A HOST-PATHOGEN STUDY OF *FUSARIUM VERTICILLIOIDES* IN RESISTANT AND SUSCEPTIBLE MAIZE INBRED LINES

by

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SUMMARY

Maize (*Zea mays* L.) is an important crop worldwide and forms the staple diet of many African countries including South Africa. Fusarium ear rot (FER) of maize is caused by a fungus, *Fusarium verticillioides,* which also produces the fumonisin mycotoxin group. The consumption of fumonisin contaminated maize grain has been associated with serious human and animal health complications. Several South African maize inbred lines exhibiting resistance to FER and fumonisin contamination have been identified. These locally adapted inbred lines could be used to generate mapping populations to identify QTLs associated with resistance to FER and fumonisin contamination. The corresponding markers could be utilised in breeding programmes through marker-assisted selection to ensure the development of commercial cultivars with resistance to FER and fumonisin contamination.

In this study, resistant and susceptible maize inbred lines were utilised to commence the development of recombinant inbred line (RIL) populations for the mapping and validation of QTLs associated with FER and fumonisin resistance. One F_2 population was phenotypically and genotypically analysed to produce a linkage map for the preliminary identification of QTLs associated with resistance to *F. verticillioides* infection and fumonisin deposition. A potential QTL for resistance to FER was detected and should be validated across several locations and years in the subsequent RIL population. Additionally, potential resistance barriers of maize to infection by *F. verticillioides* were investigated by histological studies. The importance of a closed stylar canal in determining resistance to FER was established for nine South African maize inbred lines by means of scanning electron microscopy (SEM). No significant association was observed between a closed stylar canal and the resistance/susceptible status of maize inbred lines before pollination, while the canals appeared closed in all inbred lines following pollination. The results suggest that the stylar canal architecture is not an essential factor determining resistance to *F. verticillioides* ingress in the maize inbred lines selected for this study. Furthermore, the possibility of resistance to FER and fumonisin contamination being initiated during the seedlings phase of a resistant and susceptible maize inbred line was investigated by means of confocal laser scanning microscopy (CLSM). *Fusarium verticillioides* growth originating from soil-borne or seed-borne contamination was monitored in various above and below soil plant tissues but no significant difference in the colonisation could be determined between resistant and susceptible maize seedlings. No fumonisin was produced regardless of the inoculation method or resistance status of the plant. These results suggests that the resistant and susceptible maize seedlings used in this study may not be resistant to systemic fungal ingress but may resist the deposition of fumonisins. The resistance associated with the resistant inbred line is not mediated during the seedling phase but potentially through structural and biochemical defence mechanisms during later plant developmental stages.

OPSOMMING

Mielies (Zea *mays* L.) is ‘n belangrike graangewas wat wêreldwyd geproduseer word en dien as stapelvoedsel in talle Afrika-lande, insluitend Suid-Afrika. Fusarium kopvrot (FKV) in mielies word veroorsaak deur die swam, *Fusarium verticillioides,* wat ook die fumonisien mikotoksien groepe produseer. Die inname van fumonisien-geïnfekteerde mielies gaan gepaard met ernstige gesondheidsprobleme in mense en diere. Verskeie Suid-Afrikaanse ingeteelde mielielyne, wat weerstandbiedend is teen FKV en fumonisien kontaminasie, is voorheen identifiseer. Hierdie plaaslik-aangepaste teellyne kan gebruik word om kartering populasies te genereer om kwantitatiewe eienskap loci (KEL) te identifiseer wat verband hou met weerstandbiedenheid teen FKV en fumonisien kontaminasie. Die ooreenstemmende merkers kan gebruik word in teelprogramme deur gebruik te maak van merker-geassisteerde seleksie om kommersieële kultivars, wat weerstandbiedend is teenoor FKV en fumonisien kontaminasie, te ontwikkel.

In hierdie studie is weerstandbiedende en vatbare mielie inteellyne gebruik om rekombinante inteellyn (RIL) populasies te begin ontwikkel vir die kartering en validasie van KEL’e geassosieer met FKV en fumonisien weerstandbiedenheid. Een F_2 populasie was fenotipies en genotipies geanaliseer om ‘n koppeling-kaart te verwek vir die voorlopige identifikasie van KEL’e geassosieer met weerstandigheid tot *F. verticillioides* infeksie en fumonisein afsetting. ‘n Potensiële KEL vir weerstandbiedenheid is geïdentifiseer, wat verdere bevestiging in die daaropvolgende RIL populasie in verskeie geografiese areas en oor addisionele seisoene, benodig. Potensiële fisiese versperrings teen *F. verticillioides* tydens mieliesaad infeksie is ook ondersoek met behulp van histologiese studies. Die belangrikheid van ‘n geslote styl-kanaal vir weerstandbiedendheid teenoor FKV is bevestig in nege Suid-Afrikaanse inteellyne deur middel van skandeer elektron mikroskopie (SEM). Geen beduidende verwandskap tussen ‘n geslote styl-kanaal en die weerstandbiedenheid/vatbaarheid van die inteellyne voor bestuiwing is gevind nie, terwyl die kanaal in alle inteellyne gesluit was na bestuiwing. Die resultate dui daarop dat die styl-kanaal argitektuur nie ‘n noodsaaklike faktor is in die bepaling van weestand tot *F. verticillioides* besmetting in die suiwer mielielyne wat geselekteer was in hierdie studie nie. Verder is die moontlikheid dat weestand tot FKV en fumonisien kontaminasie geïnisieer kan word gedurende die saailing-fase ondersoek in beide ‘n weerstandbiedende en vatbare mielie inteellyn met behulp van konfokale laser skandering mikroskopie (CLSM). Die groei van *F. verticillioides* afkomstig vanuit die grond of saad is gemonitor in verskeie bo- en ondergrondse plantweefsels, maar geen beduidende verskille in kolonisasie kon opgespoor word tussen weerstandbiedende en vatbare mielie saailinge nie. Geen fumonisien produksie is waargeneem nie, ongeag die innokulasie metode of weerstand-status van die plant. Hierdie resultate dui daarop dat die weerstandbiedende en vatbare mielie saailinge wat in hierdie studie gebruik is moontlik nie weerstandbiedend is teen sistemiese swaminfeksie nie, maar wel weerstand kan bied tot afsetting van fumonisiene. Die

weerstand geassosieër met die weerstandbiedende inteellyn word nie bemiddel gedurende die saailingfase nie maar waarskynlik deur strukturele en biochemiese verdedigingsmeganismes tydens latere plant ontwikkelings-stadia.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
ANOVA	Analysis of variance
ARC	Agricultural research council
CIMMYT	The international maize and wheat improvement centre
CLSM	Confocal laser scanning microscopy
cM	Centimorgans
DH	Double haploid
DNA	Deoxyribonucleic acid
EC	Electric conductivity
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assays
ES	Embryo sac
F_1	Filial generation 1
F_2	Filial generation 2
F_3	Filial generation 3
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FDA	Food and drug administration
FER	Fusarium ear rot
GC	Gas chromatography
GWAS	Genome-wide association studies
GWS	Genome-wide selection
HPLC	High performance liquid chromatography
IM	Interval mapping
KW	Kruskal-Wallis
LC/MS	Liquid chromatography/mass spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LG	Linkage group
LOD	Log of odds
LRR	Leucine-rich repeat
LSD	Least significant difference
MAS	Marker-assisted selection
MRC	Medical research council
NB	Nucleotide binding
NTC	Non-template control

PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
ppm	Parts per million
PROMEC	Programme on mycotoxins and experimental carcinogenesis
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
rpm	Revolutions per minute
SAGL	South African grain laboratory
SADC	Southern African developing community
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SIM	Simple interval mapping
SNP	Single nucleotide polymorphisms
SSR	Simple sequence repeat
SU	Stellenbosch University
TLC	Thin-layer chromatography
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
WHO	World health organization

CHAPTER 1

Resistance in maize against Fusarium ear rot and fumonisin contamination: A review

INTRODUCTION

Maize is one of the most important staple foods for many people in Africa including most South Africans (Morris, 1998). On average, maize production around the world has increased, despite the decline in the acreage planted. This can largely be attributed to improved maize cultivars and production practices (Hallauer *et al.*, 2010). Maize is the largest field crop produced in Southern Africa with South Africa as the main producer of maize in the Southern African Development Community (SADC) region, producing an average of 10 million tons per annum between 1999 and 2011 (SAGL, 2011).

Sustainable maize production is still threatened by numerous biotic and abiotic stresses. Adverse abiotic stresses such as drought, extreme temperatures (high or low) and low to variable rainfall could all lead to major yield losses (Acquaah, 2007). Biotic stresses of maize are induced by several insect pests, microorganisms as well as nematode infestations (White, 1999). Ear rot diseases caused by *Fusarium* species are the most common of which Fusarium ear rot (FER) of maize is considered amongst the most important diseases of maize (Munkvold, 2003a). Fusarium ear rot of maize is caused by *F. verticillioides* (Sacc) Nirenberg (synonym *F. moniliforme* Sheld.) along with *F. proliferatum* (Matsush.) Nirenberg [teleomorph *G. intermedia* (Kuhlman) Samuels, Nirenberg & Seifert] and *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (White 1999; Leslie and Summerell, 2006).

In South Africa, the maize stalk borer (*Busseola fusca*) is one of the most important insect pests of maize; causing damage to maize ears which can lead to yield losses of up to 75% (Annecke and Moran, 1982). Insect pests of maize may also facilitate pathogen infection by creating a wound as an entry point (Munkvold and Desjardins, 1997). Pre-harvest plant pathogenic fungi, bacteria and viruses cause yield losses by reducing the plants ability to grow normally while post-harvest disease symptoms occurring on commercial products (i.e. ears showing deformity or moulds) reduces the marketability of the crop (White, 1999). Most ear rot fungi produce and contaminate maize grain with mycotoxins (White, 1999). The most common ear rot diseases of maize are: i) *Aspergillus* ear rot, caused by *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare, ii) *Diplodia* ear rot (synonym Stenocarpella ear rot), caused by *Stenocarpella maydis* (Berk.) Sutton [synonym *Diplodia maydis* (Berk.) Sacc. and *D. zeae* (Schwein.) Lév.], iii) FER, iv) *Gibberella* ear rot (synonym Red ear rot) caused by *F. graminearum* Schwabe, v) *Nigrospora* ear rot (synonym

Cob rot) caused by *Nigrospora oryzae* (Berk. & Broome) Petch and vi) *Penicillium* ear rot (synonym Blue Eye rot) caused by *Penicillium oxalicum* Currie & Thom.

The fumonisin mycotoxin group is predominately produced by *F. verticillioides* and *F. proliferatum* and several historical mycotoxicosis outbreaks in humans and livestock (Marasas *et al*., 1981; Marasas *et al*., 1988; Harrison *et al*., 1990; Kellerman *et al*., 1990; Sydenham *et al*., 1990; Colvin and Harrison, 1992; Rheeder *et al*., 1992; Nelson, 1993; Morgavi and Riley, 2007) has urged some countries to apply regulatory measures in maize grain intended for human consumption (Rheeder *et al*., 2009). In South Africa, no regulation for fumonisin levels in maize grain is employed despite maize being an important food and feed crop. One of the effective means to control FER and fumonisin contamination in maize grain is by utilising maize cultivars with resistance to the disease and toxin accumulation (Parsons and Munkvold, 2010).

The objective of this review is to firstly provide a comprehensive overview of FER and fumonisin contamination of maize including current management strategies. Furthermore, the resistance mechanisms to FER and fumonisin contamination will be discussed with particular focus on the efforts toward developing maize cultivars with enhanced resistance through conventional and molecular plant breeding strategies.

THE ORIGIN OF MAIZE

Maize (*Zea mays* L.), a domesticated grain crop thought to have originated in Mexico or Central America (Acquaah, 2007), is a monocotyledonous plant species belonging to the grass family (Poaceae synonym Gramineae). It was cultivated by the Mayan and Aztec Indian civilizations as early as 6000 BC (Acquaah, 2007). Today, seven groups of maize [Dent (*Z. mays indentata*), Flint (*Z. mays indurate*), Flour (*Z. mays amylacea*), Popcorn (*Z. mays everta*), Sweet corn (*Z. mays saccharata*), Waxy and Pod corn (*Z. mays tunicate*)] exist, with five of these groups (Dent, Flint, flour, Sweet, and Waxy corns) being produced commercially around the world (Acquaah, 2007).

Maize is generally considered a domesticated version of teosinte [*Z. mexicana* (Schrad.) Kuntze], a wild grass that still grows in Mexico and Guatemala (Acquaah, 2007). The most notable morphological difference between modern maize and teosinte is that modern maize grows short lateral branches tipped by ears, while teosinte grows long lateral branches tipped by tassels (Doebley *et al*., 1995) (Fig. 1). The tassel of maize plants usually matures before the ears and the pollen is primarily wind dispersed during warm dry mornings resulting in maize being predominately cross-pollinated in nature (Acquaah, 2007).

In 1909, George Shull made the first clear scientific-based proposal that maize offspring, produced from crosses between two inbred lines (a single-cross hybrid), exhibited uniform and

higher yielding cultivars (Acquaah, 2007). Single-cross hybrids have been observed to produce larger number and heavier weight kernels with increased resistant to insects and diseases, both of which result in higher yield (Jugenheimer, 1976). Weak inbred lines, however, produced such few seed (i.e. inbreeding depression) that single-cross hybrid breeding became impractical and uneconomical to implement in breeding programmes (Acquaah, 2007; Hallauer *et al*., 2010).

The employment of double-cross hybrids easily overcame the problems associated with weak inbred lines. However, the significance in genetic variation observed in such hybrids made it unclear whether the offspring would express favourable quantitative traits to the same degree each time (Hallauer *et al*., 2010). Presently, single-cross hybrids are planted in almost all maize-production areas of the world (Acquaah, 2007). This is only possible due to improved technologies in farming (e.g. use of more powerful tractors and better implements), improved agricultural practises and a better understanding of the maize plant and the genetics thereof (e.g. better selection schemes and implementation of molecular and mutation plant breeding).

FUSARIUM EAR ROT OF MAIZE

Fusarium ear rot of maize is caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (White, 1999; Leslie and Summerell, 2006). The *Fusarium* species causing FER can be morphologically distinguished from one another with the most distinguishable characteristic being the mode by which they produce their microconidia, when grown on carnation leaf agar. The fungi, *F. verticillioides* and *F. proliferatum* both produce microconidial chains and false heads of microconidia, while *F. subglutinans* does not produce chains of microconidia and form false heads only. The main difference between *F. verticillioides* and *F. proliferatum* is that *F. verticillioides* produces microconidia from monophialides, while *F. proliferatum* form mono- and polyphialides. Furthermore, *F. proliferatum* usually produces shorter microconidial chains than *F. verticillioides* (Leslie and Summerell, 2006). Of these *Fusarium* species, *F. verticillioides* is most commonly associated with maize worldwide as well as in South Africa (Boutigny *et al*., 2012).

Fusarium ear rot symptoms on maize ears/kernels may be characterised by: i) whitish and/or pink to lavender pigmented fungal growth on individual or clusters of maize kernels (White, 1999) and ii) starburst symptoms seen as white streaks radiating from the silk scars of individual maize kernels (Duncan and Howard, 2010). The fungus can also occur asymptomatically in the maize grain (Munkvold *et al.*, 1997) and can infect maize ears via natural openings and wounds or via systemic colonisation of the maize plant (Munkvold and Desjardins, 1997) (Fig. 2). It appears as though the *F. veriticillioides* fungus can be transmitted from infected seeds to subsequently causing asymptomatic infected ears at maturity (Munkvold and Carlton, 1997). These findings were revealed by tracking strains of *F. verticillioides* growing in maize ears from inoculated seeds, by means of vegetative compatibility group analysis (Munkvold and Carlton, 1997). Additionally,

fluorescently labelled *F. verticillioides* fungal isolates have been tracked in maize seedlings using fluorescence microscopy (Oren *et al*., 2003, Wu *et al.*, 2011; Wu *et al.*, 2013). Wu *et al.* (2013) demonstrated that resistance to the disease and toxin contamination could be mediated during the seedling phase of maize plants. Infection via the silk channel and through wounded kernels, however, appears to be the most important infection pathway causing FER (Flett and Van Rensburg, 1992; Munkvold and Carlton, 1997; Duncan and Howard, 2010). Systemic colonization of the maize plant could be an important means of survival for the fungi associated with FER as the causal fungi can overwinter in the soil and survive in buried maize debris for up to 630 days (Cotten and Munkvold, 1998). The spores found in the soil and maize debris are dispersed by the wind, rain-splashed and spread by insects to cause FER and systemic infection the following maize season (Ooka and Kommendahl, 1977; Munkvold and Desjardins, 1997).

Fumonisin contamination

The most alarming aspect of *F. verticillioides* infection is the potential contamination of maize grain with the fumonisin B mycotoxin group. Fumonisins are a group of structurally-related metabolites produced by *F. verticillioides*. Of the known fumonisin metabolites, fumonisin B_1, B_2 and B_3 are considered the most important (WHO, 2000). Several historical outbreaks of mycotoxicosis have led to reduced performance and economic losses to livestock farmers (Morgavi and Riley, 2007). Researchers have by means of historical outbreaks and controlled experiments been able to link fumonisin-contaminated feed to diseases in several types of farm animals. Horses developed equine leukoencephalomalacia from feed containing a relatively low fumonisin content (Kellerman *et al*., 1990; Morgavi and Riley, 2007), pigs suffered porcine pulmonary edema, hydrothorax, liver problems, weight loss and eventually died (Harrison *et al*., 1990; Colvin and Harrison, 1992) while poultry diseases associated with diarrhea, weight loss and increased liver weight (Nelson, 1993; Morgavi and Riley, 2007) have been documented.

Fumonisin mycotoxicosis outbreaks have also shown to occur in humans as oesophageal cancer (Marasas *et al*., 1981; Marasas *et al*., 1988, Sydenham *et al*., 1990; Rheeder *et al*., 1992) and neural tube defects in new born babies (Missmer *et al*., 2006). Following extensive studies of mycotoxicoses, the U.S. food and drug administration (FDA) proposed maximum fumonisin limits in maize grain considered safe for animals and human consumption. The recommended maximum limits ranged between 5 and 100 ppm (FB_1 + FB_2 + FB_3), depending on the animal species. The lowest levels were applied to rabbit and horse feeds due to their greater sensitivity to fumonisin while maximum levels of fumonisin contamination in fresh and processed maize intended for human consumption ranges between 2 and 4 ppm depending on the product.

Only a few other countries have officially adopted regulations for fumonisin levels in food (Rheeder *et al*., 2009). In 2003, four out of six countries regulating fumonisin in human foodstuffs

applied a maximum limit of 1 ppm (Rheeder *et al*., 2009). Rheeder *et al*. (2009) further suggested that a maximum limit of 1 ppm would still put human health at risk because of the consumption of maize as a staple food in South Africa. It is noteworthy that natural fumonisin levels of unprocessed maize grown in the Transkei regions were recorded by Sydenham *et al*. (1990) and Rheeder *et al*. (1992), with some samples well above the maximum proposed limits. In addition, fumonisins can also be produced in asymptomatic maize ears (Rheeder *et al*., 1992).

MANAGEMENT OF FER AND FUMONISIN CONTAMINATION

The management practices to reduce FER and fumonisin contamination of maize grain can be categorised into pre-harvest and post-harvest approaches.

Pre-harvest approaches

Pre-harvest approaches are agricultural practices that can be applied before harvest to reduce FER and fumonisin contamination. Growing an appropriate maize hybrid and using an effective insecticide are considered amongst the most important ways to reduce both FER and fumonisin contamination in fresh maize produce (Parsons and Munkvold, 2010). In addition, maize genetically modified to be resistant to European corn borer infestation has shown to express lower disease severity and fumonisin levels than non-transgenic isohybrids, under similar levels of European corn borer infestations (Munkvold *et al*., 1997; Papst *et al*., 2005). However, these practises are not always enough to keep the fumonisin contaminants below the recommended limits.

Increased FER and fumonisin contamination may occur in maize plants grown under stressful conditions caused by adverse climatic factors and competition between plants for water and/or nutrients (Miller, 2001; Blandino *et al*., 2009; Abbas *et al*., 2012). Warm, dry conditions are associated with FER and fumonisin contaminated maize grain and irrigation could be used to reduce these levels (Miller, 2001; Abbas *et al*., 2012). The planting of maize plants at lower densities has also shown to reduce fumonisin contamination slightly (Blandino *et al*., 2009). Furthermore, the interaction of *F. verticillioides* with other non-fumonisin-producing pathogenic fungi may influence fumonisin production although the mechanism by which this occurs is poorly understood (Miller, 2001; Abbas *et al*., 2012). Studies showed that maize ears inoculated with non-fumonisin-producing fungal species such as *F. graminearum*, *F. subglutinans* (Miller, 2001) and *A. flavus* (Abbas *et al*., 2012) contained higher fumonisin levels. This suggests that maize ears infected with other ear rot pathogens may pre-dispose ears to *F. verticillioides* infection and also fumonisin contamination (Miller, 2001). Fungal populations, including *F. verticillioides*, can be managed using tillage, crop rotation and/or burning of the crops after harvest (Reynoso *et al*., 2006; Venturini *et al*., 2011).

Fumonisin contamination and FER can further be managed by harvesting soon after physiological maturity (Bush *et al*., 2004; Picot *et al*., 2011; Cao *et al*., 2013). Bush *et al*. (2004) demonstrated that FER and fumonisin contamination developed as kernels neared physiological maturity. Later harvesting causes the grain moisture to decrease, thereby making it prone to mechanical damage (Bruns and Abbas, 2004). Damage to maize ears should be avoided during harvesting processes since this damage could lead to higher FER and fumonisin levels (Fandohan *et al*., 2006).

Post-harvest approaches

Following harvest, fewer management strategies are available to limit FER and fumonisin contamination in maize. Maize grain should be stored in clean, dry and cool facilities, preferably 1-4ºC, while grain used for processing can be dried artificially at 50-82ºC before storage to reduce potential fumonisin contamination (Munkvold, 2003b). Removal of visibly diseased and broken kernels before processing has been shown to reduce the fumonisin levels in the product by 26-69% (Sydenham *et al*., 1994). Additional practices, such as exposure to heat and chemical processes, may also reduce fumonisin in maize grain (Humpf and Voss, 2004).

