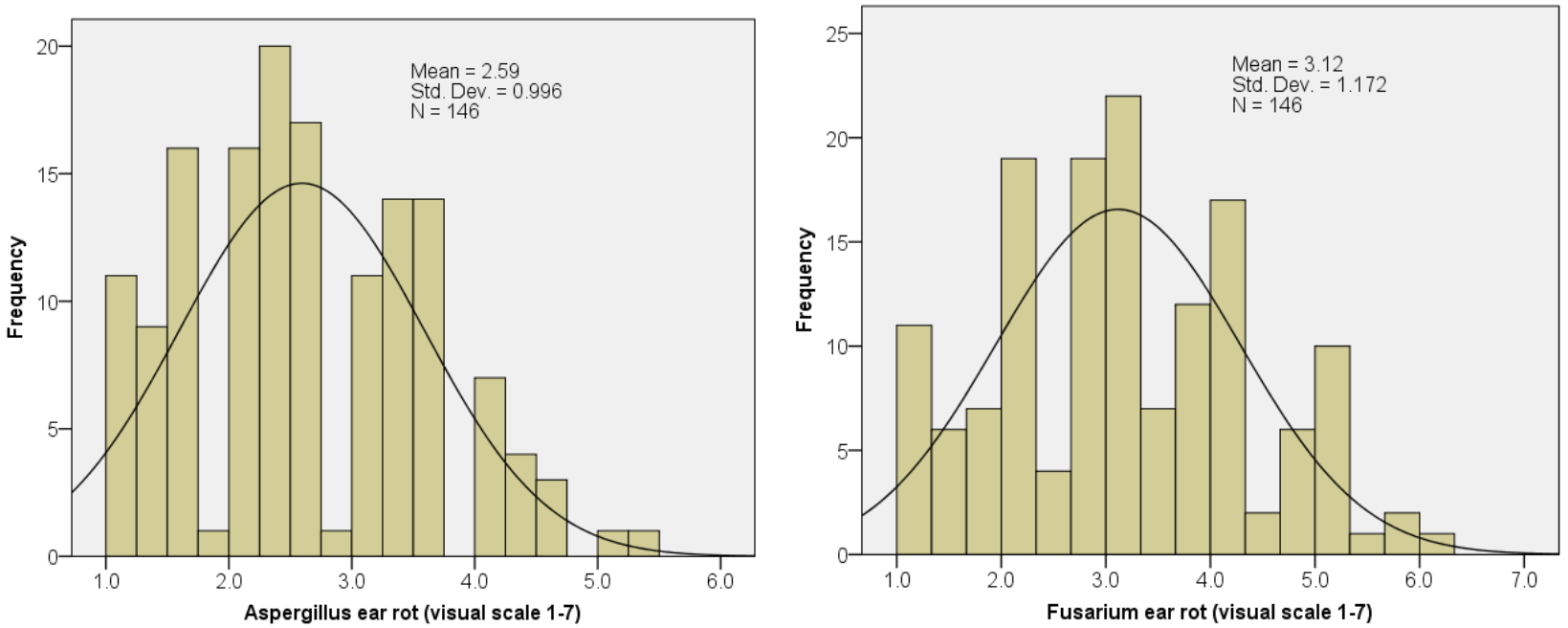

Figure 4.21 Frequency distributions of *Aspergillus* and *Fusarium* ear rot of 146 $S_{2:3}$ maize families at Cedara, during 2013/14 summer season.

4.5.4 Mycotoxin analysis

Continuous variations were observed for the frequency distributions of aflatoxin B_1 concentration and total fumonisin (Figure 4.22). A bimodal histogram was observed for aflatoxin B_1 concentration. A negatively skewed histogram was observed for total fumonisin concentration. Aflatoxin and fumonisin contamination was observed in all families and resistant controls following artificial field inoculation with *A. flavus* and *F. verticillioides*. There was no family that had complete resistance to both aflatoxin and fumonisin contamination. However, 23 families with concentration levels below the Codex Alimentarius legal limits of 5 ppb for aflatoxin B_1 were identified, while nine families with contamination levels below the set limit of 4 ppm for total fumonisin were identified.

Means for aflatoxin B_1 content among families ranged from 0.98 ppb to 39.08 ppb with a grand mean of 19.83 ppb (Table 4.14), and fumonisin content ranged from 1.76 ppm to 87.32 ppm with a grand mean of 20.08 ppm (Table 4.15). The five families, which exhibited lower levels of aflatoxin B_1 contamination compared to the resistant controls were: MTX-90, MTX-79, MTX-12, MTX-67 and MTX-36 (Table 4.14). The five families, MTX-45, MTX-90, MTX- 67, MTX- 79, MTX-36, accumulated lower levels of fumonisin contamination than the resistant controls. The families, MTX-90, MTX-79, MTX-67 and MTX-36 showed potential resistance to both aflatoxin and fumonisin contamination. Unexpectedly, the inbred line CML390, which was used as a resistant control for fumonisins, also accumulated low levels of aflatoxins.