Although a number of individual agricultural practises and post-harvest approaches could significantly reduce fumonisin levels, a combination of these practices as part of an integrated management system could be employed to sustainably reduce fumonisin levels in maize grain.

RESISTANCE TO FER AND FUMONISIN CONTAMINATION

The employment of maize cultivars with resistance to FER and fumonisin contamination is considered to be one of the most important management strategies (Parsons and Munkvold, 2010) and is considered to be an environmentally friendly and potentially economically feasible approach to reduce the disease and toxin (Small *et al*., 2012). All plants provide resistance to pathogens by conserved structural and biochemical defence mechanisms. A brief overview of some specific resistance mechanisms of maize to *F. verticillioides* and fumonisin contamination are discussed in this section.

Structural defence mechanisms

The maize plant has a number of structural barriers and morphological characteristics that potentially aid in preventing the infection and spread of pathogenic microorganisms, including *F. verticillioides*. The morphological characteristics of the maize ear are particularly important as most fungal ear rot infections originate from infection of the maize ear (Munkvold and Carlton, 1997). Maize varieties with tight husks are typically more resistant to ear rot infections than varieties with loose husks or open ears (Warfield and Davis, 1996; Parson and Munkvold, 2010). The husk is deemed to protect the inner ear from the environmental factors such as weather, insect pests and

diseases (Giles and Ashman, 1971; Wiseman *et al*., 1977; Rector *et al*., 2002). Delayed silk senescence following pollination has also been implicated in providing resistance as silks that remained green longer after pollination was associated with reduced kernel infection by *F. verticillioides* (Headrick and Pataky, 1991).

The kernel morphology has also been reported to play an important role in resistance. A thick pericarp at the early dent stage (Scott and King, 1984; Hoenisch and Davis, 1994), the presence of placento-charlazal (i.e. the black layer of the tip cap) and other properties of the tip cap have been suggested to play an important role in resistance to FER (Headrick and Pataky, 1991). However, in contrast to Hoenish and Davis (1994) and Scott and King (1984), no correlation between pericarp thickness and FER was observed by Ivić *et al*. (2008). The study by Ivić *et al*. (2008) also suggested that the contrast in results may have had to do with the type of insect pests present since damage caused by insects could increase the severity of FER symptoms. A thick pericarp was therefore suggested as a resistance trait against feeding insect pests (Ivić *et al*., 2008). The thin pericarp of sweet maize varieties, a trait intentionally bred for to improve the texture, is considered as one of the most important reasons the variety is highly susceptible to ear rot diseases (Mesterházy *et al*., 2012).

The architecture of the stylar canal (Fig. 3) has also been proposed as a potential resistance barrier to the infection by *F. verticillioides*. Duncan and Howard (2010), used a resistant and a susceptible maize inbred line to demonstrate the potential important role that a closed stylar canal has for resistance to FER. Although structural barriers and morphological characteristics may play an important role in contributing to the resistance observed, other resistance mechanisms such as biochemical defence mechanisms are considered of greater importance in determining resistance to FER and fumonisin accumulation.

Biochemical defence mechanisms

Plants induce resistance by producing chemicals that interfere with pathogenesis. Elicitors of plant pathogens are known to trigger resistance responses of their host (Chisholm *et al*., 2006). General elicitors or pathogen associated molecular patterns (PAMP) trigger defence responses in host and non-host plants, while race-specific elicitors or effectors, produced by specific pathogen races, may induce resistance responses in resistant plant varieties (i.e. gene-for-gene interaction).

Pathogens may overcome plant resistance responses by producing molecules that enable avoidance or suppression of PAMP-triggered immunity (Chisholm *et al*., 2006), while effector molecules interfere with the defence reaction of the plant so that it can gain access. Still, resistant plants encode several classes of receptor-like proteins that trigger effector-triggered immune responses (Chisholm *et al*., 2006; Sacco and Moffett, 2009). The largest class of resistance genes

are represented by a family of proteins containing a nucleotide binding (NB) site and a leucine-rich repeat (LRR) domain (Chisholm *et al*., 2006). Limited research is available of specific biochemical resistance mechanisms in maize against FER fungal invasion although Rxo1, Rp2 and Rp3 resistance receptor proteins in maize are examples of NB-LRR proteins. The Rxo1 protein confers resistance to invasion of *Xanthomonas oryzea* and *Burkholderia andropogonis*, while the Rp2 and Rp3 receptor proteins of maize confer resistance to the *Puccinia sorghi* fungus (Sacco and Moffett, 2009). A number of defense-related genes exhibiting differential gene expression in a resistant inbred line are thought to form part of the extensive biochemical defence launched against *F. verticillioides* (Lanubile *et al*., 2010).

BREEDING FOR IMPROVED RESISTANCE TO FER AND FUMONISIN CONTAMINATION

Resistance to FER and fumonisin contamination is complex and no breeding programmes for resistance exist in South Africa. Breeding for resistance to FER and fumonisin contamination relies firstly on the identification of potential sources of resistance which is evaluated by screening for the resistance under field conditions employing artificial inoculation.

Screening for resistance

The measure of resistance/susceptibility to disease is usually a comparison of disease severity between many plants treated more or less the same (Singh and Singh, 2005; Eller *et al*., 2008). The maize ears are generally inoculated to provide a more uniform distribution of inoculum among plants (Eller *et al*., 2008) and a number of inoculation techniques have been investigated, of which the silk inoculation method is the least invasive (Eller *et al*., 2008; Mesterházy *et al*., 2012). The disease symptoms of FER can be scored visually according to Clements *et al*. (2004) and Reid *et al*. (1994). However, the presence of the fungus and fumonisin contamination is not always correlated with FER symptoms (Small, 2010). The presence of the fungus can be quantified using species-specific primers in quantitative polymerase chain reactions (qPCRs) (Nicolaisen *et al.*, 2009; Boutigny *et al.*, 2012). This method of detection is sensitive and highly reproducible. Theoretically, as little as one cell equivalent nucleic acid can be quantified using qPCR (Heid *et al*., 1996).

Field evaluations can be improved by determining the quantity of fumonisins in the maize grain. Fumonisin contamination can be determined using several analytical techniques such as thin-layer chromatography, gas chromatography (GC), capillary GC, GC-mass spectrometry, liquid chromatography/mass spectrometry (LC/MS), high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assays (ELISA) (Duncan *et al*., 1998; Krska *et al*., 2008). The methods currently used to quantify fumonisin is ELISA (Eller *et al*., 2008), or immunoaffinity clean-up with HPLC, or GC in combination with a variety of detectors such as fluorescence detection with a pre- or post-column derivatisation step, UV detection, flame ionisation detection, electron capture

detection or mass spectrometry (Krska *et al*., 2008). The HPLC and GC methods require labour intensive sample preparation protocols (Krska *et al*., 2008), while the ELISA method of detection is commonly used to quantify fumonisin contamination more efficiently but with lower sensitivity (Krska *et al*., 2008; Eller *et al*., 2008).

Sources of resistance

Although little evidence of complete resistance exists for FER and fumonisin contamination (Robertson *et al*., 2006), natural sources of resistance have been identified and appear to correspond well with several dominant resistance genes (Nankam and Pataky, 1996; Clements *et al*., 2004; Ding *et al*., 2008) with moderate to high heritability (Nankam and Pataky, 1996; Robertson *et al*., 2006; Ding *et al*., 2008). Several African maize inbred lines have also been identified with potential resistance to FER and/or fumonisin contamination (Afolabi *et al*., 2007; Small *et al*., 2012; Mouton, 2014).

Molecular plant breeding techniques are being adopted to eliminate some of the time-consuming and expensive screening procedures involved in plant breeding (Holland, 2004; Collard *et al*., 2005). Molecular plant breeders utilise the information of the plant's genome to select breeding material using DNA markers. An already well-known approach, marker-assisted selection (MAS) uses DNA markers that are tightly linked to one to three quantitative traits of interest (i.e. resistance in maize to FER and fumonisin contamination) (Collard *et al*., 2005; Eller *et al*., 2008; Henry, 2013). Genome-wide selection (GWS), an alternative to MAS, has recently emerged to improve the efficiency and accuracy of selecting germplasms for specific quantitative or very complex traits (Henry, 2013; Zila *et al*., 2013).

QTL MAPPING

A number of QTL studies on maize for resistance to FER and fumonisin contamination has been conducted (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Ding *et al*., 2008; Li *et al*., 2011; Chen *et al*., 2012; Zila *et al*., 2013). QTL maps for resistance to FER and fumonisin contamination have been created by associating the phenotypic data such as visual rating of FER disease symptoms and fumonisin contamination to a genotype using DNA markers and basic genetic concepts (Brown, 2007). Gathering information about the available germplasm with regard to its resistance status is considered the earliest step of QTL mapping (Collard *et al*., 2005; Wu *et al*., 2007).

Mapping populations

Homogenous maize inbred lines of diverse genetic backgrounds with contrasting resistance statuses to FER and fumonisin contamination are crossed and used to produce genetic models or mapping populations (Collard *et al*., 2005; Wu *et al*., 2007). There are several types of mapping

populations, but the F_2 and backcross populations are the two most commonly used in preliminary QTL mapping studies since they require the shortest time to produce and provide simple powerful genetic models (Collard *et al*., 2005; Wu *et al*., 2007). The most powerful genetic models are produced by single-seed decent recombinant inbred lines (RIL) because these homogenous lines can be multiplied easily without changing their genomes (Collard *et al*., 2005). This enables trials to be conducted across several locations and years and between different laboratories to confirm QTL mapping results (Collard *et al*., 2005). The time required to produce RILs is a major drawback, but double haploid (DH) populations may be used to speed up the process in developing homogenous inbred lines. Recombinant inbred lines and DH mapping populations are considered useful to verify preliminary QTL mapping studies (Collard *et al*., 2005).

DNA markers

Various DNA marker technologies are available. The marker type can be selected depending on costs, efficiency, robustness, descriptiveness and the availability of appropriate facilities. Restriction fragment length polymorphism (RFLP) was the first DNA-based molecular marker to become widely used in mapping and population studies (Brown, 2007; Wu *et al*., 2007; Henry, 2013). During the 1980s, RFLPs were used to generate QTL maps because of their high reproducibility and co-dominant nature (Lander and Botstein, 1989). The major drawbacks of this technology include the high concentration of DNA required for detection, expenses associated with detection, as well as the labour-intensive and time-consuming nature of RFLP technology (Wu *et al*., 2007; Kesawat and Das, 2009; Mammadov *et al*., 2012).

During the 1990s, PCR-based markers overshadowed RFLP marker technology (Henry, 2013). PCR-based markers provide rapid detection of polymorphisms at a higher throughput and were considered more reliable with only a small quantity of initial DNA required (Mammadov *et al*., 2012). The most commonly used PCR-based markers in plant breeding are amplified fragment length polymorphisms (AFLPs), microsatellites or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) (Henry, 2013). The AFLP markers have great reproducibility, high level of polymorphism and represent multiple loci (Collard *et al*., 2005). Due to its high effective multiplex ratio (Varshney *et al*., 2007), AFLP markers are considered most informative for fingerprinting and genetic diversity studies. Yet, widespread application of these markers is limited due to lengthy laborious procedures and their inability to be automated (Mammadov *et al*., 2012).

Microsatellite markers, also known as simple sequence repeats (SSRs), became the marker system of choice soon after development (Mammadov *et al*., 2012). SSR loci are short tandem repeated sequences of mono-, di-, tri-, tetra- or penta-nucleotides (Powell *et al*., 1996) and are amplified by using two unique primers composed of short lengths of nucleotides that flank the SSR locus or nucleotide repeats (Powell *et al*., 1996). The amplified SSR regions can be viewed

by gel electrophoresis and staining, but has been automated to allow for higher throughput and automatic data generation. This has been achieved through the employment of multiple fluorophore-labelled primers in single PCRs (i.e. multiplex PCRs). The DNA fragments produced by these primers can be viewed using capillary electrophoresis systems (Mammadov *et al*., 2012; Henry, 2013). They are highly polymorphic and may have up to 70 or 80 alleles at a single SSR locus (Wu *et al*., 2007). The main drawback of this technology is the formation of stutter bands which can make it difficult to interpret the band profiles (Kesawat and Das, 2009). Nevertheless, several QTL mapping studies have been performed using SSRs for resistance to FER and fumonisin contamination (Robertson-Hoyt *et al*., 2006; Ding *et al*., 2008; Li *et al*., 2011; Chen *et al*., 2012).

Single nucleotide polymorphisms (SNPs) are becoming extremely useful in QTL mapping studies following the large-scale availability of sequence information and development of high throughput technologies for SNP genotyping (Jones *et al*., 2007; Takagi *et al.*, 2013). SNP markers are extremely abundant and evenly distributed in genomes, allowing for high resolution maps compared to the other marker systems (Jones *et al*., 2007). Although SNPs are less polymorphic than SSR markers (Jones *et al*., 2007; Varshney *et al*., 2007), this drawback is compensated for by the abundance of SNPs and its amenability to high- and ultra-high-throughput automation (Mammadov *et al*., 2012; Takagi *et al.*, 2013). These characteristics make SNPs suitable for genome-wide association studies (GWAS). Recently, genes associated with FER resistance in maize were studied and identified using SNPs in a GWAS (Zila *et al*., 2013). Future studies will be geared towards increasing the marker density in larger association panels to identify more SNPs that could explain the overall resistance to FER (Zila *et al*., 2013).

Linkage analysis

Linkage analysis is based on the marker order (i.e. linkage) as predicted when using the assumption that conserved Mendelian segregation ratios hold for the individuals of the mapping populations (Collard *et al*., 2005; Brown, 2007; Wu *et al*., 2007). By saturating the maize genome of the genetic model with polymorphic markers, distances between marker loci and the order can be predicted using appropriate statistical software that determine the frequency with which markers are unlinked by crossovers (Collard *et al*., 2005; Wu *et al*., 2007). The frequencies that loci are unlinked by crossovers are assumed to be directly proportional to how far apart they are on their chromosome (Brown, 2007) and a mathematical function converts the recombination fraction between loci to genetic distances between them (Collard *et al*., 2005; Wu *et al*., 2007). Several map functions have been recorded with the Morgan map function being the simplest (Wu *et al*., 2007) while the most commonly used map function is the Kosambi map function (Kosambi, 1944; Collard *et al*., 2005). An acceptable marker density is directly dependant on the QTL analysis method employed.

QTL analysis

QTL analyses can be carried out using three main methods, namely single-marker analysis for non-parametric testing using a Kruskal-Wallis (Van Ooijen, 2009) or a Wilcoxon rank sum test (Kruglyak and Lander, 1995), simple interval mapping (SIM) using a single-QTL model (Lander and Botstein, 1989), and composite interval mapping using a multiple-QTL model (Van Ooijen and Jansen, 1994). Single-marker analysis, also known as 'single-point analysis,' is the simplest method for detecting QTLs associated with single markers and analyses the phenotypic data one marker at a time (Tanksley, 1993; Collard *et al*., 2005). This method was previously used because it did not require a complete linkage map (Tanksley, 1993). However, the accuracy of QTL mapping using this method alone depends on a large number of markers to cover the whole genome with intervals of less than 15 cM (Tanksley, 1993). Today, single-marker analysis can be used together with SIM, and is especially recommended when the phenotypic data is not normally distributed (Kruglyak and Lander, 1995). Simple interval mapping was developed by Lander and Botstein (1989) to tolerate lower mapping densities with sets of linked markers analysed simultaneously with regard to their effects on quantitative traits (Tanksley, 1993). This allows for accurate analysis when linked markers are between 20 and 35 cM apart (Tanksley, 1993). Composite interval mapping is an extension of SIM and has become popular as it further improves the accuracy of QTL mapping using multiple QTL models (Jansen, 1993; Van Ooijen and Jansen, 1994; Collard *et al*., 2005).

QTL mapping for Fusarium ear rot and fumonisin resistance

Resistance to FER has been mapped to additive (Pérez-Brito *et al.*, 2001; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012; Zila *et al.*, 2013) and dominant genes (Pérez-Brito *et al.*, 2001; Chen *et al.*, 2012) of variable heritability. Pérez-Brito *et al.* (2001) mapped several QTLs in two F_3 mapping populations. Three of the QTLs were stable across four environments and common in both populations with one on chromosome 3 and two on chromosome 6. The area on chromosome 3 also corresponded to the most effective and stable QTL for FER resistance of Ding *et al*. (2008). This position is also known to contain a tight cluster of resistance factors and has been associated with resistance to the European corn borer, Fusarium stalk rot, common rust, maize mosaic virus and wheat streak mosaic virus (McMullen and Simcox, 1995).

Another QTL region of interest was identified by Li *et al.* (2011) and Chen *et al.* (2012). One QTL on chromosome 4 with stable and large effects was observed (Li *et al*., 2011; Chen *et al*., 2012) and the resistance effect was confirmed using near-isogenic lines (NIL) carrying the region (Chen *et al*., 2012). Significant resistance effects were observed with 33.7-35.2% increased resistance when the region was homozygous and 17.8–26.5% when heterozygous (Chen *et al*., 2012). In these studies, FER-resistance was tested with no fumonisin screenings.

Resistance to FER and fumonisin contamination was mapped by Robertson-Hoyt *et al*. (2006) in two populations in well replicated studies. Among the QTLs identified across all environments, three QTLs for FER-resistance (on chromosomes 2, 4 and 5) and two for fumonisin-resistance (on chromosome 4 and 5) were consistent while the two QTLs on chromosomes 4 and 5 were also consistently associated with resistance to both FER and fumonisin contamination. This suggests that resistance to FER and/or fumonisin contamination could be conferred individually or together.

MOLECULAR PLANT BREEDING

Molecular plant breeding is a potentially valuable approach to develop maize varieties with resistance to FER and fumonisin contamination, especially considering the difficulties associated with the visual disease assessments and that the disease is highly influenced by environmental conditions (Roberson-Hoyt *et al.*, 2006). The presence or absence of one to three DNA markers tightly linked to quantitative traits of interest can be used to select suitable germplasm in breeding programmes with the process being described as MAS. Genome-wide selection, an advancement of MAS, could be used to select germplasm based on many genes/QTLs responsible for the specific phenotypic traits of interest (Henry, 2013).

Since the selection criterion is based on the plant's most fundamental property, DNA, selection is unbiased and can be performed during the seedling phase. The use of MAS is not influenced by environmental conditions thus in and/or out-of-season glasshouse trials may be utilised for growing plants for selection (Collard *et al*., 2005). Therefore, exploiting out-of-season trials and performing MAS on seedlings should make molecular plant breeding more efficient when compared to traditional plant breeding practises used to improve plant varieties.

The effectiveness of MAS and GWS for quantitative traits depends on a number of factors. Environmental differences during disease screening, experimental error in pheno- and genotyping processes, and the presence of a large distance between marker and gene of interest have large adverse effects on the accuracy of QTL mapping (Collard *et al*., 2005). Due to these influential factors, QTL mapping studies should be confirmed or verified before implementing the QTL markers in breeding programmes. Few QTLs for resistance to FER and fumonisin contamination have been validated and the potential use of MAS and GWS for resistance to FER and fumonisin contamination is still largely unknown as further research is required.

CONCLUSION

Maize is one of the most important crops produced in South Africa and serves as a staple food to many people living in Africa. Numerous factors play an important role in the successful production

of maize in South Africa including the management of abiotic and biotic factors such as *F. verticillioides* and the fumonisins produced by this pathogen. Enhancing host resistance and the planting of resistant maize cultivars as part of an integrated management system is considered one of the best management strategies for both pathogen infection and mycotoxin contamination. Following the identification of useful sources of resistance in locally adapted breeding lines, the inheritance and subsequent incorporation of resistance into high-yielding, agronomically superior female lines can be studied by means of QTL mapping. The development of markers associated with resistance to FER and fumonisin could be an invaluable tool for plant breeders applying marker-assisted selection in their breeding programmes.

The breeding of improved ear rot and mycotoxin resistant maize cultivars has been slow due to the genetic x environment interactions observed. QTL studies for resistance to FER has been performed in the USA, Mexico and Asia (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al.*, 2006; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012) but the disease and toxin is highly influenced by environmental conditions and may not always be transferable to maize varieties adapted to South African climates (Shelby *et al*., 1994), Thus, QTL studies for resistance to FER and fumonisin contamination in locally adapted maize varieties was initiated in South Africa and this work is presented in **Chapter 2**.

However, the use of molecular marker technology combined with classical plant breeding techniques still provides the most promising endeavour to accelerate the development of maize resistant to fungal and mycotoxin contamination. Understanding the hosts' resistance to the *F. verticillioides* fungus could further improve breeding strategies for resistance to the disease and toxin. A closed stylar canal was proposed to be an important resistance trait of maize to FER in a resistant and a susceptible maize inbred line (Duncan and Howard, 2010) and resistance to FER and fumonisin contamination has been suggested to also occur during the early developmental stages of the maize plant (Wu *et al.*, 2013). The importance of a closed stylar canal in mediating resistance to FER was evaluated using scanning electron microscopy and the colonisation and fumonisin deposition monitored in resistant and susceptible maize seedlings by means of a fluorescent labelled *F. verticillioides* isolate using confocal laser scanning microscopy. This work is presented in **Chapter 3**.

The development of markers for resistance to FER and fumonisin contamination through QTL mapping studies and the establishment of other important FER and fumonisin resistance traits could facilitate the development of maize cultivars with enhanced resistance to the disease and toxin. The resistance is expected to improve the quality of the maize grain with respect to fumonisin contamination which could especially benefit South African communities that consume larger proportions of maize grain.

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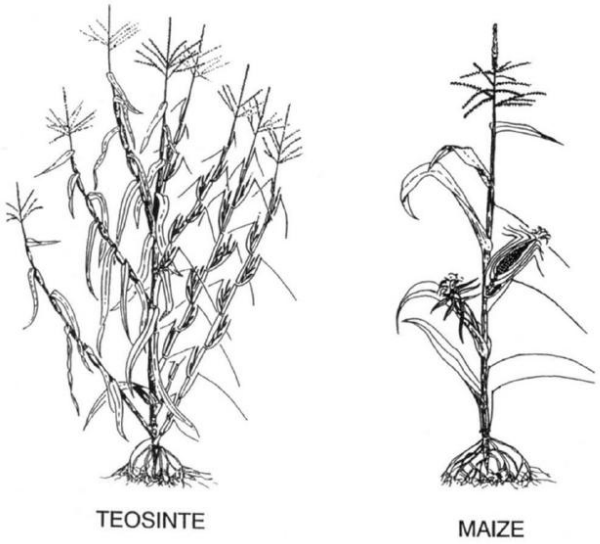

Figure 1. The morphological difference between the teosinte and modern maize. Adapted from Iltis (1983) and Doebley *et al*. (1990) by Doebley *et al*. (1995).