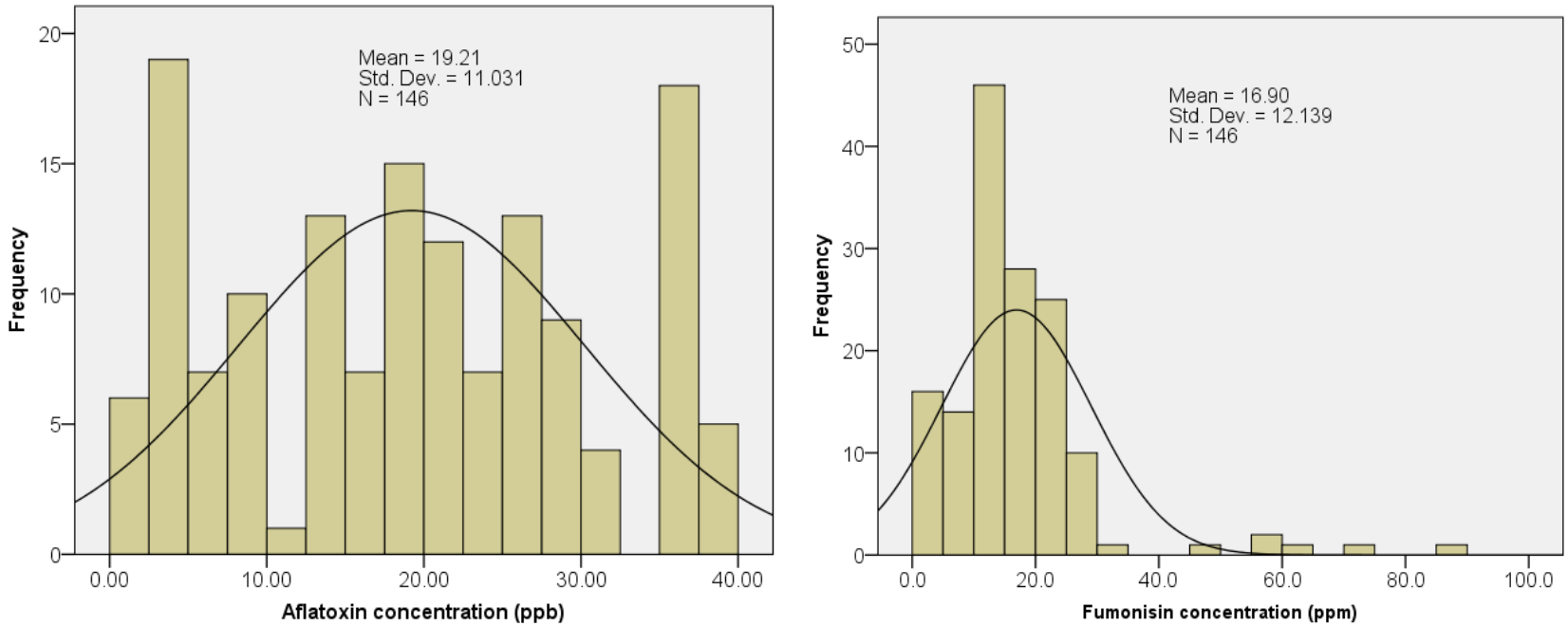

Figure 4.22 Frequency distributions of aflatoxin and fumonisin concentration of 146$S_{2:3}$ maize families at Cedara, during 2013/14 summer season.

Table 4.14 Means of aflatoxin B_1 content and agronomic traits of the top 15 and bottom 5 families relative to the controls

CODE	Pedigree	Traits measured[x]					
Top 15		**Aflatoxin B_1 conc. (ppb)**	***Aspergillus* ear rot**	**Grain texture**	**Husk cover**	**Insect damage**	**Grain moisture content**
MTX-90	(CML390/12MAKCB4123//TZAR103)-2	1.0	2.7	1	4	3	12.3
MTX-79	(CML390/12MAKCB4-48//TZAR103)-3	1.2	1.5	2	1	5	14.2
MTX-12	(CML390/12MAKCB4-110//TZAR102)-1	1.2	1.3	1	5	4	12.8
MTX-67	(CML390/12MAKCB4-22//TZAR103)-2	1.6	3.0	2	3	3	15.8
MTX-36	(CML444/08CED6-7//TZAR102)-2	1.7	2.0	1	5	4	13.3
MTX-25	(CML444/12MAKCB4-120//TZAR102)-4	3.4	3.3	2	5	5	15.0
MTX-113	(CML444/PL720//TZAR103)-1	3.5	2.7	2	1	4	15.4
MTX-103	(CML444/12MAKCB4-120//TZAR103)-2	3.9	3.3	1	3	4	13.4
MTX-99	(CML390/PAN6611//TZAR103)-4	4.0	2.4	2	3	3	12.3
MTX-118	(CML444/DTA//TZAR103)-2	4.1	2.7	2	3	2	14.2
MTX-106	(CML444/12MAKCB4-123//TZAR103)-1	4.3	4.0	1	4	2	13.7
MTX-31	(CML444/PL720//TZAR102)-2	4.3	1.0	2	3	1	14.7
MTX-45	(CML444X12MAK9-136//TZAR102)-3	4.4	1.7	2	7	6	13.8
MTX-100	(CML444/12MAKCB4-115//TZAR103)-1	4.4	2.3	2	2	2	12.0
MTX-122	(CML444/B17//TZAR103)-2	4.5	2.0	2	1	1	14.1
Bottom 5							
MTX-17	(CML390/08CED-7-2//TZAR102)-2	37.5	3.3	2	5	7	12.2
MTX-58	(CML390/12MAKCB3-2//TZAR103)-2	37.8	1.7	2	4	3	12.7
MTX-73	(CML390/12MAKCB4-44//TZAR103)-1	38.4	3.0	2	4	1	13.5
MTX-54	(CML390/12MAKCB3-1//TZAR103)-3	38.9	3.7	2	5	4	15.0
MTX-53	(CML390/12MAKCB3-1//TZAR103)-2	39.1	3.2	2	7	5	12.6
Positive control	TZAR102	2.0	1.3	1	1	1	12.7
Positive control	TZAR103/TZAR102	3.4	1.6	3	4	2	12.0
Negative control[y]	DTA/PAN6611	29.3	3.0	5	7	7	12.0
Mean		19.21	2.59	2.35	4.3	3.7	13.63
Minimum		1.0	1	1	1	1	11.1
Maximum		39.1	6	5	9	8	16.9
Range		38.18	5	4	8	7	5.8
Sig. F		<.001	<.001	<.001	<.001	<.001	<.001
l.s.d (0.05)		0.91	1.36	0.11	0.34	2.0	0.73
cv%		2.3	26.5	2.3	6.6	16.4	2.7

[x]Aspergillus ear rot 1= no infection, 7=76-100% infection, Grain texture 1=flint, 5= dent, Husk cover 1=long husk, 9= short husk, Insect damage 1= no damage, 9 = heavy damage, Grain moisture content = %

Table 4.15 Means of total fumonisin content and agronomic traits of the top 15 and bottom 5 families relative to the controls

CODE	Pedigree	Traits measured[x]					
Top 15		**Fumonisin conc. (ppm)**	**Fusarium ear rot**	**Grain texture**	**Husk cover**	**Insect damage**	**Grain moisture content**
MTX-45	(CML444X12MAK9-136//TZAR102)-3	1.8	3.7	2	7	6	13.8
MTX-90	(CML390/12MAKCB4-123//TZAR103)-2	1.9	1	1	4	3	12.3
MTX-67	(CML390/12MAKCB4-22//TZAR103)-2	2.0	1.3	2	3	3	15.8
MTX-79	(CML390/12MAKCB4-48//TZAR103)-3	2.1	4.0	2	1	5	14.2
MTX-36	(CML444/08CED6-7//TZAR102)-2	2.2	2.7	1	5	4	13.3
MTX-12	(CML390/12MAKCB4-110//TZAR102)-1	2.6	2.3	1	5	4	12.8
MTX-31	(CML444/PL720//TZAR102)-2	2.7	1.3	2	3	1	14.7
MTX-62	(CML390/12MAKCB4-3//TZAR103)-1	3.1	1.6	2	1	2	13.4
MTX-63	(CML390/12MAKCB4-3//TZAR103)-2	3.2	2.3	1	1	2	14.9
MTX-135	(CML444X12MAK9-112//TZAR103)-1	4.2	3.6	2	3	3	13.4
MTX-91	(CML390/12MAKCB4-123//TZAR103)-3	4.4	2.7	2	4	4	14.8
MTX-118	(CML444/DTA//TZAR103)-2	4.6	2.0	2	3	2	14.2
MTX-42	(CML444/09MAK24-1//TZAR102)-5	4.8	1.7	2	8	3	13.8
MTX-4	(CML390/12MAKCB4-3//TZAR102)-1	4.9	3.6	2	3	3	11.9
MTX-35	(CML444/08CED6-7//TZAR102)-1	5.1	5.3	3	5	4	13.4
Bottom 5							
MTX-126	(CML444/08CED6-7//TZAR103)-2	48.7	1.3	3	3	1	15.3
MTX-57	(CML390/12MAKCB3-2//TZAR103)-1	56.0	2.5	2	4	4	16.9
MTX-54	(CML390/12MAKCB3-1//TZAR103)-3	62.1	3.8	2	5	4	15.0
MTX-53	(CML390/12MAKCB3-1//TZAR103)-2	73.3	5.3	2	7	5	12.6
MTX-17	(CML390/08CED-7-2//TZAR102)-2	87.3	5.7	2	5	7	12.2
Positive control	CML444	2.2	2.3	3	1	1	13.7
Positive control	CML390	2.5	1.7	3	1	1	11.8
Negative control[y]	DTA/PAN6611	58.0	4.2	5	7	7	12.0
Mean		16.90	3.19	2.35	4.3	3.7	13.63
Minimum		1.76	1	1	1	1	11.1
Maximum		87.35	6.5	5	9	8	16.9
Range		85.59	5.5	4	8	7	5.8
Sig. F		<.001	<.001	<.001	<.001	<.001	<.001
l.s.d (0.05)		1.15	1.23	1.1	0.344	1.1	0.73
cv%		2.8	19.6	2.3	4.5	14.9	2.7

[x]Fusarium ear rot 1= no infection, 7=76-100% infection, Grain texture 1=flint, 5= dent, Husk cover 1=long husk, 9= short husk, Insect damage 1= no damage, 9 = heavy damage, Grain moisture content = %

A high level of contamination was observed for both aflatoxin and fumonisin in the families that were not introgressed with resistant genes. The families MTX-144 - MTX-149 and MTX-158 – MTX-164 (Negative controls, Table 4.14 and 4.15) were not introgressed. Some families that were introgressed, for example MTX-53 and MTX-54 accumulated high levels of aflatoxin and fumonisin more than the non-introgressed families (Table 4.14 and 4.15, Figure 4.20). Potential resistance to one trait and susceptibility to another trait was also observed among families. For example MTX-4 accumulated low levels of fumonisin (4.8 ppm), but high levels of aflatoxin, 13.1 ppb (Figure 4.20). In contrast, families MTX-25 and MTX-113 accumulated low levels of aflatoxin, 3.4 ppb and 3.5 ppb, respectively, but high levels of fumonisin contamination, 15.9 ppm and 21.8 ppm, respectively. Several families had a lower FER score, but a high fumonisin concentration. For example, MTX-126 had a low ear rot severity score of 1.3, but accumulated 48.7 ppm of fumonisin.

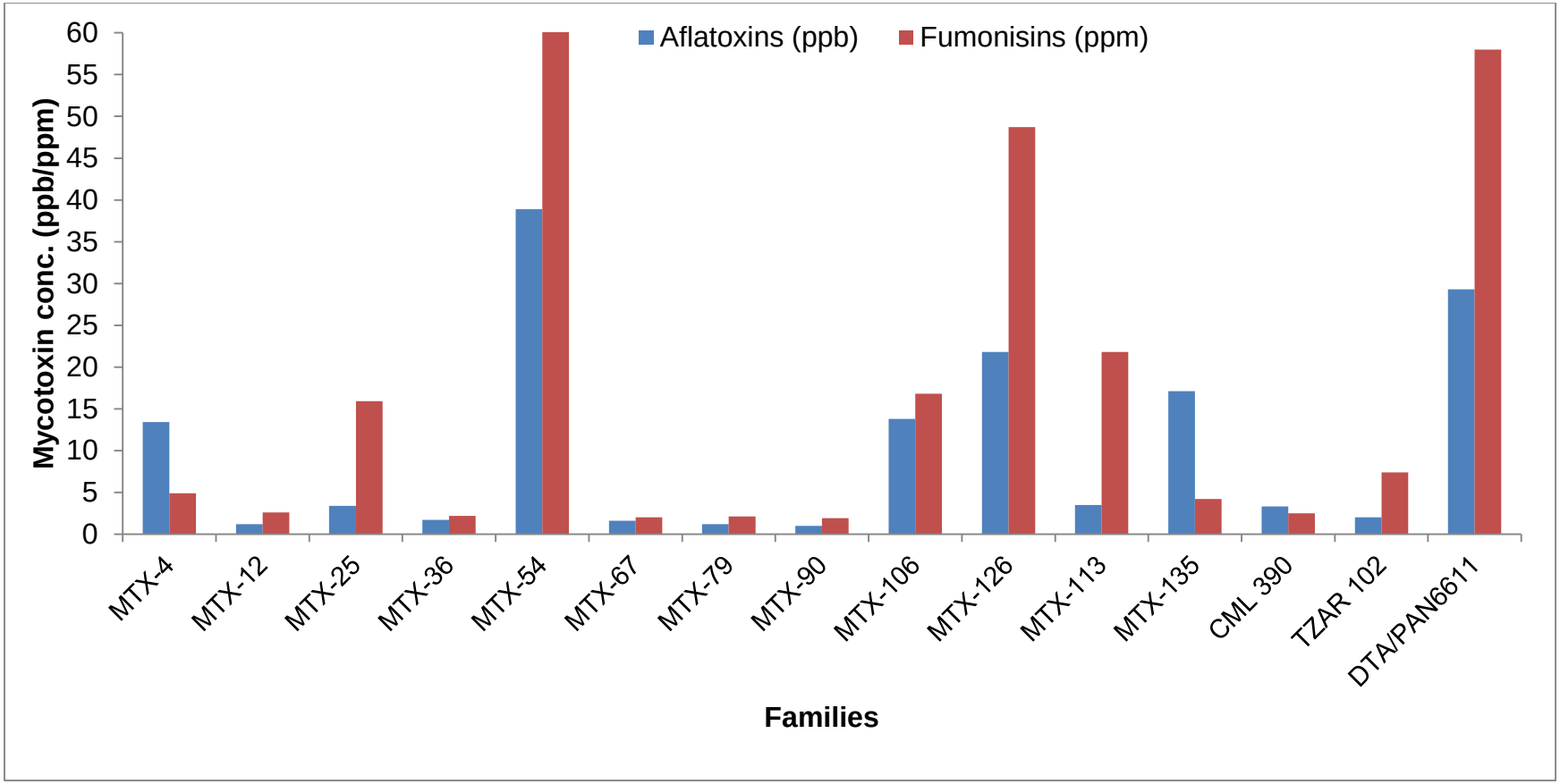

Figure 4. 23 Aflatoxin B_1 and total fumonisin (FB_1 + FB_2 + FB_3) concentration in kernels of some selected families relative to the resistant controls (CML 390 and TZAR 102) and the susceptible control (DTA/PAN6611).