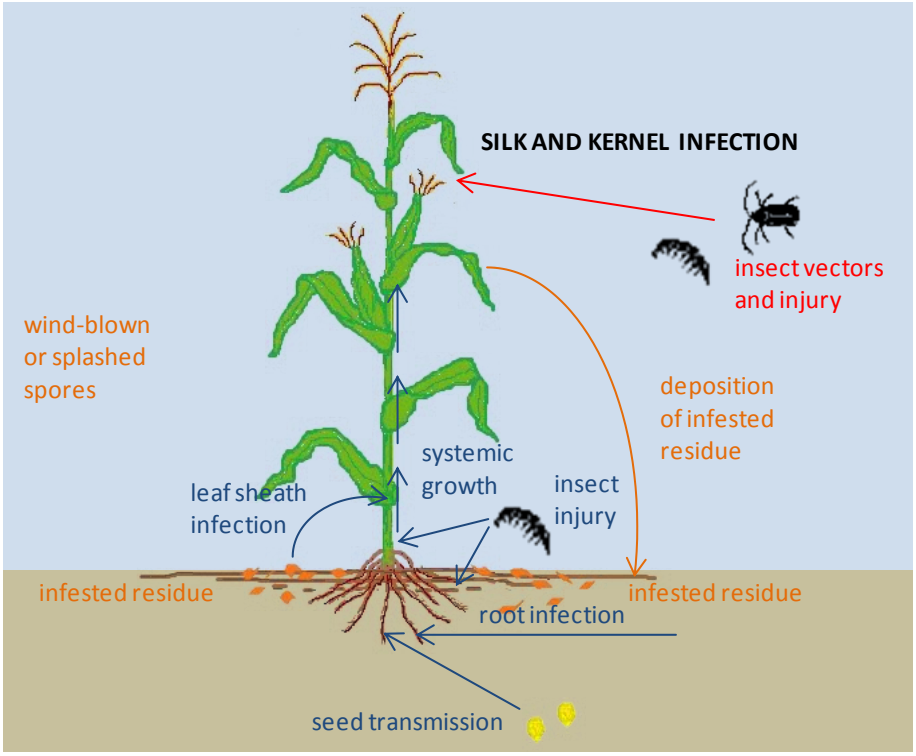

Figure 2. The disease cycle of *Fusarium verticillioides* (reported as *F. moniliforme*) on maize illustrating the various infection pathways. Modified from Munkvold and Desjardins (1997) by H.J. Vermeulen.

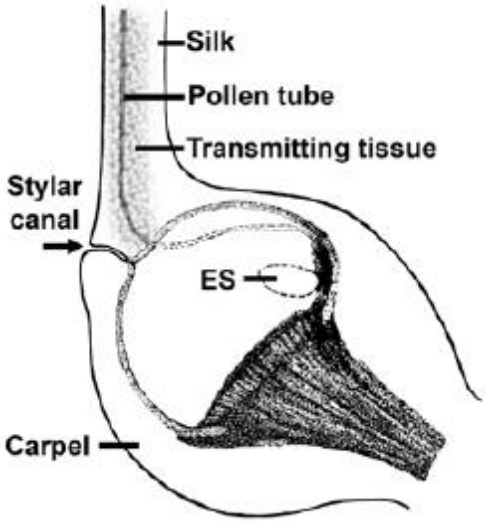

Figure 3. Diagram of a cross-section through a developing maize kernel showing the union of the silk and carpel to form the stylar canal. The pollen tube grows through the transmitting tissue of the silk (shaded area) into the embryo sac (ES) (Duncan and Howard, 2010).

CHAPTER 2

QTL mapping for resistance to Fusarium ear rot and fumonisin contamination in maize

ABSTRACT

Fusarium ear rot (FER) is an important disease of maize caused by *Fusarium verticillioides* and *F. proliferatum* fungal pathogens which produce a group of mycotoxins known as fumonisins. The consumption of fumonisin-contaminated maize grain by humans and animals have been associated with serious health implications. Fumonisin contamination is difficult to control using cultural practises, biological- and chemical controls alone. Breeding for resistance, however, is considered an important means to reduce FER and fumonisin contamination in maize. In this regard, an effort to develop markers for marker-assisted selection (MAS) is considered an important component to increase host resistance to the disease and toxins. In this study, a F_2 mapping population comprising 214 F_2 plants from the initial cross between a FER/fumonisin-resistant inbred line (CML444) and susceptible inbred (R2565y) was developed. A preliminary QTL map for resistance to FER and fumonisin contamination was generated and genotyped with 59 simple sequence repeat (SSR) markers. The plants were self-pollinated and the primary ears of the F_2 population were artificially inoculated with *F. verticillioides* to screen for resistance to FER and fumonisin contamination. The expression of disease symptoms were determined by visual assessment while resistance to *F. verticillioides* infestation and fumonisin contamination was determined using quantitative polymerase chain reaction (qPCR) and liquid chromatography tandem-mass spectrometry (LC-MS/MS), respectively. A potential minor QTL for resistance to FER was detected near marker umc1465 on chromosome 2. The marker was inherited from the maternal susceptible parent (R2565y) and explained 3.4% of the phenotypic variation. A higher density QTL map together with larger informative mapping populations and replicated screening trials would improve QTL detection for resistance to FER and fumonisin contamination.

INTRODUCTION

Fusarium ear rot (FER) of maize is caused by three closely related *Fusarium* species, namely *Fusarium verticillioides* (Sacc) Nirenberg, *F. proliferatum* (Matsush) Nirenberg and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun. Of these, *F. verticillioides* is the predominant fungus associated with maize in South Africa (Boutigny *et al*., 2012). The disease was not considered to be of major importance until the discovery and association of the mycotoxins, fumonisins, with *F. verticillioides* and *F. proliferatum* (Mesterházy *et al.*, 2012). Fumonisins are detrimental to the health of humans (Sydenham *et al.*, 1990; Rheeder *et al.*, 1992) and can cause financial loss to livestock farmers where animals are fed fumonisin-contaminated feeds (Morgavi and Riley, 2007). Considering that maize is an important staple food in South Africa and is found in most livestock diets (Morris, 1998), managing fumonisin levels in maize is of particular importance. Fumonisin contamination of maize grain is difficult to control and cultural practises are not always adequate to maintain levels below the maximum recommendation limits (Parsons and Munkvold, 2010). Breeding for resistance to FER and fumonisin is considered a powerful strategy to reduce the disease and toxin levels in maize grain.

Resistance to FER and fumonisin contamination is inherited quantitatively by additive (Pérez-Brito *et al.*, 2001; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012; Zila *et al.*, 2013) and dominant genes (Pérez-Brito *et al.*, 2001; Chen *et al.*, 2012). Although there is no known complete level of resistance (Robertson *et al.*, 2006), natural sources of resistance to the disease and toxin have been identified (Clements *et al.*, 2004; Afolabi *et al.*, 2007; Eller *et al.*, 2008). A small number of inbred lines with resistance to FER and fumonisin was determined by Small *et al.* (2012) and confirmed in a multi-site, multi-year study by Mouton (2014). These FER/fumonisin-resistant inbred lines were also further evaluated for resistance to other major ear rot pathogens with some inbred lines showing potential resistance to multiple ear rot diseases (Mouton, 2014).

Fusarium ear rot can be scored visually on mature maize ears according to Clements *et al.* (2004) and Reid *et al.* (1994) but the presence of the fungus and fumonisin contamination is not always indicated by the visual symptoms of FER (Small, 2010; Mouton, 2014). Alternatively, the extent of fungal and fumonisin contamination can be determined molecularly using species-specific primers with quantitative polymerase chain reactions (qPCRs) (Nicolaisen *et al.*, 2009; Boutigny *et al.*, 2012). Several methods can be used to recover fumonisins from maize grain (Duncan *et al.*, 1998; Kryska *et al.*, 2008). Screening for resistance to FER is time-consuming as this can only be performed on mature maize ears, while molecular screening techniques including analysis of fumonisin concentrations can be costly and is laborious (Robertson *et al.*, 2006; Eller *et al.*, 2008). The screening process is further complicated by the disease and toxin contamination being highly influenced by environmental conditions (Pérez-Brito *et al.*, 2001; Robertson-Hoyt *et al.*, 2006).

Although resistance to FER and fumonisin contamination is complex with low heritability when measured on individual plants, heritability based on family means is moderate to high (Robertson *et al.*, 2006). Selection for resistance to FER and fumonisin contamination can improve the resistance statuses of breeding material (Robertson *et al.*, 2006). Some sources of resistance could be found in older germplasms, in inbred lines not adapted to local conditions or in inbred lines exhibiting lower agronomic performances (Zila *et al.*, 2013). To improve resistance to the disease and toxin without decreasing agronomic performances, quantitative resistance alleles could be incorporated into agronomically acceptable, locally adapted germplasms, by backcrossing FER/fumonisin-resistant inbred lines to elite germplasms and selecting the rare recombinants that are adapted to the environment and resistant to FER and fumonisin contamination (Robertson-Hoyt *et al.*, 2006; Zila *et al.*, 2013). Marker-assisted selection (MAS) can form an important component for the development of resistant cultivars (Eller *et al*., 2008). The potential use of MAS for resistance to FER has been demonstrated, by verifying a QTL effect using near isogenic lines (NILs). The NILs carrying the QTL showed up to 26% increased resistance to FER when the allele was heterozygous and 35.2% when homozygous (Chen *et al.*, 2012).

The objective of this study was to generate a mapping population employing locally adapted South African inbred lines previously screened for resistance to FER and fumonisin contamination (Small *et al*., 2012; Mouton, 2014). The second objective was to produce a preliminary linkage map for QTL analysis of resistance to FER and fumonisin contamination using the F_2: R2565y x CML444 population. The mapping population would serve toward the development of a recombinant inbred line (RIL) population in which preliminary QTL could be validated across several locations over years and enable the mapping of additional QTL for resistance to FER and fumonisin contamination.

MATERIALS AND METHODS

Population development

Plant material

Five maize inbred lines; CML390, CML182, CML444, R2565y, and I137tnW; adapted to South African growing conditions and previously characterised for resistance to FER and fumonisin contamination (Small *et al.*, 2012; Mouton, 2014), were selected for this study (Table 1). The resistant inbred lines (CML390, CML182 and CML444) exhibited the highest resistance to FER and fumonisin contamination across locations (Small *et al.*, 2012; Mouton, 2014). The inbred line CML444 also exhibits tolerance to low nitrogen levels and produces high grain yield under drought conditions in Southern Africa (Messmer, 2006). Furthermore, CML444 was also shown to exhibit potential resistance to *F. graminearum* causing Gibberella ear rot, *Stenocarpella maydis* causing Diplodia ear rot and *Aspergillus flavus* causing Aspergillus ear rot (Mouton, 2014).

Development of the F_2 populations

Maize inbred lines were cultivated during two maize seasons at Welgevallen experimental farm, Stellenbosch University (SU), Stellenbosch, Western Cape, South Africa (2011/2012 and 2012/2013) to produce F_1 hybrids for the development of F_2 mapping populations. Trials were planted during November and subsequently pollinated during January-March. The time to maturity depended on plants genotype and growing conditions. Two crosses were produced, one during the 2011/2012 season (R2565y x CML444) and another during the 2012/2013 season (R2565y x CML390). Seed of the F_1 hybrid of R2565y x CML444 was planted at Makhathini research station, *Mjindi*, KwaZulu-Natal, South Africa during August 2012 and subsequently manually self-pollinated to produce the F_2 population. A field trial in Letsitele during March 2013 was initiated with approximately 300 seeds from a single F_2 ear of the R2565y x CML444 cross. The resultant plants were manually self-pollinated to produce the F_3 population for RIL population development.

A field trial was executed at Welgevallen experimental farm during November 2013 to produce F_2 ears of an additional cross produced during the 2012/2013 maize growing season. Seed of the F_1 hybrid of R2565y x CML390 was planted at Welgevallen experimental farm during 2013/2014 and manually self-pollinated to produce the F_2 ears that will form an additional F_2 mapping population.

Plant development was carefully monitored during all trials, and the ears were covered with a clear polyethylene bag before silk emergence. After silk emergence, a pollination bag was used to collect pollen to perform controlled manual cross-pollinations between resistant (CML390, CML444 or CML182) and susceptible (R2565y or I137tnW) inbred lines. Due to the synchrony of the inbred lines, resistant inbred lines were utilised as pollen donors while susceptible inbred lines were used as female parents to generate the F_1 hybrids. This was determined to be an important aspect to consider in a pilot study previously performed where F_1 hybrids, derived from crosses where the resistant inbred lines were used as the female, did not nick and therefore yielded no seed.

Growing conditions

The trial conducted in Welgevallen experimental farm during 2011/2012 was cultivated in a greenhouse monitored by the Department of Agronomy, SU. The preparation and growth conditions were similar to the greenhouse trial of Small (2010). Briefly, the surfaces of the greenhouse were sterilised with 1 ml/L Sporekill® (ICA International Chemicals, South Africa). Seeds were surface sterilized by soaking in distilled water for 4 h before undergoing a hot water treatment of 60ºC for 5 min (Leslie and Summerell, 2006). The sterilized seeds were placed on sterile moist paper towels and incubated for 5 days at 27ºC for germination before sowing in 15-L

greenhouse planting bags containing coconut coir (Vegtech 2000, South Africa). The coconut coir was prepared from dry blocks by soaking in water amended with $CaNO_3$ (1 kg/1000 L), MgS (0.3 kg/1000 L) and Sporekill® (1 L/1000 L) for 3 days. The seeds were sowed in coconut coir that was first rinsed with water to reduce the electric conductivity (EC) level to below 0.7. The seedlings were irrigated at 2 L/h using flow-regulated and pressure compensated drippers (Netafim, South Africa). Seedlings were first irrigated with 500 ml of non-fertilised water for 1 week. After this period, seedlings were irrigated with a modified Steiner nutrient solution (Steiner, 1984) with an EC of 1.5 and pH 6.5 (Combrink, 2005) for 4 weeks. A minimum of five daily irrigations were provided to allow 30% drainage over the entire day to prevent salt accumulation in the coconut coir. From the 5th week onwards, the EC and pH was maintained at 2.5 and 6.5, respectively, and the drainage was monitored regularly and adjusted according to the climate.

During the following maize growing season (2012/2013), the trial was conducted in a greenhouse in Welgevallen experimental farm, and cultivated by Agribusiness in Sustainable Natural Plant Products. Briefly, seeds were sowed directly into 10-L planting bags containing coconut coir. The trial was irrigated twice daily with 1 L of a nutrient solution containing 353.50 g/1000 L KNO_3, 68.00 g/1000 L KH_2PO_4, 115.00 g/1000 L $NH_4H_2PO_4$, 400.00 g/1000 L $Ca(NO_3)_2.2H_2O_z$, 492.00 g/1000 L $MgSO_4.7H_2O$, 9.0 g/1000 L Fe-EDTA, 1.78 g/1000 L $MnSO_4$, 1.07 g/1000 L $ZnSO_4$, 1.17 g/1000 L $Na_2B_8O_{13}.4H_2O$, 0.16 g/1000 L $CuSO_4$ and 0.10 g/1000 L Na_2MoO_4. The plants were over irrigated by 10-20% to wash out excess salts. An EC of 1.3 was maintained, and the irrigation events were widely spaced to allow sufficient time for the substrate to dry out and aerate. Spider mite infestations were monitored daily and controlled using Biomectin® (Villa Crop Protection, South Africa).

A field trial was conducted in Welgevallen experimental farm during the 2013/2014 growing season. The field was comprised of 10-m plots separated by 1-m galleys. The plots were comprised of 1-m row spacing and the seeds sowed at approximately 0.3 m intervals. The field was covered with shade net as a precaution to prevent birds such as guinea fowl from feeding off freshly sowed seeds. The net was removed as soon as the seeds started to germinate and emerge. The seedlings were drip irrigated once a day for 1.5 h at a rate of 1 L/h using Tiran drip pipe (Netafim, South Africa). Weeds were manually removed in mid-January and the field was subsequently fertilized using Nitrop [N:P:K; 2:3:2 (22) + 0.5 Zn] and Nitrophoska [1:0:0 (40) + 6 S], both with 200 kg/ha.

Additional field trials were carried out in Makhathini research station from August 2012 and Letsitele from March 2013. The field layout corresponded to the field trial carried out on Welgevallen experimental farm. The trial was overhead irrigated by central pivot irrigation systems, and the field was treated with 150 kg/ha of 2:3:4 (30) + 0.5 Zn fertiliser and a pre-emergence

herbicide, Bateleur Gold 650 EC (Syngenta, South Africa). Later, a post-emergent herbicide, Servian Cyprex 75 WG (Syngenta, South Africa) was applied, and during the sixth leaf stage the field was fertilised with 250 kg/ha Limestone Ammonium Nitrate (LAN 28) (Sasol Nitro, South Africa). Kombat Stalk borer (Kombat, South Africa) was applied at 25 g/ha during the 12th leaf stage to control stalk borer infestations. The trial conducted in Letsitele was cultivated using standard maize production practises and was maintained by PANNAR (Pty) Ltd, Greytown, KwaZulu-Natal, South Africa.

DNA fingerprinting of the parental inbred plants

The individual plants, representing inbred lines used in this study, were tested for homozygosity by CenGen (Pty) Ltd, Worcester, Western Cape, South Africa. First, DNA was extracted from healthy leaf material, sampled at four- to five-leaf stage, using a sodium dodecyl sulphate (SDS) extraction buffer. The DNA extraction protocol was initially developed for large-scale DNA extractions in 96-well plates, but has been adapted for fewer samples using individual centrifugation tubes (Agenbag, 2012). Approximately 2 x 0.5 cm^2 leaf material was sectioned into tubes containing two clean 3-mm stainless steel bearings. The extraction buffer, 600 µl SDS [0.1 M Tris (pH 7.5), 0.05 M EDTA (pH 8.0), 1.25% (w/v) SDS], was added to each tube and homogenised in a Tissue Lyser MM301 (Retsch, Germany) set at 30 mHz for 3 min. The samples were then incubated in a water bath at 65ºC for 30 min.

The samples were briefly centrifuged and subsequently cooled in a freezer for 15 min at -20ºC before adding 300 µl of 6 M NH_4OAc. The contents were mixed well by inversion and refrigerated for 15 min at 4ºC. Single tube extractions were treated with 600 µl chloroform:isoamyl alcohol (24:1 v/v) before refrigerating. The samples were centrifuged (plates: 15 min at 2 250 rpm; tubes: 5 min at 12 000 rpm), and the supernatant (approximately 600 µl) transferred to new tubes containing 360 µl iso-propanol. The contents were mixed by inversion and left to stand for 5 min at room temperature (22-25ºC). Thereafter, samples were centrifuged again (same conditions as previous). The liquid was carefully discarded and the DNA pellet was washed twice with 400 µl ice-cold 70% ethanol by centrifugation (same conditions as previous). These samples were allowed to dry completely before adding 120 µl TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)], and this DNA suspension was placed in a refrigerator (4ºC) overnight. The following day, the samples were vortexed and again centrifuged (same conditions as previous). The supernatant (i.e. DNA extract) was transferred to new tubes, leaving non-dissolved debris behind.

The DNA concentration and quality was measured using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The molecular weight and quality of the DNA (i.e. extent of DNA degradation and RNA contamination) of a subset of the samples were also determined visually by gel electrophoresis. A 0.8% SeaKem® LE agarose gel (FMC BioProducts, USA)

mixture was prepared and cooled to about 50ºC before adding 2 ml of 0.5 µg/mL ethidium bromide. The ethidium bromide was used to stain the DNA during electrophoresis for visualisation under ultraviolet (UV) lighting. The DNA samples and a lambda control (40 ng/µl) (Promega, USA) were prepared using approximately 1 µl of the undiluted DNA and lambda control combined with 4 µl colourant (Cresol) and 5 µl SABAX water. Electrophoresis was performed in the 0.8% gel at 50 V for 1 h in 0.5x TBE buffer (tris-borate-EDTA), prepared from a 5x stock solution [0.445 M Tris (pH 8.0), 0.445 M boric acid, 0.01 M EDTA (pH 8.0)]. The DNA fragments were visualised in an UVIpro Platinum documentation system (UVItec, UK).

The initial DNA extract was diluted to 25 ng/µl with SABAX water, arrayed in 96-well plates and stored at -20ºC. The DNA of the parental plants was fingerprinted using a multiplex consisting of eight highly informative simple sequence repeat (SSR) markers (Table 2). The markers were synthesised with the forward primer labelled with FAM (F), VIC (V), NED (N) or PET (P) fluorophores.

The PCR was prepared, to a total volume of 10 µl, using 5 µl of 2x Kapa2G Fast MPlex Mix (Applied Biosystems), the eight markers (Applied Biosystems) and SABAX water. The PCR conditions were controlled in a GeneAmp PCR system 9700 or a Veriti 96-well thermocycler PCR machine (Applied Biosystems). The cycling conditions consisted of 95ºC for 3 min, then 30 cycles followed with 95ºC for 30 sec, 60ºC for 15 sec and 72ºC for 1 min, with a final extension step of 72ºC for 10 min.

The PCR fragment data was collected using ABI platform electrophoresis by the Central Analytical Facility (CAF), US. First, a post-PCR clean-up was performed (i.e. PCR products were desalted while primers, nucleotides and proteins removed). A 500 LIZ® size standard (Applied Biosystems) was added to each sample and the fragments were separated using a Genome Analyzer 3730xl (Applied Biosystems). The data was collected using GeneScan® analysis software (Applied Biosystems) and was imported into GeneMapper® version 4 (Applied Biosystems) to score the allele profiles.

The allele data was imported into PowerMarker version 3.25 (Liu and Muse, 2005) to create a dendrogram, using an unweighted pair group method with arithmetic mean (UPGMA) (Nei *et al.*, 1983), for a visual representation of the purity of the inbred lines. Only crosses produced from homozygotic individuals, representative of their inbred lines, were used for developing F_2 mapping populations for QTL analysis for resistance to FER and fumonisin contamination.

Phenotypic evaluation

Inoculum preparation and inoculation

The F_2 plants grown in Letsitele were screened for resistance to FER and fumonisin. The primary ears were artificially inoculated with the *F. verticillioides* isolate, MRC 826, approximately 2 weeks after self-pollination. This isolate was obtained from infected maize in the former Transkei, South Africa, and is a prolific producer of fumonisin B_1 (Rheeder *et al.*, 2002). The inoculum was prepared in Armstrong's medium [20 g sucrose, 0.4 g $MgSO_4.7H_2O$, 1.6 g KCl, 1.1 g KH_2PO_4, 5.9 g $Ca(NO_3)_2$, 20 µl $FeCl_3$ (10 mg/ml), 20 µl $MnSO_4$ (10 mg/ml), 20 µl $ZnSO_4$ (10 mg/ml) and de-ionised water (dH_2O) to make up a volume of 1 L] incubated on a rotary shaker at 25ºC and at 100 rpm (Booth, 1971). After 4-5 days, the spores were collected in 50 ml centrifugation tubes by filtering the culture through a double layer of sterile cheese cloth. The spores were washed twice with 50-ml of sterile autoclaved dH_2O by centrifugation at 4 000 rpm for 10 min. The spores were then suspended in sterile dH_2O, and the concentration estimated using a Haemocytometer (Improved Neubauer, Germany). The maize ears were inoculated once by injecting 2 ml of a 1 x 10^6 microconidia/ml spore suspension down the silk channel using a sterile needle and syringe (Afolabi *et al.*, 2007). The pollination bags were placed back onto the ears directly after inoculation.

Visual assessment of FER disease symptoms

The ears were harvested at approximately 12% moisture content. Ears were manually harvested individually, de-husked and visually rated by estimating the percentage of infected kernels per ear and scored according to Clements *et al*. (2004). The symptoms observed included pink, purple or white mycelia as well as the starbursts symptom characterised by Duncan and Howard (2010) (Fig. 1).

Grain processing

The seeds were removed from the ears and 15 seeds were randomly selected from each F_3 ear to continue the development of the RIL population by single-seed descent. The selection was random for diseased and healthy kernels to reduce bias in the remainder kernels in which *F. verticillioides* and fumonisin contamination was quantified.

The seed was dried further in a forced air oven at 50-60ºC for 1 day. The grain was first passed through a coarse mill No8 Husqvarna (RELIANCE, Sweden) and a 20-g sub-sample *was* milled using an IKA® analytical mill A11 basic (Sigma–Aldrich, Germany) until the maize grain became finely milled flour (approximately 5 min per sample). The instruments were cleaned between each sample. The maize grain was weighed into 50 ml centrifugation tubes, with 2 g and 5 g weighed for DNA and fumonisin extractions respectively. These samples were stored at -20ºC until the extractions were performed.

Quantification of F. verticillioides *biomass in maize grain*

DNA extraction from fungal and maize samples as well as fungal biomass determination by qPCR was performed according to Boutigny *et al.* (2012). Briefly, the MRC 826 isolate (*F. verticillioides*), was grown in potato dextrose broth (PDB) in a rotary shaker for 14 days at 25ºC. The mycelia was harvested by filtration through Whatman grade no. 4 filter paper, washed twice with sterile dH_2O, freeze dried and stored at -20ºC until DNA extractions were performed. Mycelia (20 mg) and 1 ml CTAB/PVP lysis buffer [1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris (pH 8.0), 0.02 M EDTA (pH 8), 1% (w/v) PVP] was added to 2 ml centrifugation tubes containing glass beads (the PVP was added to the CTAB on the day of extraction). The samples were homogenised for 5 min using a Tissue Lyser MM301 (Retsch, Germany). After shaking, 4 µl of proteinase K (10 mg/ml) was added, and the samples were placed in a water bath for 2 h at 65ºC and vortexed every 15 min. After the incubation period, samples were centrifuged at 12 000 rpm, and 400 µl of the supernatants transferred to new tubes. The samples were further treated with 30 µl of RNase (10 mg/ml) and incubated in a water bath for 15 min at 65ºC. Thereafter, the extraction process was continued using the DNeasy® Plant Mini Kit protocol starting from step 9. An additional phenol:chloroform:isoamyl alcohol (25:24:1) extraction step and two chloroform:isoamyl alcohol (24:1) extraction steps were added after samples passed through the QIA shredder column provided by the DNeasy® Mini Spin Column kit (Fredlund *et al.*, 2008; Boutigny *et al.*, 2012).

DNA was extracted from 2 g of milled maize using the modified version of a DNeasy® Plant Mini extraction kit (QIAGEN) according to Boutigny *et al.* (2012). Briefly, 10 ml of a CTAB/PVP lysis buffer [1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris (pH 8.0), 0.02 M EDTA (pH 8), 1% (w/v) PVP] as well as 40 µl Proteinase K (10 mg/ml) were added to each sample. The samples were placed in a Labcon shaking incubator for 2 h at 65ºC and at 200 rpm. The tubes were then centrifuged for 10 min at 4 000 rpm, and 1 ml of the supernatant was transferred to new 1.5 ml centrifugation tubes containing 30 µl RNase (10 mg/ml). These samples were placed in a water bath at 65ºC for 15 min. The samples were then centrifuged for 10 min at 12 000 rpm, and 400 µl of the supernatant transferred to new centrifugation tubes. The DNA extraction process was continued using the DNeasy® Plant Mini Kit protocol from step 9.

DNA concentrations were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) and diluted to 10 ng/µl with autoclaved dH_2O. The fungal DNA and a constant maize DNA sample, described to be free from *F. verticillioides* DNA contaminants, was used to set up the standard curve. *Fusarium verticillioides* DNA (10 ng/µl) was diluted 4-, 16-, 64-, 256- and 1024-fold using the constant maize DNA (10 ng/µl). A qPCR was set up according to Boutigny *et al.* (2012) in triplicate using the dilution series including the constant maize DNA (10 ng/µl). A species-specific primer set Fver356/Fvert412 fwd/rev [Integrated DNA Technologies

(IDT), USA] was used together with 1x SensiMix™ SYBR (Bioline) and the dilution series including the constant maize DNA to set the standard curve for quantification of *F. verticillioides* biomass (Nicolaisen *et al.*, 2009; Boutigny *et al.*, 2012). The reaction took place in a Rotor-gene TM 6000 (Corbett Life Science) machine set for 95ºC for 10 min, followed by 40 cycles of 95, 60 and 72ºC for 15 sec at each temperature. The presences of inhibitors were analysed by plotting the C_T values (i.e. fractional cycle number at the point where the amplification curve crosses the threshold of detection) of the dilution series, measured by the Rotor-gene software version 1.7 (Corbett Life Science), against the logarithm of the DNA concentrations of the dilution series obtained by the NanoDrop measurements. The C_T of the constant maize DNA was extrapolated from the linear graph and compared with the measured C_T value. The acceptance criteria of sufficient DNA quality were as per CRL-EM-01/08 (2008).

Finally, the *F. verticillioides* biomass was determined in the maize grain. Each DNA sample (10 ng/µl) of the contaminated maize grain was run in duplicate/reaction. A triplicate of the 16x dilution, used to set up the standard curve, was included in the reaction as a known control. A non-template control (NTC) was also included in the reaction to determine whether any *F. verticillioides* contaminants existed in the water or reagents used to set up the reaction. The concentration of *F. verticillioides* DNA was calculated from the standard curve by the Rotor-Gene software. The average concentration of each duplicate was calculated, and these values were used to describe or quantify the *F. verticillioides* infestation which developed from the initial inoculation. Where the C_T values were larger than 30, *F. verticillioides* concentrations were considered too low for an accurate estimation and it was also considered low enough to manually set the concentration to zero. The standard deviation of the C_T values between each duplicate was accepted if < 0.16 and the reaction repeated where necessary.

Quantification of fumonisin contamination in maize grain

Fumonisin was extracted according to Small *et al.* (2012). The finely milled maize samples (5 g) were subjected to an extraction buffer consisting of 70% methanol (AR grade) and 30% water (HPLC grade). The extraction buffer (20 ml) was added to each sample and the samples were subsequently placed in an incubation shaker at 200 rpm for 30 min at 25ºC. After the incubation period, samples were centrifuged at 2 113 rpm (4ºC) for 10 min and 3 ml of the supernatant was extracted using a syringe. The extract was passed through a 0.25 µm Minisart® RC syringe filters [with hydrophilic, solvent-resistant regenerated cellulose (RC) membranes] into new 2-ml centrifugation tubes up to the 2 ml mark. The samples were refrigerated overnight at 4ºC. The following day, samples were centrifuged for 10 min at 14 000 rpm and diluted two-fold (extract:HPLC water) into 1.8-ml vials for analysis.

A serial dilution of fumonisin standards (FB_1, FB_2 and FB_3) purchased from the Medical Research Council-Programme on Mycotoxins and Experimental Carcinogenesis (MRC-PROMEC), Tygerberg, Western Cape, South Africa was made to establish a standard curve for the quantification of these compounds. Six standards, ranging from lowest (vial 1: FB_1 = 0.0504, FB_2 = 0.0505 FB_3 = 0.0052) to highest concentration (vial 6: FB_1 = 20.1600, FB_2 = 20.2000, FB_3 = 2.0800) were used for the generation of the standard curve.

The concentrations of the fumonisins were determined using an ACQUITY Ultra Performance Liquid Chromatography (UPLC®) system (Waters, USA). The separation was carried out using an Ethylene Bridged Hybrid (BEH) C18 (Waters, USA) analytical column (3.5 µm, 2.1 x 100mm) with a SunFire C18 sentry guard (Waters, USA) cartridge (3.5 µm, 4.6 × 20 mm). Water with formic acid (0.1%) was used as mobile phase A and methanol as mobile phase B. After an isocratic step of 62% mobile phase A and 38% mobile phase B, the proportion of the mobile phases A and B changed linearly so that mobile phase A decreased to 0% while mobile B increased to 100% over 5.50 min. Afterwards, the initial conditions (mobile phase A = 62%; mobile phase B = 38%) were maintained for the rest of the runtime. The total runtime was 7 min and the flow rate was maintained at 0.35 ml/min. Fumonisin B_3 was separated from FB_1 and FB_2 during this step by the differences in their molecular mass.

A Quattro Micro triple quadrupole mass spectrometer (Waters/Micromass, UK) was used to measure the proportions of FB_1 and FB_2. An electrospray ionisation probe and Mass Lynx NT software 4.1 were used to capture and process the data. The electrospray ionization source was used in the positive mode and the settings were as follows: capillary voltage, 3.50 kV; cone voltage, 50 V; source temperature, 120ºC; desolvation temperature, 450ºC; desolvation gas (nitrogen, 99.99% purity) flow, 600 L/h; cone (gas flow) 50 L/h. The mass spectrometer was operated in multiple-reaction monitoring (MRM) mode and argon was used as the collision gas. The following parent-daughter ion transitions were used to detect fumonisin B_2: *m/z* 706.30 → 318.30 at 40 V and *m/z* 706.30 → 336.30 at 40 V; and fumonisin B_1: *m/z* 722.30 → 334.30 at 40 V and *m/z* 722.30 → 352.30 38 V. A dwell time of 0.1 seconds were used for all MRM transitions and the retention window for FB_1 and FB_2 was 7 min. Masslynx processing software was used to integrate peak areas and quantify the FB_1, FB_2 and FB_3 concentrations.

The concentrations of FB_1, FB_2 and FB_3 in the maize samples were calculated using the following formula:

C (mg/kg) = (A × D)/(W)
C = concentration of fumonisin in maize sample (mg/kg or ppm)
A = concentration obtained from Masslynx (mg/ml)

D = dilution factor used (2)

W = sample equivalent weight (0.005 kg)

V = volume of extract solvent (20 ml)

Statistical analysis

The Kolmogorov-Smirnov test was used to test whether the data of the visual disease ratings (%), fungal biomasses of *F. verticillioides* (ng/µl) and the total fumonisin content (ppm) followed a normal distribution. The correlation between visual disease rating (%), fungal biomass (ng/µl) and fumonisin contamination (ppm) of maize grain was analysed using Spearman's correlation tests in STATISTICA version 12 (StatSoft), by the Centre for Statistical Consultation of SU.

Genotypic evaluation

SSR markers

SSR primers sets were randomly selected from the maize genetics and genomics database (www.maizegdb.org) to cover each of the 10 maize chromosomes. Additional SSRs were selected from chromosome regions that showed potential QTLs for resistance to FER and/or fumonisin contamination (Robertson-Hoyt *et al.*, 2006; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012) (Table 3). All primers were synthesised by Applied Biosystems with the forward primer labelled with fluorophores. Eighty eight SSR markers were optimized and tested for polymorphism on the parental plants by CenGen (Pty) Ltd. Multiplex assays were optimized where possible and used to genotype the F_2 mapping population.

Genotyping of the F_2 mapping population

DNA was extracted from healthy leaf material collected at the four- to five-leaf stage. The leaves were first dried in a VirTis® benchtop freeze-dryer (SP Scientific, USA). Approximately 2 x 1 cm^2 leaf material was sectioned into 2-ml centrifugation tubes containing two 3-mm clean steel bearings. The samples were placed in a Tissue Lyser MM301 (Retsch, Germany) for 2 min at 50 mHz. A solution of 750 µl 2% CTAB extraction buffer [1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris (pH 8.0), 0.02 M EDTA-disodium (pH 8), 1% (w/v) PVP] containing 2 µl/ml beta-mecaptoethanol was added to each tube containing the powdered leaf material. The beta-mecaptoethanol was added to the extraction buffer just before extraction. Thereafter, 500 µl of chloroform:isomyl alcohol (24:1) was added and samples were incubated for 1 h at 65ºC in a water bath. After the incubation period, samples were centrifuged at 12 000 rpm for 5 min. The supernatant was transferred to a new set of tubes containing 500 µl of ice-cold iso-propanol. The samples were incubated for 20 min at room temperature before centrifuging again at 12 000 rpm for 5 min. The liquid was discarded carefully and replaced with 500 µl of 70% ice-cold ethanol. The samples were centrifuged again at 12 000 rpm for 5 min, the liquid carefully discarded, samples left to dry at room

temperature and 200 µl of TE buffer [Tris (pH 8.0), EDTA-disodium (pH 8.0)] was added to dried samples before storing overnight at 4ºC.

DNA samples were further purified with 20 µl of 7.5 M NH_4OAc and 200 µl of the chloroform:isoamyl alcohol (24:1) mix. The contents were mixed by inversion before centrifuging at 12 000 rpm for 5 min. The supernatant was transferred to a new set of tubes containing 500 µl of ice-cold 100% ethanol. The samples were kept at -20ºC for 2 h, subsequently centrifuged at 12 000 rpm for 15 min, and the pellet washed twice with ice-cold 70% ethanol (washing was by centrifuging at 12 000 rpm for 10 min and discarding the liquid carefully each time). The tubes were inverted and left to dry thoroughly before adding 40 µl of the TE buffer [Tris (pH 8.0), EDTA-disodium (pH 8.0)]. The samples were vortexed and the concentrations determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA). The molecular weight and quality of the DNA was tested as previously described for the genetic fingerprint section of this chapter.

The PCRs were set up and carried out by CenGen (Pty) Ltd using 59 polymorphic SSR markers (Table 4) divided into 13 multiplexes. Each reaction was performed in a total volume of 10 µl consisting of 2x Kapa2G Fast MPlex Mix and 1 µl which was DNA (25 ng/µl). The PCR cycling conditions were set for 95ºC for 3 min, 30 cycles of 95ºC for 15 sec, ºC for 30 sec and 72ºC for 1 min, then at 72ºC for 10 min. The annealing temperature ( ) was adjusted according to the specific multiplex. The PCR machines used for the multiplex PCRs and the data capture processes were the same as described earlier for the fingerprint section in this chapter. Fragment analysis was performed on a Genome Analyzer 3730xl (Applied Biosystems) and the data collected using GeneScan® analysis software (Applied Biosystems). The data was imported and allele profiles analysed by GeneMapper® version 4 (Applied Biosystems) with manual adjustments made where required.

QTL mapping

A linkage map was generated using JoinMap® version 4 (Van Ooijen, 2006) using the marker data obtained from GeneMapper®. First, the file format was adjusted according to the specifications for the JoinMap® software. JoinMap® was used to calculate the segregation ratios of the markers, and the markers displaying segregation distortion were excluded from the analysis. Linkage groups were generated using maximum likelihood estimation and a log of odds (LOD) equal to 20. The distances between the markers were calculated in centimorgans (cM) using the Kosambi mapping function. Each linkage group (LG) was assigned a chromosome number by assessing the marker chromosome positions stored on the Maize Genetics and Genomics Database (http://www.maizegdb.org – verified 28 July 2014).

The linkage map and phenotypic data was imported into MapQTL® version 6 (Van Ooijen, 2009). First, the nonparametric Kruskal-Wallis (KW) test was employed to detect the association of markers with resistance to the: i) visual FER disease symptoms (%), ii) *F. verticillioides* infestation (ng/µl) and iii) fumonisin contamination (ppm). Interval mapping (IM) analysis was employed to investigate putative QTL regions. The LOD significance thresholds of the IM analysis were determined for each LG for each trait using a permutation test of 10 000 iterations.

RESULTS

Population development

Maize inbred lines resistant and susceptible to FER and fumonisin contamination were successfully cross pollinated to produce F_1 ears. The F_1 hybrids were produced from resistant inbred lines (CML390, CML444 or CML182) as the pollen donors and the susceptible inbred lines (R2565y or I137tnW) as the female parent.

The DNA fingerprint analysis of individual plants representative of the inbred lines used in this study indicated residual heterozygosity and impurity of some of the inbred lines. The inbred lines CML444, CML390 and R2565y exhibited adequate homogeneity with homozygotic individuals. The inbred line CML390 was homogenous with homozygotic individuals, while inbred lines CML444 and R2565y exhibited two homogenous groups each (Fig 2). The smaller homogenous group of CML444 exhibited residual heterozygosity by one marker. The majority of the individuals of CML444 were homozygotic, and pollen was collected from a random individual that fell within the larger homogenous group. The inbred line R2565y formed two separate homogenous groups with homozygotic individuals that differed at one marker by one nucleotide length. A single parental plant (R2565y) was selected from each of the two groups for the production of the two mapping populations (Fig. 2).

Two crosses were produced by homozygotic parental plants, representative of their respective inbred lines. The F_1 hybrids R2565y x CML444 and R2565y x CML390 were produced and successfully self-pollinated. The F_2: R2565y x CML444 produced 214 plants from a single F_2 ear for the further development of a RIL population. Currently, seed of the F_3: R2565y x CML444 and F_2: R2565y x CML390 is available for the continuation of the development of RIL populations.

Phenotypic evaluation

The F_2: R2565y x CML444 plants were inoculated and evaluated for resistance to FER and fumonisin contamination. However, the phenotypic data was not normally distributed and was skewed to the left ($P < 0.05$) (Fig. 3). The percentage of FER disease symptoms ranged from 0-100% (mean = 21.05%). The concentrations of *F. verticillioides* in the maize grain was determined

to be between 0-1.6 ng/µl (mean = 0.07 ng/µl), while the total fumonisin concentration (FB_1+ FB_2 + FB_3) was determined to be 0.13-74.08 ppm (mean = 2.78 ppm).

Significant positive correlations were found between the phenotypic variables. A significantly high correlation was found between total fumonisin and fungal biomass ($r = 0.87$; $P < 0.05$), while a significant but moderate correlation was obtained between fungal biomass and visual rating ($r = 0.60$; $P < 0.05$) as well as total fumonisins and visual rating ($r = 0.61$; $P < 0.05$) (Fig. 4).

Genotypic evaluation

The parental plants were screened with 88 SSR markers for polymorphisms. Of these markers, 59 were polymorphic and used to genotype the 214 F_2: R2565y x CML444 plants. Markers umc1472, umc1590, bnlg1662, umc1807, umc1034, umc1562, umc1268 and bnlg1655 showed significant segregation distortion and were removed from the analyses to obtain the best order possible for the linkage group analysis (Table 5). A sparse linkage map was generated with 41 markers distributed over nine of the 10 maize chromosomes (no markers linked to chromosome 7) (Fig. 5).

QTL mapping

By the KW test, resistance to the visual FER disease symptoms were associated with markers umc1465 (LG2.2), umc1908 (LG3), mmc0371 (LG4), bnlg1621a (LG4), bnlg1740 (LG6.2), bnlg1782 (LG8), umc1149 (LG8), bnlg240 (LG8), umc1336 (LG10) and umc1506 (LG10) at a low significance level of $P < 0.05$. The KW test showed that umc1622 (LG2.1) may be associated with resistance to *F. verticillioides* infestation ($P < 0.05$). The KW analysis also showed that resistance to total fumonisin accumulation may be associated with markers umc1622 (LG2.1), umc1465 (LG2.2), umc1861 (LG2.2) and umc1908 (LG3) ($P < 0.05$) (Table 6). By this single marker association analysis, markers associated with resistance were detected at a very low significance level (Table 6).

Being more stringent, the IM test did not detect any QTLs at a highly significant level and thus few of the single marker associations detected at a low significance level proved to be true. The IM analysis detected a potential minor QTL (LOD = 1.6; $P < 0.05$) for resistance to the visual disease symptoms of FER by umc1465 (LG2.2) (Fig. 6). This putative QTL explained 3.4% of the phenotypic variance by dominant and additive effects.

Interval mapping analysis associated resistance to i) *F. verticillioides* infestation to marker, bnlg1740 (LG6.2) (LOD = 1.5), with an additive effect that explained 4.4% of the phenotypic variance; ii) resistance to fumonisin contamination by bnlg1740 and umc1653 on LG6.2 (LOD = 1.5) that explained 3.9-4.6% of the phenotypic variance by an additive effect; and iii) resistance to fumonisin contamination by umc1336 on LG10 (LOD = 1.8) that explained 4.2% of the phenotypic

variance by both dominant and additive effects. These markers, associated with resistance by the IM analysis only, was not supported by the KW test and were therefore not considered as QTLs.