4.5.5 Correlations between ear rot and mycotoxin contamination

Aflatoxin B_1 contamination showed a significant ($P < 0.001$) and positive correlation with AER as shown for example by MTX-79, which accumulated 1.2 ppb and an ear rot rating of 1.5 (Table 4.14). A significant ($P < 0.001$) and positive correlation was also observed between insect damage and aflatoxin contamination. This result is exemplified by MTX-17 which had a high insect damage score of 7 and a high aflatoxin contamination level of 37.5 ppb. A weak but significant ($P < 0.001$) positive correlation was observed between aflatoxin B_1 contamination and grain texture. The relationship between aflatoxin B_1 with husk cover was significant ($P < 0.001$) and positively correlated. A weak, negative correlation but significant ($P < 0.05$) relationship was observed between aflatoxin B_1 contamination and grain moisture content (Table 4.16).

A significant ($P < 0.001$) and positive correlation was observed between fumonisin contamination and FER. This result is shown for example by MTX-90, which had a mean FER rating of 1.0 and fumonisin contamination of 1.9 ppm (Table 4.15). The relationship between insect damage and fumonisin contamination also showed a significant ($P < 0.001$) and positive correlation. This finding is exemplified by MTX-17, which had a high insect damage score of 7 and a resultant high fumonisin contamination level of 87.3 ppm (Table 4.15). Fumonisin contamination also showed a significant ($P < 0.001$) and positive correlation with grain texture and aflatoxin B_1 contamination. A significant ($P < 0.001$) but negative correlation was observed between fumonisin contamination and grain moisture content. No significant correlation ($P > 0.05$) was observed between fumonisin content and husk cover (Table 4.15).

Table 4.16 Pearson's correlation coefficients showing pair-wise association among eight traits in 146 $S_{2:3}$ maize families

	Aflatoxin conc.	**Fumonisin conc.**	***Aspergillus* ear rot score**	***Fusarium* ear rot score**	**Insect damage**	**Husk cover**	**Grain texture**	**Grain moisture content**
Aflatoxin conc.	-							
Fumonisin conc.	0.49***	-						
***Aspergillus* ear rot score**	0.20***	0.19***	-					
***Fusarium* ear rot score**	0.21***	0.33***	0.18***	-				
Insect damage	0.36***	0.37***	0.24***	0.56***	-			
Husk cover	0.26***	0.09ns	0.17***	0.27***	0.42***	-		
Grain texture	0.15***	0.51***	0.07ns	0.19***	0.16***	0.10ns	-	
Grain moisture content	-0.12**	-0.28***	-0.05ns	-0.21***	-0.15***	-0.13**	-0.39***	-

*, **, *** indicates the term is significant at P<0.05, P<0.01, P<0.001, respectively.

4.5.6 Heritability estimates and genetic gain

When the top 10% resistant families were selected, very high genetic gain was realised for aflatoxin and fumonisin contamination resistance. Low genetic gains were realised for AER and FER. Low genetic gains were predicted for aflatoxin and fumonisin contamination, AER and FER. Heritability estimates were also very high for aflatoxin and fumonisin contamination resistance, AER and FER (Table 4.17).

4.6 Conclusion

This chapter described the outcomes of this study and highlighted the patterns observed. Results revealed a high incidence of ear rot diseases caused by mycotoxin associated fungi among southern African maize hybrids. Significant variation was observed between single cross hybrids, three-way cross hybrids and $S_{2:3}$ families for both ear rot and mycotoxin contamination resistance. New maize hybrids and families which were superior to the controls were identified. The implications of these findings to maize breeding are discussed in the next chapter.

Table 4. 17 Breeding gains and heritability in $S_{2:3}$ families

Trait	**Observed mean of $S_{2:3}$ selected families ($\bar{x}$)**	**Population mean ($\bar{x}$)**	**Realised genetic gain ($\bar{x}$-μ)**		**Predicted genetic gain**		**Heritability (h^2)**
			(Mean)	**(%)**	**(Mean)**	**(%)**	
Aflatoxins (ppb)	3.2	19.8	-16.6	-84	-2.4	-12	0.99
Fumonisins (ppm)	3.3	20.6	-17.3	-84	-8.3	-40	0.99
Aspergillus ear rot score	2.4	2.6	-0.2	-8	-1.1	-42	0.74
Fusarium ear rot score	2.6	3.2	-0.6	-19	-2.0	-63	0.92

[x]1 = no infection, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75%, 7 = 76 -100%

CHAPTER FIVE

GENERAL DISCUSSION

5.1 Introduction

This chapter presents the discussion and interpretation of the study. It begins with an analysis of ear rots incidence among hybrids of different maturities and its implication for future breeding programs. This is followed by the dissection of results obtained from evaluation of hybrids and their progenies. It also discusses the relationships between ear rots, mycotoxin contamination and agronomic traits. A conclusion on the chapter is drawn.

5.2 Survey of natural incidence of ear rots in southern African maize hybrids

Results from this study showed that ear rots are of economic importance and there is need to initiate programs to address them. *Stenocarpella maydis* (Diplodia ear rot) accounted for the highest incidences of ear rot diseases in 2012/13 compared to the 2013/14 season. This can be explained by the variability of weather patterns between the two seasons. Ears are most susceptible to *Diplodia* infection during the first three weeks of silking and when the green silks start to turn brown (White, 1999). Dry weather prior to silking followed by wet weather during silking seems to increase the incidence and severity of Diplodia ear rot (Steckel, 2003). The coordinated timing of spore release and plant silking could be responsible for this relationship (Agrios, 1997). In this study, a period of dry spell with high temperatures was experienced in February to early March, during the 2012/13 summer season. High rainfall and high relative humidity was recorded in the rest of March providing a conducive environment for *S. maydis* development. The period coincided with silking dates of the hybrids. In the 2013/14 summer season such a pattern was not observed.

In 2013/14, the most isolated fungus was *Fusarium verticillioides*. Dry conditions at silk emergence are known to favour the spread of *F. verticillioides* microconidia (Munkvold, 2003). Little rainfall coupled with higher temperatures and relative humidity in March 2014 compared to March 2013 was recorded. Conditions in 2013/14 were, therefore more favourable for *F. verticillioides* than *Diplodia* compared to the 2012/13 season. Overall, *F. verticillioides* accounted for the highest incidences of ear rot diseases in hybrids. Results in the present study are in agreement with previous studies that reported the high occurrence of *F. verticillioides* and fumonisins in maize and maize based food in Zambia (Mukanga et al., 2010), Limpopo and Eastern Cape provinces of South Africa (Ncube et al., 2011; van der Westhuizen et al., 2010) .

Results from the present study showed that late maturing hybrids are more susceptible to ear rots. As the water available for fungal growth plays a key role, late-maturing maize genotypes in which grain moisture content decreases slowly are most likely to be susceptible (Eller et al., 2008). Xiang et al. (2010) also reported presence of direct relationship between kernel dry down rates of a given maize genotype and the extent of ear rot severity symptoms. The occurrence of late season drought also contributes to the high incidence of ear rots caused by *Fusarium* and *Aspergillus* species (Wagacha and Muthomi, 2008). Hence late maturing genotypes are most likely to be infected. For example, a late season decline in rainfall in April and May 2013 might explain the high incidences of *A. flavus* in 2012/13 season compared to the 2013/14. Although low ear rot incidences were observed in early maturing hybrids, the hybrids displayed a high negative correlation between yield and ear rot incidences compared to the medium and late hybrids. Gasura et al. (2010) reported that early maturing maize genotypes yield 20-30% less than late maturing genotypes. Thus despite their ability to escape ear rots infection and provide food early, the challenge is to increase yield while maintaining earliness.

The biotic causes of kernel wounds result in increase of fungal species infection as shown by a high correlation between insect damage and incidences of ear rots in this study. Insects play a significant role in the vectoring of fungal spores and also provide entry holes to fungal organisms through their tunnelling activity, both prior to and after

harvest (Cardwell et al., 2000). For example, insects such as thrips (*Frankliniella occidentalis*) and the African maize borer (*Busca fuseola*) have been related to high incidences of disease caused by *Fusarium* spp. (Ncube et al., 2011). Munkvold (2003) also showed that control of European corn borer using transgenic maize reduces the amount of injury to ears, and subsequently lower the severity and incidences of ear rots.

Dent genotypes were more susceptible than the flint as shown by a positive and significant correlation between ear rot incidences and grain texture. Czembor and Ochodzki (2009) also found higher ear rot resistance in flint genotypes than in dent genotypes. Previous studies emphasized the role of kernel structure, especially thickness of pericarp, wax and aleurone layers (Clements et al., 2004). The hard kernel coat, typical of flint maize, can make genotypes less susceptible to fungal infection than dent maize (Bennetzen and Hake, 2009). Moreover, flint kernels were found to contain more amylose. The endosperm component together with the physical properties of kernels, contribute to their hardness (Robutti et al., 2000) and in consequence with the susceptibility to biological damage. Dent maize is therefore soft and can be easily damaged by insects creating channels for fungal proliferation.

In summary, the experimental maize hybrids were susceptible to diseases caused by *S. maydis, F. verticillioides, A. flavus* and *F. graminearum*. The fact that a high number of hybrids were susceptible has implications for both breeders and consumers of maize grain. First, it is a matter of great concern that millions of people might be consuming contaminated maize grain and maize-based foods daily without being aware of the danger. The Table 5.1 indicates the potential mycotoxins that can be caused by these fungi. In terms of breeding, the way forward is to continue screening more experimental hybrids and breeding populations to find safe and high yielding varieties. Further research should include artificial inoculation and mycotoxin evaluation of the experimental hybrids to minimize problems of escape by some hybrids and thereby maximizing repeatability.

Table 5.1 Identified fungi and their potential mycotoxins

Identified causal fungi	Potential mycotoxins
Fusarium verticillioides	Fumonisin B_1,B_2,B_3
Fusarium graminearum	vomitoxins (deoxynivalenol) and zearalenone
Stenocarpella maydis	Diplodiotoxins
Aspergillus flavus	Aflatoxin B_1,B_2, G_1,G_2 and M_1 (derivative of AFB_1 found in milk)

5.3 Development of mycotoxin resistant hybrids

Single cross maize hybrids with consistently low levels of fumonisins in grain were identified in the field and greenhouse experiment, giving hope that resistant commercial hybrids can be developed, and that shuttling the breeding between the greenhouse and field testing would be effective. Use of greenhouses would be important to bridge the summer seasons, because the logistics of conducting off- station winter nurseries are very expensive. Three-way cross hybrids with low levels of both fumonisin and aflatoxin contamination were also identified in the field experiment, adding further credence that resistance can be stacked in the end product. The three-way cross hybrids are the predominant form of hybrids which are grown in SSA. However, single cross hybrids provide the greatest opportunity for expression of hybrid vigour and usually have higher yields than other types of hybrids. They also provide maximum uniformity for seed characteristics, height and maturity (Fehr and Hadley, 1980). The main disadvantage of single cross maize hybrids is that the inbred line used as the female parent usually produces lower seed yields than the types of females used for producing other hybrids (, 1978). Thus, the purpose of three-way and double cross hybrids is to reduce seed cost by use of a more productive non-inbred female parent. Therefore the development of three-way crosses would be ideal for the resource poor farmers in SSA, who are the target for the deployment of mycotoxin resistant maize hybrids. However for the large scale commercial farmers in South Africa the single cross would be the hybrid of choice.

A survey of the literature, suggests that this could be the first study to report identification of maize hybrids with potential resistance to both fumonisin and aflatoxin contamination in a single hybrid and also adapted to southern African production conditions in subtropical and temperate environments. Previous studies have reported on the development of maize inbred lines for either fumonisin or aflatoxin but not both in a single genotype (Afolabi et al., 2007; Small et al., 2012). However, the hybrids with potential mycotoxin resistance, which were identified in this study, will be subjected to further evaluation across seasons and over an extended range of conditions, representative of the localities where they will eventually be grown, to further confirm their resistant status and value for cultivation.

5.3.1 Nature of resistance and genotype x environment interaction

Seemingly mycotoxin resistance is quantitative, because none of the hybrids evaluated in this study were completely resistant to fumonisins or to both fumonisin and aflatoxin contamination. The involvement of polygenic inheritance can also be inferred from the continuous distributions of fumonisin and aflatoxin data in both single cross and three-way cross hybrids. This has implication for breeding strategy. The observation of a lack of complete resistance is in agreement with previous studies (Abbas et al., 2006; Henry et al., 2009). It is suggested that both parents of single cross hybrids or all three parents of the three-way cross should be improved for resistance. As a result resistance should be improved in all heterotic groups which will be used to make hybrids among them.