DISCUSSION

None of the South African commercially available cultivars have immunity to FER or fumonisin accumulation (Rheeder *et al*., 1990; Schjøth *et al.*, 2008). In this study, maize inbred lines, resistant and susceptible to FER and fumonisin contamination (Small *et al*., 2012; Mouton, 2014) were crossed to produce segregating F_2 populations for preliminary QTL mapping. From the F_2 populations, RIL populations will be developed for QTL validation and identification of additional QTLs associated with resistance to FER and fumonisin contamination. Markers derived from QTL studies could be used in MAS for the development of maize cultivars resistant to FER and fumonisin contamination (Holland, 2004; Robertson-Hoyt *et al.*, 2006).

The F_1 hybrids generated from homogenous and homozygotic individuals ensured the integrity of the germplasms utilized to develop the mapping populations. Genetic fingerprinting can be used to test inbred lines for purity and homozygosity (Heckenberger *et al.*, 2002) and these genetic statuses are important to consider for the development of most mapping populations (Collard *et al*., 2005; Wu *et al*., 2007). The variation in one marker by one nucleotide in individuals representing inbred line R2565y could be the result of a mutation but the difference was most likely due to a technical error rather than genetic variation. Several technical contributions that could cause this variation have been investigated (Schlötterer and Tautz, 1992; Hatcher *et al.*, 1993; Smith *et al.*, 1997; Nataraj *et al.*, 1999; Heckenberger *et al.*, 2002).

Two F_2 mapping populations R2565y x CML444 and R2565y x CML390 were developed in this study. Only the population R2565y x CML444 was used to generate a preliminary QTL map for resistance to FER and fumonisin contamination. A number of QTL studies for resistance to FER and fumonisin contamination have been conducted in the USA, Mexico and Asia (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al.*, 2006; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012). However, QTL studies for resistance in locally-adapted breeding material could be useful since the disease and toxin accumulation is influenced by environmental factors. Maize varieties grown outside of its production region tend to be more susceptible to fumonisin contamination (Shelby *et al*., 1994). Thus, the QTL identified in studies around the world using varieties adapted to their specific production regions may not always be transferable in locally adapted varieties of different countries.

A sufficient level of disease development is deemed important for reliable resistance screening, but the disease level should not be so severe that the differences are difficult to observe (Mesterházy *et al.*, 2012). In this study, the disease level was low with the distributions of the

phenotypic data severely skewed. The omission of the parental inbred lines and susceptible controls from the F_2 screening trial affected the ability to interpret the phenotypic data obtained as little variation existed between the individuals of the F_2 population. Furthermore, the trial site has not previously been utilised for screening for resistance to *F. verticillioides* and fumonisins even though the climatic conditions were deemed optimal for disease development. This limited the ability to accurately map QTLs as specific markers could not easily be associated to phenotypic plant responses due to the absence of parental phenotypes. Phenotypic variation between the individuals of a mapping population is essential for the accurate mapping of putative QTLs. Therefore, an effort to increase the disease levels would have to be employed in subsequent QTL mapping studies by either introducing more inoculum, employing a double inoculation system (Robertson-Hoyt *et al*., 2006), or by performing screening trials in environments more suited to the proliferation of the pathogen.

The level of disease development in this study corresponded to studies that employed the same inbred lines and inoculation method over different years and various locations in South Africa (Small, 2010; Mouton, 2014). The method of inoculation was similar to the study of Robertson-Hoyt *et al.* (2006), however, five times more inoculum was used and the ears were inoculated twice to reduce the chance of escapes in the aforementioned study. Other QTL studies for resistance to FER and fumonisin contamination utilised the sponge and nail punch inoculation method, described by Drepper and Renfro (1990) (Pérez-Brito *et al.*, 2001; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012) as injury by the nail punch can be used to simulate insect injury and provide an even distribution of injury leaving the fungus with an even chance of infection through the wounds (Eller *et al.*, 2008). The method of inoculation is an important measure of the type of resistance and could therefore be useful to use both methods during parental selection and in QTL mapping trials (Mesterházy *et al.*, 2012).

High genotypic correlation between FER disease severity and fumonisin contamination across replications and environments was found by Robertson *et al*. (2006), which suggests that genotypes that are most resistant to FER tend to be more resistant to fumonisin contamination. Thus, from a breeder's point of view, selecting against FER may be a useful strategy for selecting genotypes with less genetic susceptibility to high fumonisin concentration (Robertson *et al*., 2006). In this study, fumonisin contamination in maize grain correlated significantly positive with *F. verticillioides* biomass. Similar results were obtained by Boutigny *et al*. (2012) and Mouton (2014). The FER disease severity and fungal biomass correlated positively and these results corresponded to the findings of Mouton (2014), while FER disease severity and fumonisin contamination correlated positively as well, as was determined by Small *et al*. (2012) and Mouton (2014).

The results from KW and IM should correspond where marker density is high with intervals <20 cM while deviations between the two results may indicate substandard phenotypic data (Stuber *et al*., 1992; Kruglyak and Lander, 1995). In this study, a sparse linkage map was generated which caused the results of the KW and IM to differ considerably (Tanksley, 1993). The IM analysis method is quite robust against deviation from normality (Van Ooijen, 2009). As the marker density decreases, the KW method quickly becomes less accurate, while IM can still tolerate distances between markers up to 35 cM (Tanksley, 1993). The marker density of this study was extremely low and marker distances were often more than 35 cM.

A sparse linkage map was generated with markers that showed expected Mendelian segregation ratios. Segregation distortion commonly occurs naturally in stretches of the maize genome (Lu *et al.*, 2002; Zhang *et al.*, 2006) but it can also be due to incorrect scoring of difficult marker profiles. Distorted marker profiles were re-evaluated following the generation of the linkage map but remained significantly distorted. It is not known what causes natural segregate distortion but it is probably due to gametophytic factors such as competition during pollen tube developments, pollen lethality, preferential fertilisation and the selective elimination of zygotes (Lu *et al.*, 2002; Zhang *et al.*, 2006). In some cases, inclusion of segregated markers can greatly reduce the accuracy of QTL detection, especially for sparse maps (Xu, 2008). An increase in marker density could provide the opportunity to cover the distorted regions that may also be associated with resistance to FER and fumonisin contamination without greatly affecting the accuracy of the QTL analysis (Xu, 2008). In this study a linkage map covering only 461.2 cM was obtained as opposed to 1969 and 1993 cM by Roberson-Hoyt *et al*. (2006), indicating insufficient genome coverage of the linkage maps due to the extremely low marker density. This has limited the chances of detecting QTL substantially as the biggest portion of the genome could not be analysed.

The QTL analysis for resistance to FER and fumonisin contamination was insufficient due to poor phenotypic data and inadequate genome coverage. This may have been further compounded by the use of a mortal mapping population (F_2 population) of moderate size and the inability to obtain phenotypic data from replicated screening trials. The results of the QTL analysis were therefore assessed cautiously. One QTL for resistance to FER visual symptoms was associated with both the KW and IM analysis, albeit at a very low LOD threshold. The marker associated with this QTL, umc1465 (bin 2.04), explained a small proportion of the phenotypic data (3.4%) and was inherited maternally from the susceptible inbred line, R2565y. A minor QTL effect can come from the susceptible parents but the majority of the effect is usually associated with the resistant parent through maternal inheritance (Scott and King, 1984; Headrick and Pataky, 1991; Nankam and Pataky, 1996). This could indicate that the putative QTL detected in this study is not true and requires further validation. We also hesitate to speculate on the specific resistance

mechanisms employed or candidate genes for resistance to FER and fumonisin contamination because some of the QTL regions spans more than 20 cM representing a large area of the maize genome which could contain genes for resistance to *F. verticillioides* and fumonisin contamination.

The resistance associated with regions by bin 2.04 has not yet been identified in other QTL mapping studies. Resistance to FER can be validated in QTL mapping studies using single-seed decent RILs of the F_2: R2565y x CML444 in replicated trials across several environments. This would also enable the study of genotype x environmental interactions in determining QTL stability across diverse geographic environments in determining durable resistance over several environments (Pérez-Brito *et al.*, 2001; Robertson-Hoyt *et al.*, 2006; Ding *et al.*, 2008; Li *et al.*, 2011; Chen *et al.*, 2012). Following validation, the position for the QTL can be determined by fine mapping procedures (Collard *et al.*, 2005).

In addition to QTL validation and fine mapping, further studies using other unique mapping populations generated from different parental inbred lines can be developed to establish QTLs conserved in maize genotypes resistant to FER and fumonisin contamination (Ding *et al.*, 2008; Chen *et al.*, 2012). Informative QTL maps for resistance to FER and fumonisin contamination could assist in the development of MAS breeding programmes and provide stepping stones for genome-wide association studies for the resistance to FER and fumonisin contamination (Laidò *et al.*, 2004; Zila *et al*., 2013).

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Table 1. Resistant and susceptible maize inbred lines employed in developing F_2 mapping populations.

Inbred Line	Resistance status[a]	Source[b]
CML390	Resistant	CIMMYT-Zimbabwe
CML444	Resistant	CIMMYT-Zimbabwe
CML182	Resistant	CIMMYT-Zimbabwe
R2565Y	Susceptible	ARC-South Africa
I137tnW	Susceptible	ARC-South Africa

[a]The maize inbred lines were characterised as resistant (R) and susceptible (S) to Fusarium ear rot by Small *et al.* (2012) and Mouton (2014)

[b]CIMMYT: The International Maize and Wheat Improvement Centre; ARC: Agricultural Research council

Table 2. Simple sequence repeat (SSR) markers used for DNA fingerprinting of individual plants representing five South African maize inbred lines.

Marker	Repeat	Chromosome	Arm	bin
umc1508	(ATG)4	1	Long	1.06
umc2217	(TG)6	1	Short	1.03/04
umc1165	(TA)6	2	Short	2.01/02
umc1058	(GC)7	4	Long	4.11
umc1287	(CCGTGC)4	8	Long	8.06
umc1370	(CGGG)5	9	Short	9.01
umc1982	Not available	9	Long	9.07/08
phi452121	Not available	Not available	Not available	Not available

Table 3. Markers established with potential resistance to Fusarium ear rot (FER) and/or fumonisin contamination.

Marker	Chromosome	Resistance trait[a]	Publication
bnlg1953	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
phi001	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2096	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1811	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1884	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1335	1	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc33a	1	FER	Pérez-Brito *et al*., 2001
csu164a	1	FER	Pérez-Brito *et al*., 2001
npi282b	1	FER	Pérez-Brito *et al*., 2001
bnl17.18b	1	FER	Pérez-Brito *et al*., 2001
umc161a	1	FER	Pérez-Brito *et al*., 2001
bnlg1347	1	FER	Robertson-Hoyt *et al*., 2006
bnlg2331	1	FER	Robertson-Hoyt *et al*., 2006
npi287a	2	FER	Pérez-Brito *et al*., 2001
bnlg1662	2	FER and fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1606	2	FER and fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1520	2	FER and fumonisin	Robertson-Hoyt *et al*., 2006
umc1696	2	FER	Robertson-Hoyt *et al*., 2006
umc2256	3	FER	Li *et al*., 2011
bnlg1523	3	FER	Ding *et al*., 2008
bnlg1144	3	FER	Li *et al*., 2011
bnlg1904	3	FER	Ding *et al*., 2008
umc1012	3	FER	Ding *et al*., 2008
phi029	3	FER	Ding *et al*., 2008
umc1742	3	FER	Ding *et al*., 2008
bnlg1452	3	FER	Ding *et al*., 2008
umc1025	3	FER	Ding *et al*., 2008
umc92a	3	FER	Pérez-Brito *et al*., 2001
umc1908	3	FER	Ding *et al*., 2008
umc50a	3	FER	Pérez-Brito *et al*., 2001
umc10a	3	FER	Pérez-Brito *et al*., 2001
bnlg1063	3	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1160	3	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1489	3	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1594	3	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1294	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc87a	4	FER	Pérez-Brito *et al*., 2001
umc2082	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2281	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2280	4	FER and fumonisin	Robertson-Hoyt *et al*., 2006
umc1117	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2061	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1511	4	FER	Chen *et al*., 2012
bnl5.71b	4	FER	Pérez-Brito *et al*., 2001
bnlg1621a	4	FER	Chen *et al*., 2012 and Li *et al*., 2011
bnlg1137	4	FER	Li *et al*., 2011
bnlg2244	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1086	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1101	4	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
bnl8.29b	5	FER	Pérez-Brito *et al*., 2001
nc007	5	FER	Li *et al*., 2011
umc1766	5	FER	Li *et al*., 2011
bnlg1879	5	FER	Ding *et al*., 2008

Marker	Chromosome	Resistance trait[a]	Publication
phi008	5	FER	Ding *et al*., 2008
umc1935	5	FER	Ding *et al*., 2008
umc1355	5	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2111	5	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1060	5	FER	Chen *et al*., 2012
bnlg2323	5	FER	Ding *et al*., 2008
umc1332	5	FER	Ding *et al*., 2008
mmc0081	5	FER	Chen *et al*., 2012
umc1524	5	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1941	5	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
phi048	5	FER and fumonisin	Robertson-Hoyt *et al*., 2006
umc59a	6	FER	Pérez-Brito *et al*., 2001
umc65a	6	FER	Pérez-Brito *et al*., 2001
umc1979	6	FER	Li *et al*., 2011
phi031	6	FER	Li *et al*., 2011
umc138a	6	FER	Pérez-Brito *et al*., 2001
umc132a(chk)	6	FER	Pérez-Brito *et al*., 2001
bnlg1740	6	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2059	6	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc1066	7	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc2098	7	FER and Fumonisin	Robertson-Hoyt *et al*., 2006
umc149a	7	FER	Pérez-Brito *et al*., 2001
umc1193	7	FER and fumonisin	Robertson-Hoyt *et al*., 2006
umc1034	8	Fumonisin	Robertson-Hoyt *et al*., 2006
umc1460	8	FER	Ding *et al*., 2008
umc1172	8	Fumonisin	Robertson-Hoyt *et al*., 2006
umc1360	8	Fumonisin	Robertson-Hoyt *et al*., 2006
phi115	8	FER	Ding *et al*., 2008
umc1040	9	Fumonisin	Robertson-Hoyt *et al*., 2006
dupssr6	9	Fumonisin	Robertson-Hoyt *et al*., 2006
umc1191	9	Fumonisin	Robertson-Hoyt *et al*., 2006
bnlg1655	10	FER	Ding *et al*., 2008
bnlg640	10	FER	Ding *et al*., 2008
umc1336	10	FER	Ding *et al*., 2008
npi232a	10	FER	Pérez-Brito *et al*., 2001

[a]Durable resistance to FER and/or fumonisin contamination established by the authors in the following column

Table 4. Polymorphic markers of the parental inbred lines, R2565y and CML444, used to genotype the F_2: R2565y x CML444 mapping population.

Marker	Chromosome	Arm	bin	Resistance trait[a]	Reference
umc1452	1	Short	1.03/04	N/A	No
umc1472	1	Short	1.04	N/A	No
umc2217	1	Short	1.03/04	N/A	No
umc1917	1	Short	1.04	N/A	No
umc1988	1	Long	1.06	N/A	No
umc1754	1	Long	1.06	N/A	No
umc1812	1	Long	1.06	N/A	No
umc1590	1	Long	1.06	N/A	No
bnlg2057	1	Long	1.06	N/A	No
umc2100	1	Long	1.12	N/A	No
umc1622	2	Short	2.00/01	N/A	No
umc1165	2	Short	2.01/02	N/A	No
umc1265	2	Short	2.02	N/A	No
umc1465	2	Short	2.04	N/A	No
umc1861	2	Short	2.04	N/A	No
bnlg1662	2	Long	2.08	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
bnlg1267	2	Long	2.07/08	N/A	No
umc1042	2	Long	2.07	N/A	No
bnlg1606	2	Long	2.08	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
bnlg1144	3	Short	3.02	FER	Li *et al.*, 2011
umc1012	3	Short	3.04	FER	Ding *et al.*, 2008
umc1025	3	Short	3.04	FER	Ding *et al.*, 2008
umc1908	3	Short	3.04	FER	Ding *et al.*, 2008
umc2262	3	Short	3.04	N/A	No
phi053	3	Long	3.05	N/A	No
bnlg1063a	3	Long	3.06	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
bnlg1160	3	Long	3.06	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
umc1594	3	Long	3.09/10	N/A	No
umc1008	4	Short	4.01	N/A	No
umc2082	4	Short	4.03	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
phi021	4	Short	4.03	N/A	No
bnlg1621a	4	Long	4.06	FER	Chen *et al.*, 2012 and Li *et al.*, 2011
mmc0371	4	Long	4.05/06	N/A	No
umc1086	4	Long	4.08	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
umc1650	4	Long	4.09	N/A	No

Marker	Chromosome	Arm	bin	Resistance trait[a]	Reference
umc1058	4	Long	4.11	N/A	No
umc1761	5	Short	5.02	N/A	No
umc1332	5	Long	5.04	FER	Ding *et al.*, 2008
umc1143	6	Short	6.00	N/A	No
umc1887	6	Long	6.03/04	N/A	No
bnlg1740	6	Long	6.07	FER and fumonisin	Robertson-Hoyt *et al.*, 2006
umc1653	6	Long	6.07/08	N/A	No
umc1760	7	Long	7.05	N/A	No
umc1327	8	Short	8.01	N/A	No
umc1807	8	Long	8.03	N/A	No
umc1034	8	Long	8.02/03	fumonisin	Robertson-Hoyt *et al.*, 2006
umc1457	8	Long	8.03	N/A	No
bnlg1782	8	Long	8.05/06	N/A	No
bnlg240	8	Long	8.06	N/A	No
umc1562	8	Long	8.05	N/A	No
umc1149	8	Long	8.05/06	N/A	No
umc1268	8	Long	8.07	N/A	No
umc1370	9	Short	9.01	N/A	No
dupssr6	9	Short	9.02	fumonisin	Robertson-Hoyt *et al.*, 2006
umc1982	9	Long	9.07/08	N/A	No
bnlg1655	10	Long	10.03	FER	Ding *et al.*, 2008
umc1336	10	Long	10.03	FER	Ding *et al.*, 2008
umc1506	10	Long	10.05	N/A	No
umc1196a	10	Long	10.07	N/A	No

[a]Durable resistance to Fusarium ear rot and/or fumonisin contamination

Table 5. Marker (allele) segregation ratios and markers exhibiting significant segregation distortion in the F_2:R2565y x CML444 mapping population.

Marker	Maternal[a]	Heterozyote[b]	Paternal[c]	Significance
umc1982	60	100	54	-
umc1370	55	97	62	-
umc1058	49	107	57	-
umc2217	53	101	60	-
umc1165	50	117	47	-
umc1457	54	119	41	-
umc1008	53	114	47	-
umc1268	51	25	138	*******
umc1887	53	99	54	-
bnlg1782	46	116	52	-
umc1622	52	115	47	-
bnlg240	47	109	57	-
umc1196	51	109	54	-
umc1327	57	112	45	-
umc2262	62	106	46	-
umc1653	53	99	62	-
umc1807	49	125	40	**
umc1143	60	96	58	-
umc2100	56	109	49	-
umc1149	47	110	57	-
umc1265	55	107	52	-
umc1754	44	114	56	-
umc1761	44	107	63	-
umc1760	54	104	56	-
umc1452	54	109	50	-
umc1590	25	132	56	*******
bnlg2057	43	116	53	-
umc1042	49	111	53	-
umc1917	51	111	51	-
umc1506	51	102	61	-
umc1472	12	185	17	*******
umc1861	50	114	50	-
umc1562	40	131	43	****
mmc0371	51	105	58	-
phi053	63	103	48	-
umc1650	51	107	56	-
umc1465	57	106	51	-
umc1988	45	115	54	-
Bnlg1662	52	154	8	*******
Bnlg1063	57	108	49	-
umc1812	44	114	56	-
phi021	43	112	59	-
bnlg1740	56	99	59	-
bnlg1267	56	106	52	-
bnlg1144	59	108	46	-
bnlg1606	48	114	50	-
umc1594	48	120	44	-
umc1025	60	107	45	-
umc1332	37	117	58	**
dupssr6	51	101	62	-
bnlg1621a	48	112	54	-
umc1086	49	108	57	-
umc1012	65	104	45	-

Marker	Maternal[a]	Heterozyote[b]	Paternal[c]	Significance
umc1336	58	109	47	-
umc1908	62	102	50	-
bnlg1160	51	109	54	-
umc1034	70	109	35	****
bnlg1655	57	88	69	**
umc2082	46	119	48	-

[a]Number of F_2 individuals that inherited both alleles from the maternal inbred parent (R2565y)

[b]Number of F_2 individuals heterozygous for alleles from R2565y and CML444 inbred parents

[c]Number of F_2 individuals that inherited both alleles from the paternal inbred parent (CML444)

**: P = 0.05; ***: P = 0.01; ****: P = 0.005; *****: P = 0.001; ******: P = 0.0005; *******: P = 0.0001

Table 6. Markers associated with resistance in maize to Fusarium ear rot, *Fusarium verticillioides* infestation and total fumonisin (FB_1 + FB_2 + FB_3) contamination by the Kruskal-Wallis test.