Another challenge in breeding for resistance which is common with quantitatively inherited traits would be genotype x environment interaction effects. The current study indicates that GxE would present fewer complications in selection of hybrids, because there was a significant and positive correlation between results from the greenhouse and the field. However several single cross maize hybrids showed significant differences in fumonisin content between the field experiment at Makhathini and the greenhouse tunnel. Such an observation supports reports of the effect of genotype x environment interaction in fumonisin contamination. This is exemplified by FUMH 23,

FUMH 34, FUMH 63 and FUMH 69, which had high fumonisin content at either Makhathini and low content in the greenhouse or vice-versa. Previous studies have reported high GxE interactions for grain contamination by aflatoxins and fumonisins contributing to low heritability estimates for ear rots and mycotoxin contamination (Abbas et al., 2002). Genotype x environment interactions especially drought stress has therefore been attributed as the main reason for the lack of consistency of the performance of maize genotypes for resistance to mycotoxin contamination (Abbas et al., 2002; Cotty and Jaime-Garcia, 2007). In sharp contrast, results from this study displayed high heritability estimates for ear rots and mycotoxin contamination. Thus results from the present study indicate that, although ear rots and mycotoxin resistance are complex traits, selection of best performing genotypes could speed up breeding for resistance to mycotoxin contamination in hybrids or populations.

5.3.2 Associations between ear rots and mycotoxin contamination

Rapid and cheaper screening methods would be required to scale up breeding for mycotoxin resistance in maize hybrids. In this regard, the findings from the current study reveal a promising trend. Total fumonisins and disease severity on single cross hybrids were positively correlated in both the field and greenhouse experiments. Similar results were observed in three-way crosses. The strong correlations between Fusarium and Aspergillus ear rots and with fumonisin and aflatoxin contamination observed in this study suggest that selection against Fusarium and Aspergillus ear rots should also result in reduced susceptibility to fumonisin and aflatoxin contamination. This is a favourable situation because ear rots are easy to score in the field, relatively across environments, and much less expensive to phenotype than mycotoxin contamination.

The findings from the current study are consistent with previous investigations. Previous studies have reported a similar correlation between fumonisin and ear rot severity (Robertson et al., 2006). A study by Robertson et al. (2006) showed that, due to high heritability and strong genetic correlation between ear rot and mycotoxin resistance, selection for ear rot resistance should result in lines with greater resistance to fumonisin contamination. Although this relationship describes a general trend, a number of hybrids

in the present study (e.g FUMH52 and FUMH81 in the greenhouse experiment) had low ear rot severity with very high levels of fumonisin content in grain. A similar observation that identified high levels of fumonisins in apparently symptomless kernels has previously been reported (Mukanga et al., 2010; Small et al., 2012). This might be difficult to explain but Bacon et al. (2008) reported on the endophytic colonization of maize plants by *F. verticillioides* and presumed that this can contribute to mycotoxin contamination of grain. Murillo-Williams and Munkvold (2008) reported that systemic infections from the stalk to the ear leading to more asymptomatic infections and toxin accumulation may be a problem predominantly in hot regions. As global warming seems to be durable, this problem may become more serious in the tropics and subtropical areas mainly sub-Saharan Africa. Another possible explanation for outliers observed in this study is that the visual rating was unable to separate between visual disease symptoms caused by non-fumonisin producing fungal species, resulting in high visual rating and low fumonisin concentration. This underscores the need for fumonisin analyses when evaluating maize resistance. Unfortunately laboratory assays are still very expensive which can impact negatively on research, especially in developing countries.

Results from this study suggest the existence of some common resistance mechanisms to these two fungi, but many questions remain to be addressed to further elucidate these relationships. The possibility of common host resistance mechanisms of maize, as well as common infection and virulence mechanisms of the fungi, should be investigated. Other factors that impact fungus-plant interaction, such as insect resistance or damage, should be considered. Abbas et al. (2006) found that both Aspergillus and Fusarium ear rot were significantly correlated with insect damage. Also, genes that control plant stress reactions may also contribute to the correlation between resistances to these two fungi. For example, stress due to weather conditions influences fumonisin and aflatoxin contamination (Lobell et al., 2008; Murillo-Williams and Munkvold, 2008). This will be recommended for investigation in future studies.

5.4 Evaluation of $S_{2:3}$ families

5.4.1 Germplasm resistance

Families with potential resistance to both aflatoxin and fumonisin contamination in grain were identified. This is the first report on identification of maize families with potential resistance to both fumonisin and aflatoxin contamination adapted to southern African conditions. For effective selection, resistant traits need to be evaluated in more than one environment. On the other hand, inoculation and mycotoxin analysis procedures are labour intensive, time consuming and expensive. Therefore, in the present study with segregating $S_{2:3}$ families, evaluations of mycotoxin accumulation were conducted in only one environment. The objective was to eliminate only the highly susceptible ones. Subsequently, progenies of the families with potential resistance identified in this study will be advanced for further evaluation across seasons and over an extended range of conditions to further confirm their resistant status.

The relationship between aflatoxin B_1 and total fumonisin concentration was significant and positively correlated. Similar correlations have been reported previously (Robertson et al., 2006). Such observations have prompted previous studies to suggest that a common resistance might exist between aflatoxin and fumonisin contamination (Abbas et al., 2006; Robertson-hoyt et al., 2007). Consistent with that observation, the maize inbred line CML 390, which was used as a resistant control for fumonisin accumulation in this current study also showed resistance to aflatoxin contamination. However, some families with high aflatoxin B_1 concentration accumulated low levels of fumonisin concentration and *vice versa*. Therefore selecting for aflatoxin resistance does not necessarily result in fumonisin resistant genotypes, hence grain must be evaluated separately for both aflatoxin and fumonisin contamination.

5.4.2 Association between ear rot and mycotoxins

Findings from this study also agree with previous studies that found significant and positive correlations between aflatoxin and fumonisin accumulation with ear rots (Abbas et al., 2006; Robertson et al., 2006). This suggests that selection against Fusarium ear

rot and Aspergillus ear rot can aid in the identification of aflatoxin and fumonisin resistant genotypes. Thus, removal of diseased kernels destined for human consumption or livestock feed can significantly reduce levels of aflatoxin and fumonisin concentration in maize grain. However, low correlation coefficients observed in this study indicate that aflatoxin and fumonisin accumulation levels cannot be determined solely by the severity of Aspergillus ear rot and Fusarium ear rot, but chemical analysis is essential to confirm the levels of contamination. Some inconsistencies were observed in the relationship between fumonisin concentrations and Fusarium ear rot where families with symptomless ears had a high concentration of fumonisins. A survey in several Zambian districts by Mukanga et al. (2010) also reported such inconsistencies which may be explained by the presence of non-fumonisin producing endophytic *F. verticillioides* (Bacon et al., 2008; Small et al., 2012). Furthermore, the error of visual assessment of Fusarium ear rot is higher compared to Aspergillus ear rot due to the random distribution of single infected kernels.

5.4.3 Association between insect damage and mycotoxins

A significant correlation was observed between aflatoxin B_1 and fumonisin concentration with insect damage and grain texture. Insects are common vectors for spreading *A. flavus* and creating openings for *F. verticillioides to* colonise maize kernels. Several previous studies are in agreement that the aflatoxin and fumonisin problem in maize could be partially remedied by conventional breeding or transgenic solutions to the insect vectors that introduce *A. flavus* and *F. verticillioides* spores directly into the developing maize ears (Abbas et al., 2007; Munkvold 2003; Wu, 2006). Unfortunately, it has since been shown that *A. flavus* and *F. verticillioides* can infect maize ears via the silk channel and other entry points (Warburton and Williams, 2014) other than insect damage. Thus, elimination of aflatoxin and fumonisin has not been achieved in previous research efforts simply by combating insect vectors for transmission of *A. flavus* and *F. verticillioides.*

Insects probably play a more important role in the *A. flavus* and *F. verticillioides* infection process when conditions are less favourable for the fungus (Mayfield et al.,

2012). Under conditions more favourable to *A. flavus* and *F. verticillioides* (high temperatures and drought stress), the role of insect injury in aflatoxin and fumonisin contamination probably diminishes. The pathogens might even display limited parasitic abilities under such conditions (Guo et al., 2001). At Cedara Research Station, during the 2013/14 summer season, the level of humidity was very high with an average of 75% for the duration of this study. High levels of humidity are conducive for fungal development but not for aflatoxin and fumonisin accumulation (Warburton and Williams, 2014). Rainfall and average temperature were moderate and under artificial inoculation, significant differences in aflatoxin and fumonisin accumulation were observed among the families. Research efforts focusing on development of germplasm with resistance to insect injury acting along with resistance against fungal growth and mycotoxin production could prove beneficial.

5.4.4 Association between agronomic traits and mycotoxins

Earlier studies evaluated maize germplasm for drought tolerance and concluded that this character appeared tightly linked with mycotoxins reduction (Henry et al., 2013; Williams and Windham, 2001). In the current study, drought tolerant lines which were not introgressed with aflatoxin and fumonisin genes were included. None of these inbred lines were resistant. They accumulated high levels of both aflatoxin and fumonisin concentration. It is, however, imperative to evaluate sources of aflatoxin and fumonisin contamination resistance identified in this study under drought conditions.

Previous studies have shown that harder (flint) kernels minimise insect damage and impede fungal penetration compared to the soft (dent) kernels (Wit et al., 2011). In the present study, sources of aflatoxin and fumonisin resistance were all flint and this might probably explain why resistant families identified in this study were also flint. The non-introgressed lines were predominantly dent and showed high susceptibility. However a high correlation was observed between *Fusarium* ear rot and fumonisin accumulation compared to *Aspergillus* ear rot and aflatoxin accumulation, suggesting that selecting for the flint trait might prove valuable for fumonisin accumulation resistance. This

requires further investigation because there was limited variation among families for grain texture in the current study. Validation is required.

Earlier studies have suggested that a tight husk and non-upright ear prevent entry of spores and keep the ear dryer, making it a less conducive environment for fungal growth (Warburton and Williams, 2014). This suggestion is also in agreement with results obtained in the present study which showed a positive correlation between husk cover, Aspergillus ear rot, Fusarium ear rot and aflatoxin contamination. However there was no significant correlation between husk cover and fumonisin accumulation among families. This is because artificial inoculation was used. The pathogen was administered into all the maize plants.

A negative correlation was observed between grain moisture content with aflatoxin and fumonisin accumulation suggesting that late maturing families have valuable resistance than early maturing families. This can be explained by the use of tropical resistant inbred lines as sources of resistance in this study which are late maturing. Maturity plays a role in reduction of aflatoxin and fumonisin, but only in some environments. It is therefore not possible to predict in all cases if early or late maturing varieties will have less aflatoxin and fumonisin accumulation, because accumulation depends on the location and the weather (Abbas et al., 2007). Although insect and drought resistance always play a positive role in reducing aflatoxin levels when insects or drought is present, other traits may be less useful, because the ideal maturity to escape *A. flavus* and *F. verticillioides* or mycotoxin production cannot be predicted in advance, a problem further exacerbated by global climate change. Tight husks are not preferred by many South African farmers, and optimal grain hardness is dictated by market pressures and end use, and not only by resistance. Thus, more targeted options for *A. flavus* and *F. verticillioides* resistance are necessary.

5.4.5 Heritability and breeding gains

Heritability estimates of resistance to aflatoxin and fumonisin contamination, low severity of Aspergillus ear rot and Fusarium ear rot were high in the germplasm. These

results indicate that selection of potentially resistant families identified in this study provide a greater chance of attaining genotypes with complete resistance to both mycotoxin accumulation and ear rots severity. Robertson et al. (2006) also found high heritability estimates for Fusarium ear rot and fumonisin contamination of inbred lines on two maize populations. A high genetic gain realized in aflatoxin and fumonisin contamination also suggest a significant breeding progress made in the present study through introgression of resistant genes into adapted lines. Although results predict low genetic gains, further research efforts to improve aflatoxin and fumonisin accumulation through selection would result in genotypes with complete mycotoxin resistance. Strategies that increase genetic variation in the base population would be employed to enhance breeding gains in the program.

5.5 Conclusion

This chapter discussed the findings observed in the current study and correlates them with findings from previous studies on the subject. The overall conclusions for the study, implications and recommendations for future breeding programs are drawn in the next chapter.

CHAPTER SIX

CONCLUSION, IMPLICATIONS AND RECOMMENDATIONS

6.1 Introduction

Previous chapters have highlighted the economic importance of mycotoxin contamination on maize yield losses and impact on human and livestock health. Introgression of resistance genes into adapted germplasm was therefore pursued in order to enhance grain quality by developing aflatoxin and fumonisin resistant maize hybrids. The objective of this chapter is to highlight the major objectives, findings, challenges and implications of the study for breeding. The recommendations for the future studies are also discussed.

6.2 Summary of objectives and research approach

The main aim was to prove the concept that resistant hybrids and new maize inbred lines can be developed by stacking genes for aflatoxin and fumonisin resistance, at the hotspot site in South Africa. The objectives were tackled by firstly carrying out a survey on regional experimental maize hybrids, secondly by crossing aflatoxin and fumonisin resistant inbred lines. This was followed by evaluation of the single, three-way crosses and $S_{2:3}$ families in the greenhouse and field environments. The overview of the findings is presented in the next section.