Marker	Visual disease	Fungal biomass	Fumonisin
umc1452	-	-	-
umc2217	-	*	*
umc1917	-	*	*
umc1988	-	-	-
umc1812	-	-	-
umc1754	-	-	-
bnlg2057	-	-	-
umc1622	-	**	**
umc1165	-	-	-
umc1265	-	-	-
umc1465	**	-	**
umc1861	*	-	**
bnlg1267	-	-	-
umc1042	-	-	-
bnlg1606	-	-	-
bnlg1144	-	-	-
umc1012	-	-	-
umc1025	-	-	-
umc2262	*	-	*
umc1908	**	-	**
phi053	-	-	-
bnlg1063	-	-	-
bnlg1160	-	-	-
umc1008	-	-	-
phi021	-	-	-
mmc0371	**	-	-
bnlg1621a	**	-	-
umc1761	-	-	-
umc1332	-	-	-
umc1143	-	-	*
umc1887	-	-	-
bnlg1740	**	-	-
umc1653	-	-	-
bnlg1782	***	-	-
umc1149	***	-	-
bnlg240	***	-	-
umc1370	-	-	-
dupssr6	-	-	-
umc1336	**	-	-
umc1506	**	-	-
umc1196	-	-	-

*: P= 0.1; **: P = 0.05; ***: P= 0.01

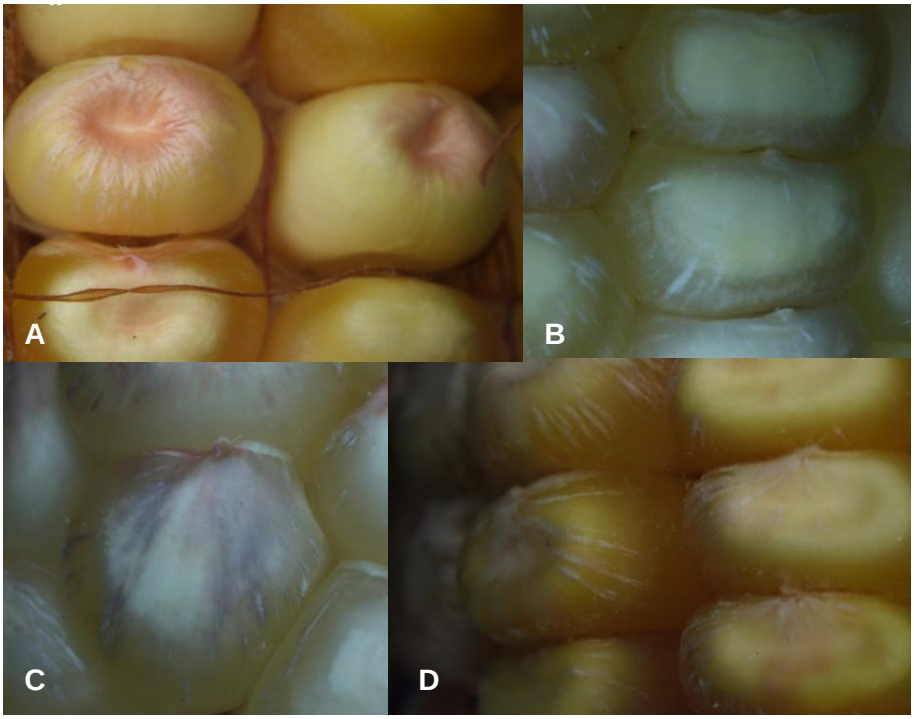

Figure 1. Disease symptoms of Fusarium ear rot of maize. **(A)** Pink mycelia growing on yellow maize kernels, **(B)** white starbursts symptom with white mycelia radiating from the kernel cap, **(C)** purple, pink and white mycelia on white maize kernels and **(D)** white to pinkish mycelia growing from starbursts symptoms on yellow kernels.

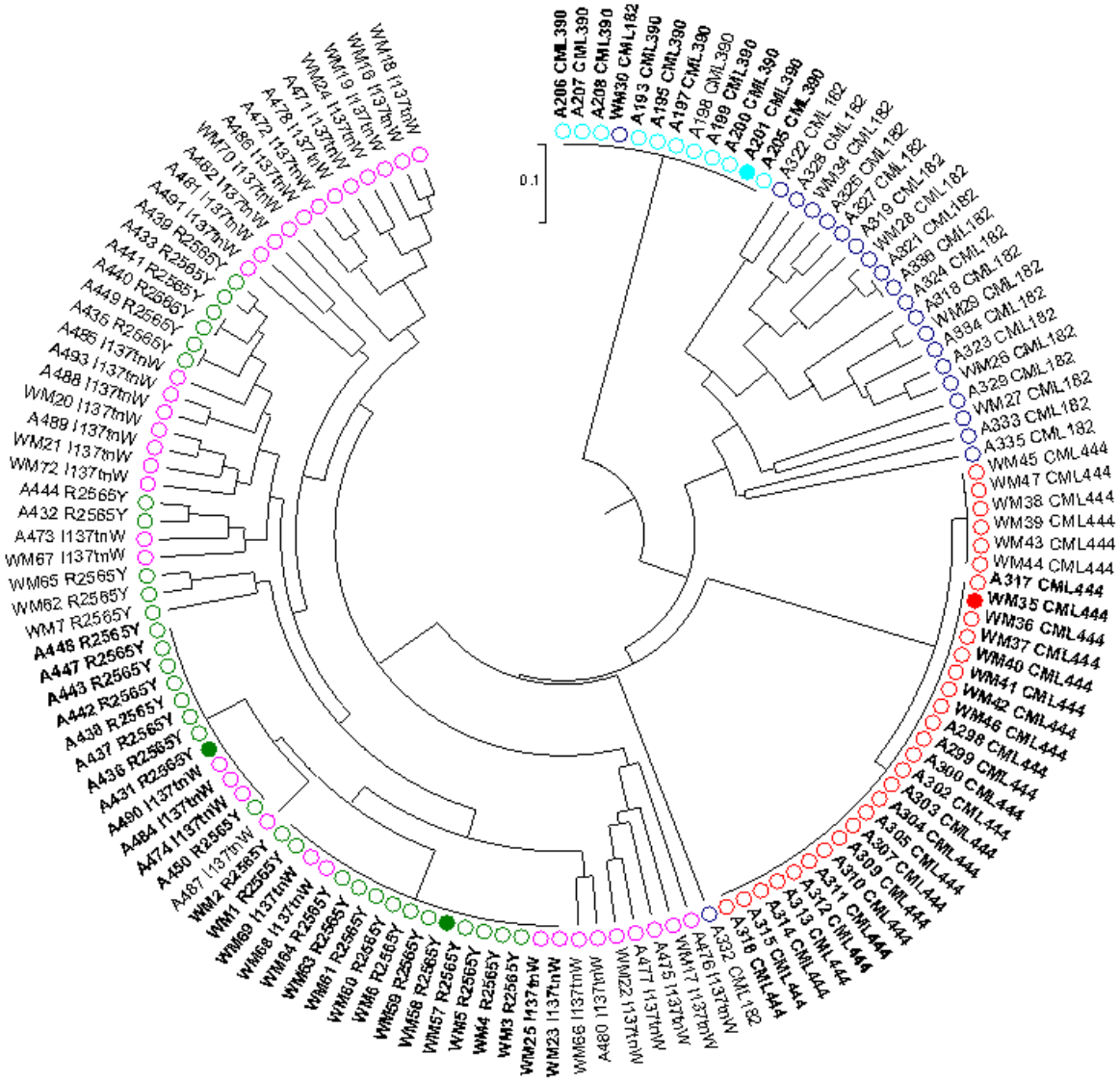

Figure 2. Dendogram of the resistant (CML390, CML444 and CML182) and susceptible (R2565y and I137tnW) maize inbred lines. Inbred lines are represented by different colours while filled circles represent the individuals of the initial crosses that were used for the development of the mapping populations R2565y x CML444 and R2565y x CML390.

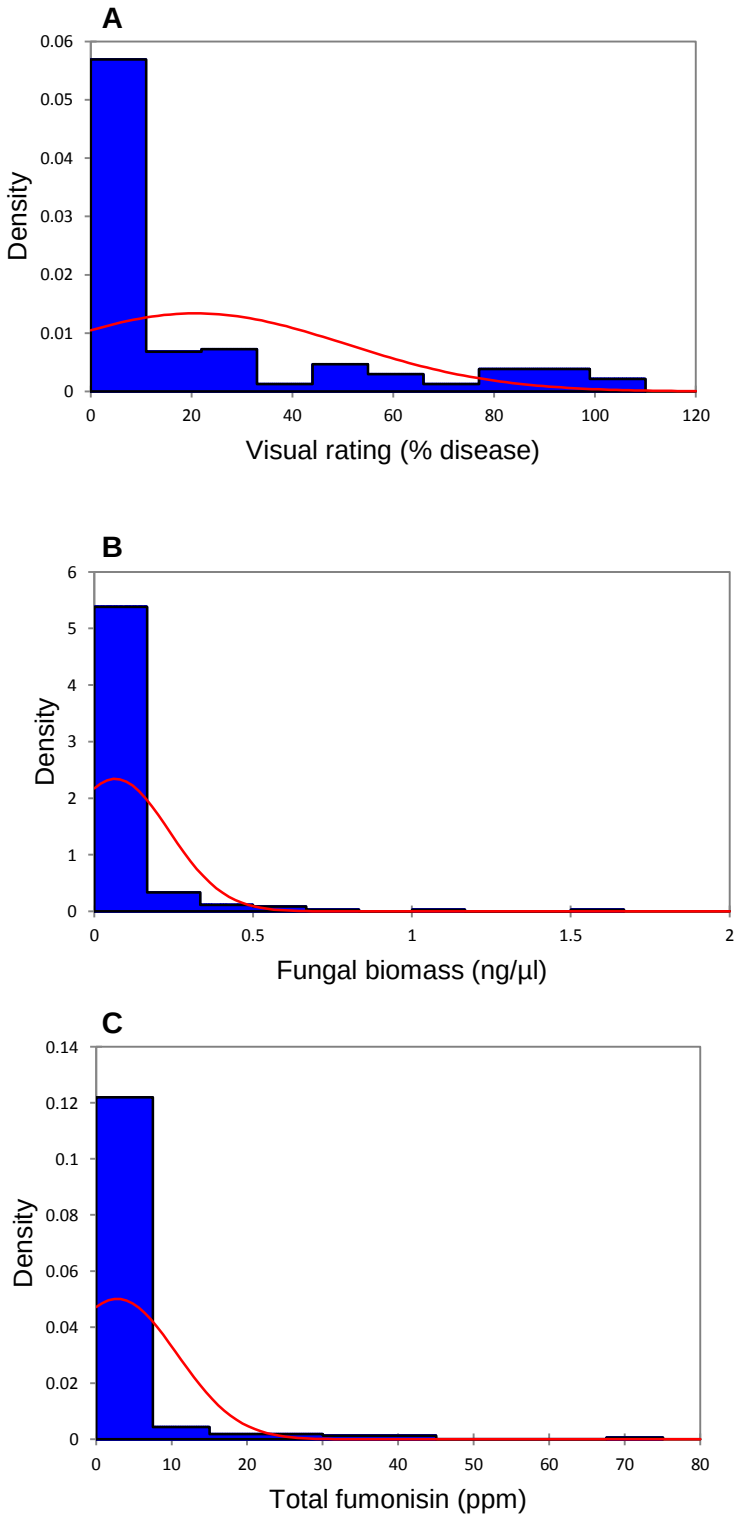

Figure 3. Histograms of the distribution of the phenotypic data obtained from the F_2 population evaluated. **(A)** Visual rating (%), **(B)** *Fusarium verticillioides* biomass (ng/µl) and **(C)** total fumonisin (FB_1 + FB_2 + FB_3) contamination (ppm).

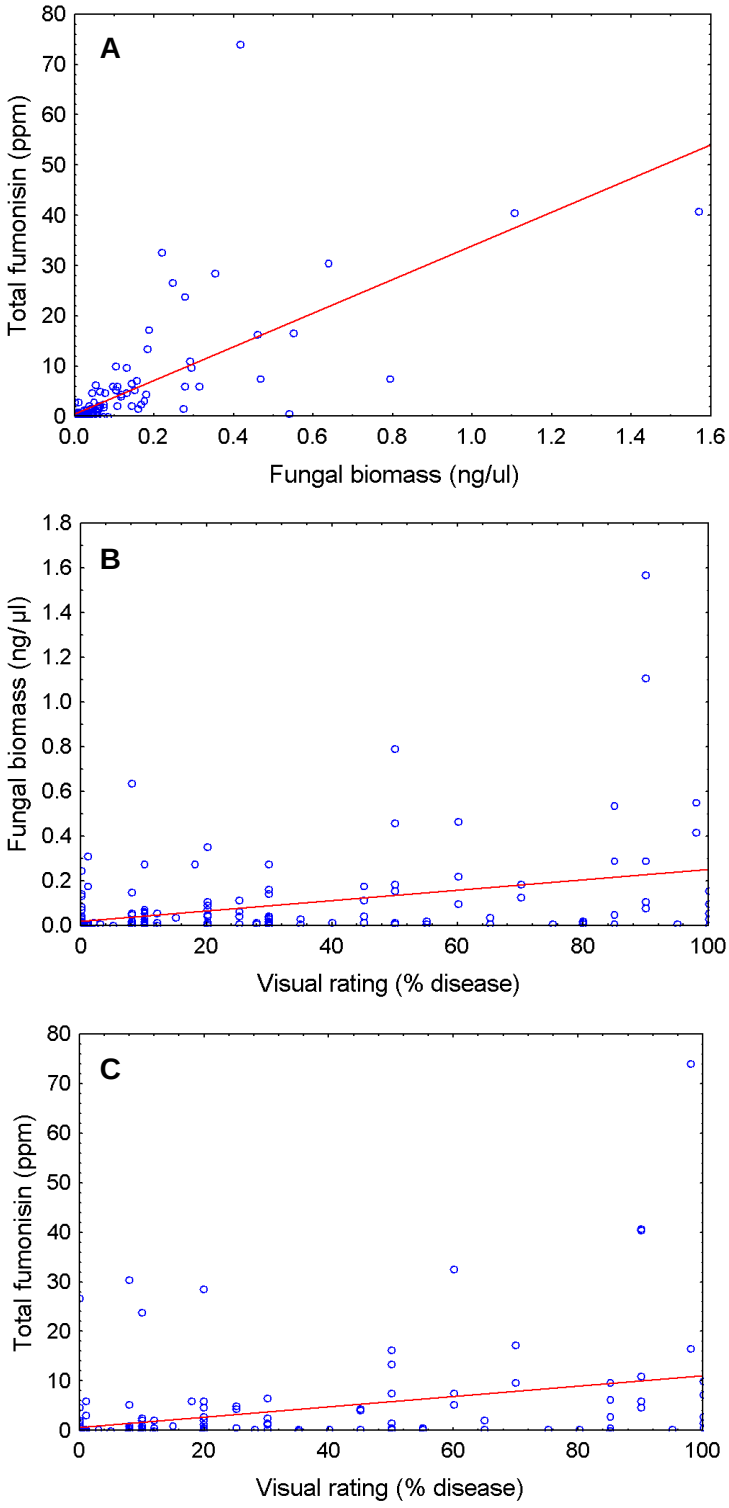

Figure 4. Correlation of fumonisin contamination, *Fusarium verticillioides* fungal biomass and visual Fusarium ear rot (FER) disease symptoms. **(A)** Total fumonisin (FB_1 + FB_2 + FB_3) (ppm) contamination against fungal biomass of *F. verticillioides* (ng/µl) (R = 0.87), **(B)** fungal biomass against visual FER diseases symptoms (%) (R = 0.60) and **(C)** fumonisin contamination against visual FER symptoms (R = 0.61).

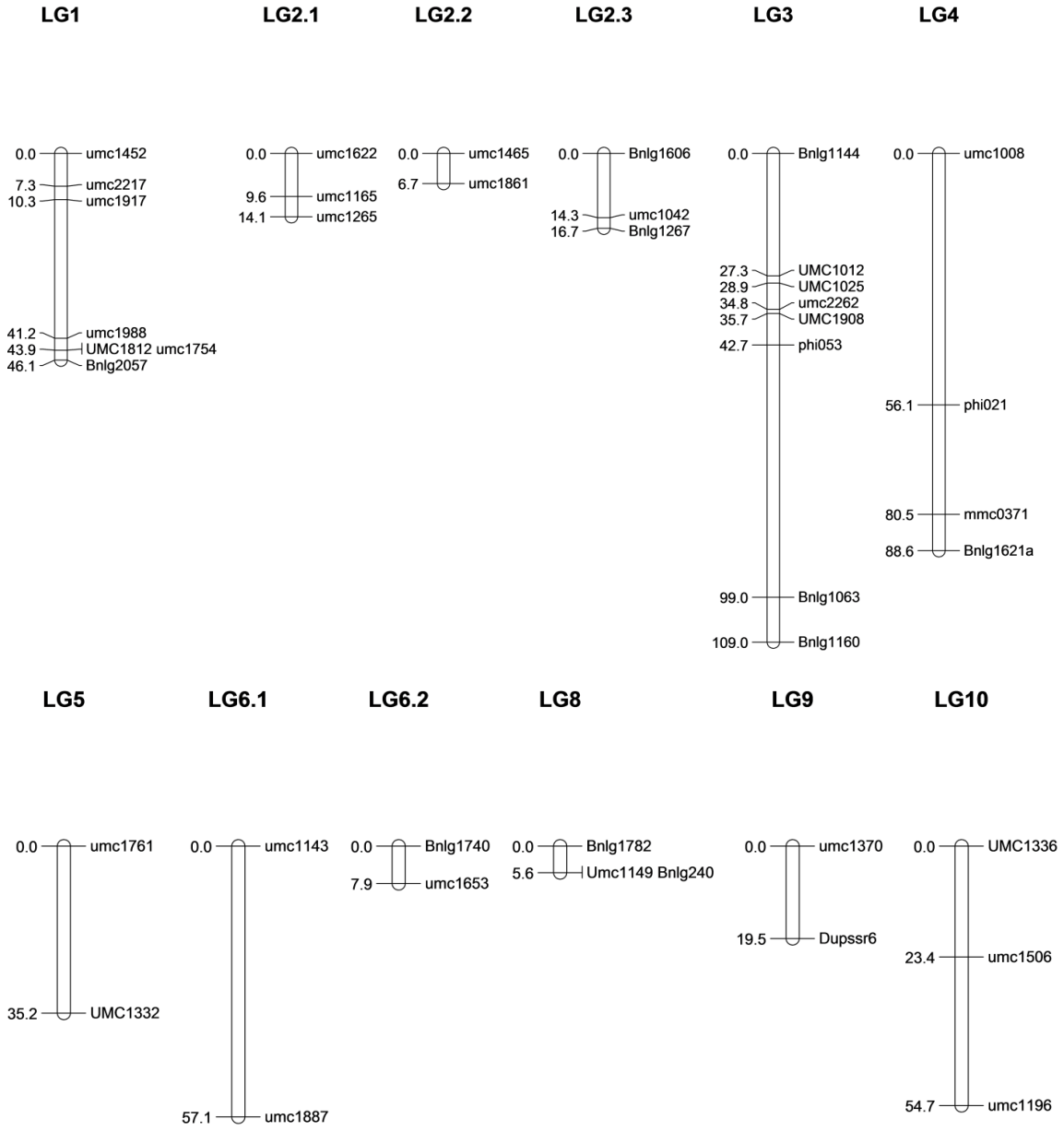

Figure 5. Linkage map of 41 markers distributed over nine maize chromosomes. The linkage group (LG) numbers were assigned according to chromosome numbers.

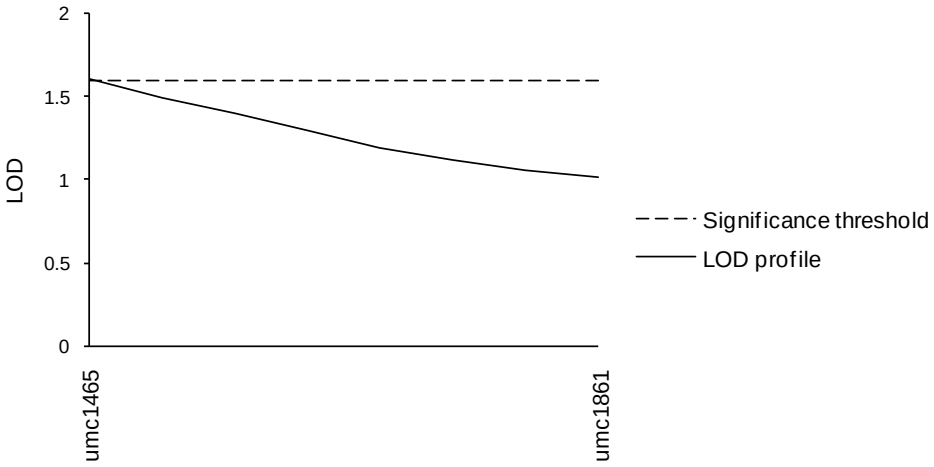

Figure 6. The log of odds (LOD) profile of linkage group 2.2 for resistance to Fusarium ear rot disease symptoms. The marker umc1465 surpasses the LOD threshold at $P < 0.05$.

CHAPTER 3

Histological evaluation of potential resistance barriers of maize against infection by *Fusarium verticillioides*

ABSTRACT

Fusarium verticillioides is an important pathogen of maize that causes Fusarium ear rot (FER) and produces toxic, secondary metabolites called fumonisins. The fungus can infect the plant systemically via natural openings, wounds and roots but may also be present in asymptomatic maize tissue. Host resistance to *F. verticillioides* infection is reliant on several structural and biochemical resistance mechanisms. In this study, the importance of the stylar canal architecture in determining resistance to FER in nine South African maize inbred lines was investigated by means of scanning electron microscopy (SEM). In addition, the colonisation and fumonisin deposition by a *F. verticillioides* isolate, expressing red fluorescence constitutively and green fluorescence when fumonisin is produced, was monitored in a FER- and fumonisin-resistant and susceptible maize inbred line by means of confocal laser scanning microscopy (CLSM). The stylar canal of young maize ears, sampled at 1-2 weeks before pollination and kernels, sampled at approximately 1 month after self-pollination, were evaluated. Only three inbred lines, CML390 (resistant), RO544W (intermediately resistant) and R2565y (susceptible) predominantly exhibited closed stylar canals with 91.3, 95.2 and 60% of canals viewed as closed, respectively. All other inbred lines (CML444, VO617Y-2, US2540, RO549W, R119W and I137tnW) evaluated predominately exhibited open stylar canals prior to pollination. The stylar canals of all inbred lines appeared closed 1 month after pollination and there was no significant association between stylar canal architecture and the FER resistance status of the plants before or after pollination ($P > 0.05$). This suggests that the stylar canal may not play a pivotal role in resistance to *F. verticillioides* ingress. A genetically engineered *F. verticillioides* isolate was inoculated on resistant (CML390) and susceptible (R2565y) maize seedlings, and its growth and fumonisin deposition visualised in roots, stems and leaves. No significant difference in the incidence could be determined regardless of the inoculation method and resistance status of the plant ($P > 0.05$). In addition, no green fluorescence was detected indicating that little to no fumonisins were produced in the maize seedlings. The resistance associated with CML390 is probably not conferred from seedling stage but potentially mediated by structural and biochemical defence mechanisms later in the plant's development.