6.3 Summary of the major findings

Literature confirms the significance of ear rots and mycotoxins but has some gaps that need to be filled, such as knowledge on the natural incidences of the causal pathogens in maize hybrids, and whether or not mycotoxin resistance can be effectively stacked in commercial hybrids.

6.3.1 Survey on natural incidences of ear rots in southern African maize hybrids

The study revealed significant variation among hybrids for ear rot incidence.

- The vast majority of maize hybrids were susceptible to the ear rots diseases caused by *S. maydis, F. verticillioides, A. flavus* and *F. graminearum*. The program should emphasize selection for resistance to these pathogens and their potential mycotoxins, such as diplodiotoxins, fumonisins, aflatoxins, vomitoxins and zearalenone.
- Ear rots caused by *F. verticillioides* and *S. maydis* pose the most significant food and feed safety threat as well as maize production in southern Africa, hence they will be given special attention in the breeding programme.
- Seemingly early maturing hybrids are less susceptible to ear rots infection whereas late maturing hybrids are more susceptible. This is still subject to further investigations.
- The dent texture of maize grain and insect damage has a positive and significant correlation with incidence of ear rot, while flint grain hybrids appears more resistant.
- Consistent with the literature, the weather patterns played a significant role in the development of ear rots, a dry season favours mostly *Fusarium* and *Aspergillus* ear rots, whereas a wet season is more conducive for *Diplodia* ear rots.

6.3.2 Stacking of aflatoxin and fumonisin resistance in single and three-way cross hybrids

The study was successful at stacking the genes in maize hybrids, and also reveals significant variation for resistance.

- The five single cross hybrids: FUMH03, FUMH10, FUMH30, FUMH47, FUMH73 exhibited low grain fumonisin contamination (< 4 ppm) qualifying them as candidates for advancement in the programme.
- Greenhouse screening is capable of predicting hybrid disease resistance in the field, indicating that the shuttling between the greenhouse (winter) and field trials (summer) would be effective for breeding resistant hybrids.
- Three three-way cross hybrids: FUMH/AFTX11, FUMH/AFTX12 and FUMH/AFTX 18 displayed combined resistance for both aflatoxin (< 5 ppb) and fumonisin (< 4 ppm). This indicates that aflatoxin and fumonisin resistance can

be effectively stacked in the end product, which is predominantly grown by the target smallholder farmers in southern Africa.

- There was a significant correlation between aflatoxin and fumonisin contamination suggesting a possible common resistance to aflatoxin and fumonisin contamination
- Ear rot severity was significantly correlated to mycotoxin contamination indicating that resistance to mycotoxin contamination could be predicted on the basis of ear rot severity in the field. However a significant number of hybrids did not follow this trend.
- Aflatoxin and fumonisin contamination resistance is a highly heritable trait suggesting that selection of best performing genotypes could speed up breeding of hybrids for resistance to mycotoxin contamination.
- Results confirmed that stacking of mycotoxin resistance genes in maize hybrids could be achieved.

6.3.3 Introgression of resistance to aflatoxin and fumonisin contamination in germplasm lines

The study demonstrates that introgression of both aflatoxin and fumonisin resistance in adapted lines would be effective.

- Four $S_{2:3}$ families; MTX-36, MTX-67, MTX-79 and MTX-90 displayed combined resistance for aflatoxin and fumonisin contamination, accumulating low mycotoxin content than the resistant controls. New maize inbred lines will be derived from these families.
- High heritability estimates were observed for both mycotoxin contamination resistance, but heritability was lower for the ear rot severity, indicating that strategies that focus on selection for ear rot resistance will be less successful than those that focus on direct selection for mycotoxin contamination resistance.
- The fact that high genetic gains were realised and four families performed better than the resistant controls demonstrates the significant contribution made by this study in breeding for mycotoxin resistance in maize. This should be taken further.

- Non-introgressed genotypes accumulated high levels of mycotoxin contamination than the majority of introgressed families indicating that stacking of resistance genes was successful.
- Flint genotypes have valuable resistance to mycotoxin contamination and ear rots damage compared to dent genotypes; hence selection for the hard grains will be emphasized in the program.
- Insect damage and grain texture coupled with favourable environment contribute significantly to mycotoxin and ear rots diseases in maize. The two traits should be incorporated in the selection index for mycotoxin resistance breeding in the programme.

6.3 Recommendations and the way forward

The study reveals strategies that can be employed in developing high yielding and aflatoxin and fumonisin resistant maize hybrids.

i. Resistance alone does not add economic value to farmers but yield matters most; therefore hybrid seed for yield trials should be produced from the identified resistant genotypes. They should be tested in multi-location environments that include the target smallholder farmers.
ii. The possibility of common host resistance mechanisms of maize, as well as common infection and virulence mechanisms of the fungi, should be investigated.
iii. Drought stress influences fumonisin and aflatoxin contamination, thus evaluation of introgressed drought tolerant germplasm is recommended in future studies.
iv. Use of double haploid technology to speed up inbreeding thereby reducing breeding expenses encountered when evaluating several generations is recommended. However effective inducers which work with the flint resistant backgrounds should be identified.
v. Because of significant environmental effects and genotype × environment interactions, multi-location and multi-year evaluations are critically important in developing maize germplasm lines and populations with adequate levels of stable resistance to aflatoxin and fumonisin accumulation.

vi. Research efforts focusing on development of germplasm with resistance to insect injury acting along with resistance against fungal growth and mycotoxin contamination could also prove beneficial.

vii. Due to the labour intensive and time consuming of artificial inoculation in maize fields, marker assisted selection for the identification of QTL markers for use during early generation selection is recommended.

6.4 Conclusions

The concurrent goals of the present study were to determine the extent of ear rots incidence in southern Africa maize hybrids and introgress aflatoxin and fumonisin contamination resistance into subtropical and temperate maize inbred lines. The ultimate goal is to produce genotypes which have good agronomic characteristics with combined resistance to both aflatoxin and fumonisin contamination. This study confirmed that there is a high incidence of ear rots in the current maize hybrids mainly contributed by *F. verticillioides* and *S. maydis*. The present study has succeeded in introgressing mycotoxin resistance but this is not enough for the product to be accepted by the farmers. Agronomic qualities, such as maturity, good combining ability for yield and good grain characteristics need to be determined in the aflatoxin and fumonisin resistant maize lines before they can be used to breed hybrids to be deployed in farmers' fields. The identified four $S_{2:3}$ families which displayed resistance to both aflatoxin and fumonisin contamination will be advanced in the breeding programme. Overall, the present study succeeded in determining the incidence levels of ear rots in southern Africa maize germplasm and in the introgression of mycotoxin resistance genes. The concept of stacking aflatoxin and fumonisin resistance genes has been adequately proven. Further resources should be mobilised to upscale the effort to breed new varieties which can reach the farmer. Breeding is not complete unless the variety is grown by the target farmers.

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