INTRODUCTION

The fungus, *Fusarium verticillioides*, is amongst the most important pathogens of maize and causes Fusarium ear rot (FER) and fumonisin contamination of maize grain. This fungus can infect the maize plant through natural openings (Duncan and Howard, 2010), wounded tissue (Flett and van Rensburg, 1992; Munkvold and Carlton, 1997; Duncan and Howard, 2010), and can grow systemically within the plant (Munkvold and Carlton, 1997; Oren *et al.*, 2003; Murillo-Williams and Munkvold, 2008; Wu *et al.*, 2011; Wu *et al.*, 2013). Fusarium ear rot can occur from systemic infection through roots and infected kernels but is most frequently associated with damaged ears as a result of feeding insects and/or birds or mechanical damage (Flett and van Rensburg, 1992; Munkvold and Carlton, 1997; Bruns and Abbas, 2004; Fandohan *et al.*, 2006).

Host resistance to *F. verticillioides* is considered the most important and effective means to control FER and fumonisin contamination of maize. Both structural and biochemical resistance traits can impede *F. verticillioides* infection. Tight husks (Warfield and Davis, 1996; Parson and Munkvold, 2010), a thick pericarp (Scott and King, 1984; Hoenisch and Davis, 1994) and the presence of a closed stylar canal (Duncan and Howard, 2010) have been considered important structural resistance barriers to *F. verticillioides* infection. The resistance observed in plants with tight husks and thick pericarps appears to be largely facilitated by insect pest resistance (Warfield and Davis, 1996; Ivić *et al.*, 2008; Parson and Munkvold, 2010), while a closed stylar canal has been proposed as a direct resistance barrier to *F. verticillioides* infection (Duncan and Howard, 2010).

Fusarium verticillioides produces mould and starburst symptoms on maize kernels, but can also occur in the maize plant asymptomatically (Munkvold and Desjardins, 1997). Fumonisin contamination in asymptomatic ears could come from systemic infection of *F. verticillioides* that may originate from contaminated soil and/or maize seeds (Munkvold and Carlton, 1997; Wu *et al.*, 2013). Furthermore, resistance to systemic infection by *F. verticillioides* and subsequent fumonisin contamination has been suggested to be initiated during the early developmental stages of the maize plant (Wu *et al.*, 2013).

In this study, the importance of a closed stylar canal in conferring resistance to *F. verticillioides* was evaluated in nine maize inbred lines using scanning electron microscopy (SEM). Furthermore, genetically engineered *F. verticillioides* isolates, expressing the red fluorescent protein (*DsRed*) constitutively and an enhanced green fluorescent protein (*eGFP*) when fumonisin is produced were characterised and the growth- and fumonisin-deposition subsequently monitored in a resistant and susceptible maize seedling using confocal laser scanning microscopy (CLSM). The proficiency of *F. verticillioides* to infect and deposit fumonisins in maize seedlings, systemically from inoculated-seeds and soil was also investigated.

MATERIALS AND METHODS

Stylar canal architecture investigation

Planting material

The stylar canal architecture of four resistant (CML390, CML444, VO617Y-2 and US2540), two intermediately resistant (RO549W and RO544W) and three susceptible (R119W, R2565Y and I137tnW) maize inbred lines were investigated in this study (Table 1). These inbred lines were grown both in a greenhouse and field trial at the Welgevallen experimental farm, Stellenbosch University (SU), Stellenbosch, Western Cape, South Africa, during the 2012/2013 and 2013/2014 maize production seasons, respectively.

Growing conditions

The trial conducted in Welgevallen experimental farm during 2011/2012 was cultivated in a greenhouse monitored by the Department of Agronomy, SU. The preparation and growth conditions were similar to the greenhouse trial of Small (2010). A field trial was conducted in Welgevallen experimental farm during the 2013/2014 growing season. The field was comprised of 10-m plots separated by 1-m galleys. The plots were comprised of 1-m row spacing and the seeds sowed at approximately 0.3 m intervals. The field was covered with shade net as a precaution to prevent birds such as guinea fowl from feeding off freshly sowed seeds. The net was removed as soon as the seeds started to germinate and emerge. The seedlings were drip irrigated once a day for 1.5 h at a rate of 1 L/h using Tiran drip pipe (Netafim, South Africa). Weeds were manually removed in mid-January and the field was subsequently fertilized using Nitrop [N:P:K; 2:3:2 (22) + 0.5 Zn] and Nitrophoska [1:0:0 (40) + 6 S], both with 200 kg/ha.

DNA fingerprinting

The maize inbred lines were DNA fingerprinted to ensure that all plants were genetically homogenous. The DNA was extracted using a sodium dodecyl sulphate (SDS) extraction protocol, and the molecular weight and quality of the DNA were determined by gel electrophoresis according to Agenbag (2012). The parental plants were then fingerprinted at CenGen (Pty) Ltd, Worcester, Western Cape, South Africa by using a multiplex of eight simple sequence repeat (SSR) markers. These include phi452121 P, umc1058 N, umc1165 P, umc1287 P, umc1370 V, umc1508 F, umc1982 F and umc2217 N (Table 2). The forward primer of the markers where labelled with FAM (F), VIC (V), NED (N) or PET (P) fluorophores. The PCR reactions were set up in a total volume of 10 µl, using 5 µl of 2x Kapa2G Fast MPlex Mix (Applied Biosystems), the eight markers, 1 µl of DNA (25 ng/µl) and SABAX water. The PCR conditions were controlled in a GeneAmp PCR system 9700 or a Veriti 96-well thermocycler (Applied Biosystems) PCR machine. The conditions

were held at 95ºC for 3 min, then 30 cycles followed with 95ºC for 30 sec, 60ºC for 15 sec and 72ºC for 1 min. In the final step, samples were held at 72ºC for 10 min before the machines cooled to 15ºC.

The PCR fragment data was collected using ABI platform electrophoresis. A 500 LIZ® size standard (Applied Biosystems) was added to each sample and the fragments were separated using a Genome Analyzer 3730xl (Applied Biosystems). The data was collected using GeneScan® analysis software (Applied Biosystems) and was imported into GeneMapper® version 4 (Applied Biosystems) to score the allele profiles. This fingerprint data was imported into PowerMarker version 3.25 (Liu and Muse, 2005) to create a dendrogram, using an unweighted pair group method with arithmetic mean (UPGMA) (Nei *et al.*, 1983).

Sampling and sample preparation

Samples for microscopic observations were taken from each inbred line at two time points. These include young maize ears 1-2 weeks before pollination and maturing maize kernels were sampled at approximately 1 month after self-pollination. The young maize ears and maturing maize kernels were kept on ice until dissection to further narrow the areas of interest for microscopic observations. Young maize ears were sliced longitudinally and this was sectioned to 1 x 1 cm sample sizes. The silk scars of maturing maize kernels were sampled to obtain the stylar canals of kernels after self-pollination. The dissected samples were placed in centrifugation tubes, super-cooled in liquidised propane, drenched in liquid N_2 and then stored at -80ºC until fixation.

Samples were fixed in 2.5% glutaraldehyde for 3-6 h at room temperature (22-25ºC) or overnight at 4^0C. The maize ovaries and kernels were then rinsed with 2 ml of 0.1 M cacodylate buffer before fixing the lipids in 2 ml of 1% osmium tetroxide for approximately 1 h at room temperature. The samples were rinsed again with 2 ml of 0.1 M cacodylate buffer and finally in 2 ml of de-ionised water (dH_2O).

Immediately after rinsing, ovaries and kernels were dehydrated in an alcohol series consisting of 30, 50, 70, 90, 95 and 100% ethanol with 10-minute intervals. The samples were then transferred to a critical point drier Polaron E3000 (Quorum technologies) containing acetone. The critical point drier was operated according to the manufacturer's recommendations using liquid CO_2, and samples were soaked in CO_2 for 2 h. The dried samples were sputter coated S150A (Edwards) in gold palladium alloy and viewed using a LEO 1450VP SEM. Three to ten stylar canals were documented per individual before self-pollination and four to 12 maturing maize kernels were documented per individual after self-pollination.

Statistical analysis

The data was analysed using STATISTICA version 12. An analysis of variance (ANOVA) test was used to determine whether differences in stylar canal closure existed between inbred lines, and also to determine whether a closed stylar canal can be correlated to a FER/fumonisin resistant phenotype. The least significant differences (LSD) were calculated to compare differences of closed stylar canal architecture represented by the different inbred lines.

Monitoring *Fusarium verticillioides* and fumonisin deposition in maize seedlings

Isolates

Genetically engineered *F. verticillioides* isolates were kindly provided by Dr. R.H. Proctor (United States of Department of Agriculture; USDA) (Table 3). These include TEF Red1, which constitutively expressed the *DsRed* gene, and M3125-G6-1 which expressed the *eGFP* gene only when fumonisins are produced. The *DsRed* gene was integrated into the genome of the M-3120 isolate under the constitutive promoter of the translation elongation factor gene (*TEF1*), while the *eGFP* gene was fused to the promoter of the *FUM8* gene of M-3125 to monitor expression of fumonisin biosynthesis. The resultant transformants TEF Red1 and M3125-G6-1 were crossed to produce three isolates, 5-7ss4, 5-8ss4 and 5-9ss5, expressing *DsRed* constitutively and *eGFP* only when fumonisins are being produced (Fig. 1). The geneticin resistance gene, *GenR*, was used as a selective marker and the mutant isolates were stored in media containing G418 (Invitrogen, USA).

Characterisation

The genetically modified *F. verticillioides* isolates and their wild types were included in a characterisation study. The well-characterised, prolific fumonisin producing *F. verticillioides* isolate, MRC 826 (Rheeder *et al.*, 2002), was included for comparative purposes. All isolates were characterised according to colour, growth-rate, morphology, microconidia producing potential and fumonisin producing potential. The trials were performed three times with three replications per isolate.

To determine differences in colour and growth-rates, isolates were grown on potato dextrose agar (PDA) under 12 h light 12 h dark cycles at 25ºC for 14 days. The growth-rates of the fungal isolates were compared over 7 days at three temperatures. PDA plugs (5-mm-diameter) of *F. verticillioides* obtained from 4-day-old cultures were placed in 90-mm dishes containing PDA. The isolates were incubated in the dark at 15, 25 and 35ºC for 7 days and fungal growth was measured every 24 h. The growth was recorded as the average of two perpendicular diameter readings and this was used to calculate the total area of the culture. The area of the fungal growth

(total area – plug area) was calculated and used to determine the growth-rate (area of the fungal growth/day) of each isolate.

Microconidiophores and the size of microconidial spores of each isolate were evaluated from cultures grown on carnation leaf agar. The plates were incubated under 12 h light 12 h dark cycles at 25ºC for approximately 10 days. The presence of microconidial chains and false-heads were determined under 400x magnification. The average spore size of 15-20 microconidia were recorded at 1000x magnification and the length and width of the spores were measured using ImageJ version 1.47v (Rasband, 1997–2014) and multiplied to obtain a rough estimate of the area of each spore.

The microconidia producing potential of the *F. verticillioides* isolates were determined as follows: a spore suspension was prepared by adding 5 ml of dH_2O to PDA dishes containing 5-day-old *F. verticillioides* cultures. The spore suspension was transferred from the dishes to 2-ml centrifugation tubes and mixed well. The concentration of the spore suspensions was estimated using a Haemocytometer (Improved Neubauer, Germany), adjusted to 1×10^6 microconidia/ml and 300 µl of the spore suspension was added to each Erlenmeyer flask containing Armstrong's medium (Booth, 1971). The isolates were incubated in a rotary shaker set at 25ºC and at 100 rpm. After the 4th day, spores were filtered though sterile cheese cloth and collected into two 50-ml centrifugation tubes, washed twice in dH_2O and the number of microconidia determined using a Haemocytometer (Improved Neubauer, Germany).

The total fumonisin (FB_1 + FB_2 + FB_3) producing potential of each isolate was determined as follows: rice (25 g) was soaked overnight in 15-ml of autoclaved dH_2O in sealed 500-ml Erlenmeyer flasks. The flasks containing the rice were autoclaved for 30 min and allowed to cool. Plugs made from the young growth of 4-day-old cultures grown on PDA were placed onto the rice and incubated for 4 weeks at room temperature. Fumonisin B_1, B_2 and B_3 were extracted using 100 ml of extraction buffer (70:30; methanol:HPLC grade water). The extraction buffer was mixed well with the rice medium and incubated on an incubator shaker for 60 min at 25ºC and at 200 rpm. The extract was decanted into 50 ml centrifugation tubes and centrifuged at 4ºC for 10 min at 4 000 rpm. The supernatant was transferred to clean 50-ml centrifugation tubes and centrifuged again at 4ºC for 10 min at 4 000 rpm. A sterile syringe was used to transfer 2-ml of the extract through a 0.25-µm Minisart® regenerated cellulose (RC) filter into 2-ml centrifugation tubes. The samples were refrigerated at 4ºC overnight, centrifuged for 10 min at 14 000 rpm and diluted 30 times using HPLC grade water. Six standards, ranging from lowest (vial 1: FB_1 = 0.0504, FB_2 = 0.0505 FB_3 = 0.0052) to highest concentration (vial 6: FB_1 = 20.1600, FB_2 = 20.2000, FB_3 = 2.0800) were used for the generation of the standard curve. The diluted samples were transferred into glass vials and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantification.

Species verification

Quantitative PCR (qPCR) with the Fver356/Fvert412 fwd/rev [Integrated DNA Technologies (IDT), USA] species-specific primer sets (Nicolaisen *et al.*, 2009) were performed as per Boutigny *et al.* (2012). The DNA was first extracted from 2-week-old cultures grown on PDA. The fungi were then transferred to 2-ml centrifugation tubes containing glass beads. DNA was extracted from each isolate using the Wizard® Genomic DNA Purification Kit extraction protocol for plant tissue with minor modifications. The extraction buffer was added to the tubes and the samples were transferred to a vortex equipped with an attachment plate and were vortexed on high for 30 min. The samples were then incubated in a water bath for 1h at 65ºC.

The rest of the extraction process was performed according to the Wizard® Genomic DNA Purification Kit manufacturer's recommendations for plant tissue. The DNA concentration was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) and was adjusted to 20 ng/µl with autoclaved dH_2O. The diluted DNA was used in a qPCR assay together with a standard 64x dilution of *F. verticillioides* (i.e. 10 ng/µl fungal DNA diluted 64x in a 10 ng/µl solution of known and constant maize DNA) in triplicate and non-template control (NTC). The standard was used as a positive control to compare the DNA melting points of all samples in order to verify that all isolates were *F. verticillioides*.

Pathogenicity trial

Ears of the susceptible maize inbred line (R2565Y) were inoculated with microconidia of the *F. verticillioides* isolates using the silk inoculation technique (Afolabi *et al.*, 2007). The ears of five plants were inoculated with either TEF Red1, M3125-G6-1, cocktail mix (5-7ss4 + 5-8ss4 + 5-9ss5) and MRC 826. Control plants were inoculated with sterile dH_2O. Approximately 1 month after inoculation, ears were harvested and the numbers of FER infected ears per treatment were determined.

The infected ears were studied using fluorescence microscopy to verify that the FER symptoms observed were caused by the transformed isolates. For verification, four symptomatic kernels of the infected ears were surface sterilised for 2 min in 70% ethanol and subsequently placed onto dishes containing PDA. After 3 days at room temperature, mycelia growing from the seeds were transferred to clean PDA dishes and these were incubated for 5 days at room temperature. After 5 days, the fungal growth was viewed using a fluorescence Axioskop microscope (Zeiss, Germany) equipped with an epifluorescence condenser, a high-pressure mercury lamp, Neofluar objectives and Zeiss filters 02, 06 and 18. The filter set included excitation levels of G 365, BP 436/8 and BP 395-425, respectively with the green fluorescence observed with filter 06 and the red fluorescence observed with filter 18.

Monitoring of Fusarium verticillioides *in maize seedlings*

The transformed isolate 5-9ss5 was selected to monitor the growth and fumonisin deposition in resistant (CML390) and susceptible (R2565y) maize seedlings using fluorescence microscopy. The proficiency of *F. verticillioides* infestation in maize seedlings from sterilised seeds sown in inoculated soil and inoculated seeds grown in sterilised soil were evaluated. The soil was sterilised by autoclaving for 30 min, while seeds were sterilised by a hot water treatment of 60ºC for 5 min (Leslie and Summerell, 2006).

A microconidial spore suspension was prepared according to Small *et al.* (2012). After 4 days in Armstrong's medium, the spores were collected by filtration, washed and the concentration adjusted to 1 x 10^6 microconidia/ml. Five resistant and five susceptible maize seeds were inoculated by planting sterile seeds in autoclaved soil containing 0.8% (w/v) inoculum (soil-inoculated). Another five-resistant and susceptible seeds were inoculated by planting seeds that were first immersed in a spore suspension (1 x 10^6 microconidia/ml) for 5 minutes, into autoclaved soil (seed-inoculated). Sterile seeds planted directly into autoclaved soil served as control plants for the experiment. The inoculated and control seedlings were allowed to grow for 3 weeks before the microscopic observations were made.

The leaves, stems and roots of seedlings were sectioned longitudinally into 20 µm thick slices using a cryostat CM 1100 (Leica, Germany). Microscope slides of the sections were prepared using fluorescent mounting medium (Dako, USA) and the sections were viewed using an inverted CLSM 510 meta (Zeiss, Germany) at 561 nm/585-600 nm and 488 nm/500-520 nm, separately, to tracked and green fluorescence produced by the mutant *F. verticillioides* isolate.

Statistical analysis

The data was analysed by the Centre for Statistical Consultation, SU, using STATISTICA version 12. Separate ANOVA's were used to determine whether one or more isolates differed significantly in terms of growth-rate, microconidial spore sizes, microconidia producing potential and fumonisin producing potential. The isolates were compared and the significant differences between each isolate were determined using LSD tests. The data of the spore producing potential was log10 transformed for the analysis.

Fisher's exact test was used to determine whether there was a difference in fungal growth in soil- and seed-inoculated resistant (CML390) and susceptible (R2565y) plants by analysing the fungal incidence in the roots, stems and leaves.

RESULTS

Stylar canal architecture

Genetic variation existed within some of the inbred lines evaluated (Table 4). Five inbred lines CML390, VO617Y-2, US2540, RO549W and RO544W formed distinct genetic clusters, while the remaining inbred lines R119W, R2565Y and I137tnW formed one large cluster. The stylar canal architecture was evaluated from individual plants that fell within the clusters as these were considered representatives of their particular inbred line (Fig. 2).

The architecture of the stylar canals could be visualised by SEM (Table 5, Figs. 3-6). The stylar canals of the young ears seemed to be closing as the ovaries aged (Fig. 3), while some inbred lines exhibited open stylar canal architectures for longer during development (Fig. 4). The stage of development could be indicated by the length of the silks and were scored according to the developmental stage where the stylar canal is closed in figure 3.

Significant differences of stylar canal architecture were observed between inbred lines before pollination ($P < 0.05$) (Fig. 7). One resistant inbred line CML390 and one intermediately resistant inbred line RO544W exhibited a significantly higher percentage of closed stylar canals with 91.3 and 95.24% closure, respectively (Table 5). Two susceptible inbred lines R119W and R2565Y exhibited a significantly lower percentage closure with 40% and 60% closure respectively. The lowest percentage of closed stylar canals were observed from three resistant CML444 (0%), VO617Y-2 (14.29%) and US2540 (25%), one intermediately resistant, RO549W (0%), and one susceptible, I137tnW (7.69%), inbred line with no significant differences between them ($P > 0.05$). There was no significant difference in stylar canal architecture with respect to the FER resistance status of the inbred line before pollination ($P > 0.05$).

Open and closed stylar canal architectures were exhibited after pollination (Figs. 5-6). However, the stylar canals sampled from maturing maize kernels appeared to be closed most of the time for all of the inbred lines tested, with 89.66-100% closure per inbred line (Table 5). No significant differences were observed of the stylar canal architecture after self-pollination ($P > 0.05$).

Monitoring *Fusarium verticillioides* and fumonisin deposition in maize seedlings

Characterisation

All the *F. verticillioides* isolates, with the exception of MRC 826, produced white mycelial growth before sporulation. Isolate MRC 826 produced distinct orange mycelia before sporulation. However, after 14 days all the isolates produced subtle variations in colour when viewed from above and below (Fig. 8).

The incubation temperature played a significant role on the average growth-rate of the fungi ($P < 0.05$). The fastest growth-rate was observed at 25ºC and the slowest at 15ºC. Significant differences were observed between the average growth-rate of isolates incubated at 25ºC and 35ºC ($P < 0.05$). However, no significant differences in growth-rates between isolates were observed at 15ºC ($P > 0.05$) (Fig. 9). The M-3125 isolate grew significantly faster than all the other isolates at 25ºC ($P < 0.05$) while the growth-rates of all the genetically engineered isolates, with the exception of M3125-G6-1, were not significantly different at 25ºC ($P > 0.05$). The 5-7ss4 did not differ significantly to M3125-G6-1 at 25ºC ($P > 0.05$). There were no significant differences in the growth-rates of MRC 826, M-3120 and M3125-G6-1 at 25ºC ($P > 0.05$). No significant difference was observed between wild-type *F. verticillioides* isolates (M-3120, M-3125 and MRC 826) at 35ºC ($P > 0.05$). The growth-rate between transformants TEF Red1 and M3125-G6-1 did not differ significantly from their respective wild-types M-3120 and M-3125 ($P > 0.05$) at 35ºC. Isolates, 5-7ss4 and 5-9ss5, exhibited significantly higher growth-rates when compared to the other transformed isolates ($P < 0.05$) and grew at a similar rate at 25ºC ($P > 0.05$). The growth-rate of isolate 5-8ss4 was significantly lower than 5-7ss4 and 5-9ss5 ($P < 0.05$) but did not differ significantly from M3125-G6-1 and TEF Red1 or from the untransformed parent, M-3120, at 35ºC ($P > 0.05$).

All the isolates produced both chains and false-heads of microconidia (Fig. 10). On average, MRC 826 exhibited significantly larger microconidia when compared to M-3120, M-3125, TEF Red1, M3125-G6-1, 5-7ss4, 5-8ss4 and 5-9ss5 ($P < 0.05$) (Fig. 11). No significant differences, in the average spore size per isolate were observed between the remaining isolates ($P > 0.05$).

There were significant differences between isolates when microconidia-producing potential was evaluated ($P < 0.05$) (Fig. 12). The spore-producing potential of M-3120, M-3125, M3125-G6-1 and 5-8ss4 did not differ significantly ($P > 0.05$). The spore producing potential of M-3120, TEF Red1, M3125-G6-1, 5-7ss4 and 5-8ss5 did not differ significantly from one another ($P > 0.05$). The spore-producing potential of 5-9ss5 was significantly less than M-3120, M-3125, TEF Red1, M3125-G6-1, 5-7ss4 and 5-8ss4 ($P < 0.05$). The MRC 826 isolate produced significantly less spores in comparison to all the other isolates ($P < 0.05$).

The fumonisin producing potential of isolates, M3125-G6-1 and MRC 826 were significantly higher when compared to all the other isolates evaluated ($P < 0.05$) yet did not differ significantly from each other ($P > 0.05$) (Fig. 13). All other isolates, with the exception of M-3125, did not differ significantly in their fumonisin production ($P > 0.05$) while M-3125 differed significantly from M-3120 and 5-9ss5 in their ability to produce fumonisins *in vitro* ($P < 0.05$). Additionally, the

transformed isolates were verified as *F. verticillioides* by qPCR analysis with species-specific primers.

Pathogenicity trial

Fusarium ear rot was observed in maize ears inoculated with TEF Red1, M3125-G6-1, cocktail mix (5-7ss4 + 5-8ss4 + 5-9ss5), MRC 826 and with water controls. Four of the five ears inoculated with TEF Red1 produced FER symptoms, while five of the five ears inoculated with M3125-G6-1 were symptomatic. Four of the five ears inoculated with the cocktail developed FER symptoms. Fusarium ear rot was observed in all ears inoculated with MRC 826. Two of the five ears inoculated with the water control produced FER symptoms.

The *F. verticillioides* fungi were isolated from four FER-diseased kernels per symptomatic ear. Red fluorescence was detected from the fungal isolates of three of four kernels inoculated with TEF Red1, while five of five ears inoculated with M3125-G6-1 exhibited green fluorescence. Both green and red fluorescence were detected in two of the four ears inoculated with the cocktail mix.

Monitoring Fusarium verticillioides *and fumonisin deposition in maize seedlings*

The red fluorescent signal from the *F. verticillioides* isolate 5-9ss5 was detected inside the root, stem and leaf material of both resistant and susceptible maize seedlings irrespective of the inoculation method (Fig. 14). The red fluorescence of the fungus was detected between the mesophyl cells and inside vascular bundles of both resistant and susceptible maize seedlings.

There was no significant difference in the incidence of *F. verticillioides* red fluorescent signals between the resistant and susceptible maize seedlings, independent of inoculation method and plant material investigated ($P > 0.05$). In addition, no difference in the colonisation or growth pattern of the fungus could be observed between the resistant and susceptible maize inbred lines. There was no green fluorescence detected in any of the plant material examined.

DISCUSSION

Although genetic variation existed within some of the maize inbred lines, inbreds, CML390, CML444 and R119W were mostly homogenous and homozygotic, while VO617Y-2, US2540, RO549W, RO544W, R2565Y and I137tnW were not homogenous nor homozygotic (Heckenberger *et al*., 2002). It was not possible to genetically distinguish individuals of R119W, R2565Y and I137tnW by the SSR marker data but more SSRs can be added to separate these inbred lines into individual genetically distinguishable clusters (Nei *et al.*, 1983). Although the accuracy of the phylogenetic tree could be violated due to insufficient marker data, little variation is expected within inbred lines and the fingerprinting analysis was effective in identifying complete off-type individuals,

consequently allowing the selection of stylar canals from individuals falling within the respective groups.

An intact, uninjured maize ear presents a formidable barrier to pathogens like *F. verticillioides* with the apparent absence of penetration structures such as appressoria (Duncan and Howard, 2010). In the study by Duncan and Howard (2010), the stylar canal was identified as a previously undocumented infection court and thought to play a potentially important role in conferring resistance to infection by *F. verticillioides*. However, in this study there was no significant association of closed stylar canal architecture with resistance to FER before pollination or after pollination. This is in contrast to the findings of Duncan and Howard (2010) who reported an open stylar canal of the resistant (HT1) and closed stylar canal of the susceptible (AD38) maize inbred which was evident very early in development and remained so through maturity.

Miller (1919) examined various lines of dent maize and found that the open stylar canals became closed by the time the silk was ready for pollination. In this study, stylar canals were also closing as the ovaries aged, and almost all stylar canals were closed for maize kernels by 1 month after self-pollination of all the inbred lines tested. In contrast, the susceptible inbred line used by Duncan and Howard (2010), seemed to exhibit a slow closure rate and was open even 1 month after self-pollination. The susceptibility in such an inbred line may have been largely due to the formation of the stylar canals. Furthermore, resistance to FER is generally screened for by the inoculation of maize ears at the R2 blister stage (Clements *et al*., 2004). Since the inbred lines used in this study were previously characterised in this way (Small et al., 2012; Mouton, 2014), after the stylar canal had already closed, the resistance in this study could not be based on the stylar canal architecture but rather indicated towards other structural and biochemical defence mechanisms.

Although resistance to *F. verticillioides* can be mediated by numerous resistance barriers exhibited by the maize ears, including husk tightness and pericarp thickness, infection by the fungus and subsequent fumonisin contamination can also be caused by systemic infection originating from contaminated soil and seeds (Munkvold and Carlton, 1997). Genetically engineered *F. verticillioides* isolates provide a valuable means to study the host-pathogen interaction. In this study a number of genetically engineered *F. verticillioides* isolates were characterised and one of the isolates were selected for a histological investigation in maize seedlings. The fungus infected the roots of the maize seedlings and was found growing in the stems and leaves of soil- and seed-inoculated plants. This is in agreement with numerous studies that demonstrated the ability of *F. verticillioides* to infect the maize plant systemically from contaminated soil and from infected seeds (Oren *et al*., 2003; Murillo-Williams and Munkvold, 2008; Wu *et al.*, 2011; Wu *et al*., 2013).

Furthermore, Wu *et al*. (2013) demonstrated that resistance to the FER and fumonisin contamination could to a large extent be mediated during the early developmental stages of the maize plant. Wu et al. (2013) showed that the fungus was able to colonise the roots of the susceptible plants more often, frequently filling the cells of the roots with hyphae, when compared to the colonisation in resistant plants six days after inoculating newly emerged seedlings with *F. verticillioides*. In addition, FB_1 started accumulating in the roots of the maize seedlings 2 days after inoculation and after 4 days after inoculation, the susceptible plants started to accumulate more fumonisin than the resistant plants used in their study. The results of Wu *et al*. (2013) were in contrast to the results of this study as no difference could be observed in the colonisation of resistant and susceptible maize seedlings. Furthermore, green fluorescence of the fungus was not observed in any of the plant material examined, indicating that no fumonisin was produced in the maize seedlings during the specific sampling stage. However, the monitoring of the eGFP signals for an indication of fumonisin production in this study could be unreliable due to signals being too low for detection over the auto-fluorescence of the plant tissues. It was not clear if fumonisin was produced at some stages during the seedling development or if these inbreds had resistance against fumonisin contamination during the seedling stage.

Fluorescent-expressing transgenic isolates can be visualised in living tissues without any processing or manipulation of the samples. This property makes it useful for analysis of *in planta* fungal development (Lorang *et al*., 2001). However, further research using quantitative measures should be used to clarify if the differences in resistance between inbred lines are distinguishable during the early developmental stages of the plant. The colonisation of the fluorescing fungi can be quantified by observing colony forming units of the fluorescent-labelled fungi isolated from different tissues such as root, stem and leaf (Wu *et al.*, 2011; Wu *et al.*, 2013) and fumonisin production can be quantified in these tissues using LC-MS/MS (Zitomer *et al.*, 2008) or enzyme-linked immunosorbent assay (Wu *et al.*, 2013). The effective use and monitoring of fluorescent-expression isolates of *F. verticillioides* in maize, could aid in broadening current understandings of the *F. verticillioides*-maize interaction in resistant and susceptible maize genotypes.

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Table 1. Resistant and susceptible maize inbred lines evaluated for stylar canal morphology.

Inbred Line	Resistance status[a]	Source[b]
CML390	R	CIMMYT-Zimbabwe
CML444	R	CIMMYT-Zimbabwe
VO617y-2	R	ARC-South Africa
US2540	R	ARC-South Africa
RO549W	I	ARC-South Africa
RO544W	I	ARC-South Africa
R119W	S	ARC-South Africa
R2565Y	S	ARC-South Africa
I137tnW	S	ARC-South Africa

[a]The maize inbred lines were characterised as resistant (R), intermediately resistant (I) and susceptible (S) to *Fusarium* ear rot (Small *et al*., 2012; Mouton, 2014)

[b]CIMMYT: The International Maize and Wheat Improvement Centre; ARC: Agricultural Research council

Table 2. Simple sequence repeat (SSR) markers used for DNA fingerprinting of individual plants representing nine South African maize inbred lines.

Marker	Repeat[a]	Chromosome	Arm	Bin
umc1508	(ATG)4	1	Long	1.06
umc2217	(TG)6	1	Short	1.03/04
umc1165	(TA)6	2	Short	2.01/02
umc1058	(GC)7	4	Long	4.11
umc1287	(CCGTGC)4	8	Long	8.06
umc1370	(CGGG)5	9	Short	9.01
umc1982	Not available	9	Long	9.07/08
phi452121	Not available	Not available	Not available	Not available

[a]The marker repeat and positions were obtained from the maize genetics and genomics database (www.maizegdb.org – verified 9 September 2014)

Table 3. Wild-type and genetically engineered *Fusarium verticillioides* isolates characterised morphologically and molecularly in this study.

Isolate	Background	Fluorescence[a]	Origin
M-3120	Wild type	None	California, USA
M-3125	Wild type	None	California, USA
TEF Red1	Transformed, host: M-3120	DsRed	N/A
M3125-G6-1	Transformed, host: M-3125	eGFP	N/A
5-7ss4	TEF Red1 x M3125-G6-1	DsRed + eGFP	N/A
5-8ss4	TEF Red1 x M3125-G6-1	DsRed + eGFP	N/A
5-9ss5	TEF Red1 x M3125-G6-1	DsRed + eGFP	N/A
MRC 826	Wild type	None	Transkei, SA

[a]The *DsRed* gene is expressed constitutively, while the *eGFP* gene is expressed only when fumonisins are being produced

Table 4. The DNA fingerprint data of nine maize inbred lines.

	Markers[a]							
Line	**phi452121**	**umc1058**	**umc1165**	**umc1287**	**umc1370**	**umc1508**	**umc1982**	**umc2217**
CML390								
A338	215/222	88/88	151/163	106/112	123/123	127/127	202/222	145/166
A339	215/215	88/88	151/151	112/112	123/123	127/127	222/222	145/145
A341	215/215	88/88	151/151	112/112	123/123	127/127	222/222	145/145
A342	215/216	88/98	151/151	106/112	120/124	127/127	222/222	145/154
A497	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A501	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A502	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A503	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A505	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A506	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A508	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A510	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
A511	215/215	88/88	151/151	112/112	123/124	127/127	222/222	145/145
CML444								
A298	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A299	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A300	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A302	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A303	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A304	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A305	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A307	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A309	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A310	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A311	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A312	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A313	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A314	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A315	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A316	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A317	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166
A547	227/227	104/104	149/149	106/106	123/123	130/130	202/202	166/166

Line	Markers[a]							
	phi452121	umc1058	umc1165	umc1287	umc1370	umc1508	umc1982	umc2217
VO617Y-2								
A344	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
A345	216/216	98/98	163/163	106/106	124/128	127/127	222/222	165/165
A346	215/216	88/88	163/163	112/112	128/128	127/127	222/222	165/165
A347	216/216	98/98	163/163	106/106	124/124	127/127	222/222	165/165
A348	216/216	98/98	163/163	112/112	128/128	127/127	222/222	154/154
A349	216/216	88/88	163/163	106/106	128/128	127/127	228/228	154/165
A350	216/216	98/98	163/163	112/112	124/124	127/127	222/222	165/165
A351	216/227	88/98	163/163	112/112	124/128	127/127	222/228	154/165
A353	216/216	98/98	163/163	112/112	128/128	127/127	222/222	154/154
A354	216/216	98/98	163/163	112/112	128/128	127/127	222/222	154/154
A355	216/216	88/98	163/163	106/106	120/128	127/127	222/228	154/165
A357	216/216	98/98	163/163	106/106	120/120	127/127	222/222	154/154
A358	216/216	98/98	163/163	112/112	120/120	127/127	222/228	154/154
A359	216/227	88/98	163/163	112/112	124/128	127/127	222/228	154/165
A360	216/216	98/98	163/163	112/112	128/128	127/127	222/222	154/154
A361	216/216	88/88	163/163	112/112	128/128	127/127	222/222	165/165
A362	216/216	88/88	163/163	106/106	124/124	127/127	228/228	154/154
A603	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
A604	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
A607	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
A608	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
A609	216/216	88/88	163/163	106/106	128/128	127/127	222/222	165/165
R0549W								
A363	221/222	98/104	151/151	106/112	124/128	127/127	202/222	154/154
A364	221/222	98/104	163/163	112/112	124/128	130/130	202/222	154/165
A366	216/222	98/98	161/163	112/112	123/124	127/130	222/222	154/165
A367	216/228	98/104	0/0	112/112	123/124	124/130	222/222	165/165
A368	215/216	88/98	163/163	106/112	123/124	127/127	202/228	154/165
A369	227/228	98/104	0/0	106/112	123/124	127/127	222/228	165/165
A370	222/222	98/98	163/163	106/106	123/124	130/130	202/202	165/165
A371	222/222	104/104	163/163	112/112	124/124	124/130	222/228	165/165
A372	221/222	98/104	151/163	112/112	124/124	127/127	222/222	154/165
A373	222/222	98/104	0/0	112/112	124/124	130/130	222/228	165/165
A374	210/228	98/98	149/161	106/112	123/124	127/127	222/222	166/166
A375	216/221	98/104	163/163	112/112	124/124	127/127	222/222	154/165

Line	Markers[a]							
	phi452121	umc1058	umc1165	umc1287	umc1370	umc1508	umc1982	umc2217
R0549W								
A376	227/227	98/98	0/0	112/112	124/124	127/127	222/222	154/154
A377	227/228	98/104	161/161	106/112	123/124	127/130	202/222	165/165
A378	216/228	98/104	0/0	112/112	123/124	127/127	202/228	165/165
A379	216/222	98/98	161/161	112/112	124/124	127/130	202/202	154/154
A380	216/222	88/98	163/163	112/112	128/128	130/130	202/222	165/165
A381	216/222	98/98	163/163	112/112	123/124	127/130	202/202	154/165
A382	216/222	98/98	161/163	112/112	123/124	130/130	202/222	154/154
A513	216/216	98/98	163/163	112/112	123/124	130/130	202/202	165/165
A515	222/222	98/98	163/163	112/112	124/124	130/130	202/202	154/154
A516	216/216	98/98	163/163	112/112	123/124	127/130	202/202	154/165
A520	216/222	98/98	163/163	112/112	124/124	130/130	202/202	154/165
A521	216/222	98/98	163/163	112/112	123/124	127/127	202/202	154/154
US2540								
A384	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A385	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A386	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A387	216/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A388	216/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A389	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A391	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A392	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A393	227/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A394	227/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A395	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A396	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A398	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A401	227/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A402	216/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A562	216/216	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A566	227/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
A567	216/227	88/88	161/161	112/112	124/124	127/127	222/222	154/154
R119W								
A412	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A413	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164

Line	Markers[a]							
	phi452121	umc1058	umc1165	umc1287	umc1370	umc1508	umc1982	umc2217
R119W								
A414	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A415	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A416	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A416	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A417	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A418	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A419	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A420	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A421	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A422	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A423	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A424	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A425	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A426	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A427	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A429	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A430	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A618	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A622	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A625	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A626	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
R2565Y								
A431	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A432	227/227	104/104	163/163	106/106	124/128	130/130	222/222	165/165
A433	222/227	88/104	163/163	106/106	124/128	130/130	222/222	164/165
A435	222/222	88/104	163/163	106/106	124/128	130/130	222/222	165/165
A436	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A437	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A438	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A439	222/227	88/104	163/163	106/106	128/128	130/130	222/222	164/165
A440	222/227	88/104	163/163	106/106	124/124	130/130	222/222	164/165
A441	222/227	88/104	163/163	106/106	124/124	130/130	222/222	164/165
A442	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A443	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A444	227/227	88/104	163/163	106/106	128/128	130/130	222/222	165/165

Line	Markers[a]							
	phi452121	umc1058	umc1165	umc1287	umc1370	umc1508	umc1982	umc2217
R2565Y								
A447	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A447	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A448	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A449	222/222	88/104	163/163	106/106	124/128	130/130	222/222	165/165
A450	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A583	222/227	104/104	163/163	106/106	128/128	130/130	222/222	164/165
A584	222/227	88/104	163/163	106/106	124/128	130/130	222/222	165/165
A586	222/227	88/88	163/163	106/106	124/128	130/130	222/222	164/165
A588	222/227	104/104	163/163	106/106	124/124	130/130	222/222	164/165
A589	222/227	88/104	163/163	106/106	124/124	130/130	222/222	164/165
A591	222/227	104/104	163/163	106/106	124/128	130/130	222/222	164/165
R0544W								
A451	216/216	88/88	149/149	106/106	124/124	127/130	202/202	166/166
A452	216/216	88/88	161/161	106/106	124/124	127/127	228/228	165/165
A453	216/216	88/88	149/149	106/106	124/124	127/127	202/202	165/165
A454	215/216	88/98	149/161	106/106	124/124	127/127	202/222	154/166
A455	216/216	88/88	149/149	106/106	124/124	127/130	202/228	165/165
A456	215/216	88/98	149/161	106/106	123/124	127/130	202/222	154/166
A457	216/216	88/88	149/149	106/106	123/123	127/127	202/202	166/166
A458	216/216	88/88	149/149	106/106	123/124	130/130	202/202	166/166
A459	216/216	88/88	149/149	106/106	124/124	127/127	202/202	165/166
A460	216/216	88/88	149/149	106/106	124/124	130/130	202/202	165/165
A461	216/216	88/88	149/149	106/106	124/124	127/130	202/202	165/165
A462	216/216	88/88	161/161	106/106	124/124	127/127	228/228	165/165
A463	216/216	88/88	149/149	106/106	124/124	127/127	202/202	165/165
A464	216/216	88/88	149/149	106/106	123/124	127/127	202/202	165/165
A465	216/216	88/88	149/149	106/106	123/124	127/127	202/202	165/165
A466	216/216	88/88	149/149	106/106	123/124	127/130	202/228	165/166
A467	216/216	88/88	149/149	106/106	124/124	130/130	202/202	166/166
A468	216/216	88/88	149/149	106/106	124/124	127/130	202/202	165/166
A469	216/216	88/88	161/161	106/106	124/124	127/127	202/228	165/165
A525	216/216	88/88	149/149	106/106	123/124	127/127	202/202	165/165
A527	216/216	88/88	149/149	106/106	124/124	127/127	202/202	165/165
A537	216/216	88/88	149/149	106/106	124/124	127/127	202/202	156/156
A538	216/216	88/88	149/149	106/106	124/124	127/127	202/202	165/165

	Markers[a]							
Line	**phi452121**	**umc1058**	**umc1165**	**umc1287**	**umc1370**	**umc1508**	**umc1982**	**umc2217**
I137tnW								
A471	222/227	104/104	160/163	100/100	124/124	130/130	222/222	154/154
A472	222/227	88/88	160/163	100/106	124/124	130/130	222/222	154/164
A473	215/227	98/104	163/163	106/112	120/128	130/130	222/222	165/165
A474	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A475	210/227	98/104	161/163	112/112	124/128	130/130	222/228	164/166
A476	222/222	88/104	161/161	106/112	128/128	130/130	222/228	164/166
A477	216/227	98/104	163/163	106/106	124/128	127/130	222/228	164/166
A478	222/227	88/88	160/163	100/106	128/128	130/130	222/222	164/164
A480	227/227	88/88	163/163	106/106	128/128	130/130	202/202	164/164
A481	222/222	88/88	149/163	106/106	128/128	130/130	222/222	164/164
A482	216/222	88/88	149/163	106/106	124/128	127/130	202/222	164/166
A484	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A485	222/222	88/104	163/163	100/106	124/128	130/130	222/222	164/164
A486	227/227	88/104	160/163	100/100	124/128	130/130	222/222	154/164
A487	222/227	88/88	163/163	106/106	124/128	127/130	222/222	164/164
A488	222/227	104/104	149/163	106/106	124/124	127/130	222/222	164/164
A489	222/227	88/104	163/163	100/106	128/128	127/130	222/222	165/165
A490	227/227	88/88	163/163	106/106	128/128	130/130	222/222	164/164
A491	216/222	88/98	163/163	106/112	120/128	130/130	202/222	165/166
A492	216/227	88/98	163/163	106/112	124/128	127/130	222/222	154/165
A493	222/227	104/104	149/163	106/106	124/128	127/130	222/222	164/165
A631	222/222	88/88	163/163	100/106	128/128	130/130	222/222	164/164

[a]Marker data expressed in amplified base-pair length

Table 5. The stylar canal architecture of nine maize inbred lines resistant or susceptible to Fusarium ear rot and fumonisin contamination.

		Before pollination[b]			After pollination[c]		
Inbred line	**Status[a]**	**Closed**	**Open**	**Closed (%)**	**Closed**	**Open**	**Closed (%)**
CML390	R	21	2	91.30	25	1	96.15
CML444	R	0	36	0.00	26	3	89.66
VO617Y-2	R	4	24	14.29	21	1	95.45
US2540	R	7	21	25.00	31	0	100.00
RO549W	I	0	30	0.00	21	0	100.00
RO544W	I	20	1	95.24	28	0	100.00
R119W	S	10	15	40.00	36	1	97.30
R2565Y	S	12	8	60.00	24	2	92.31
I137tnW	S	2	24	7.69	25	0	100.00

[a]Inbred lines resistant (R), intermediately resistant (I) and susceptible (S) to Fusarium ear rot (Small *et al*., 2012; Mouton, 2014)

[b]Stylar canal architecture of young maize ears sampled 1-2 weeks before pollination

[c]Stylar canal architecture of maturing maize kernels sampled approximately 1 month after self-pollination

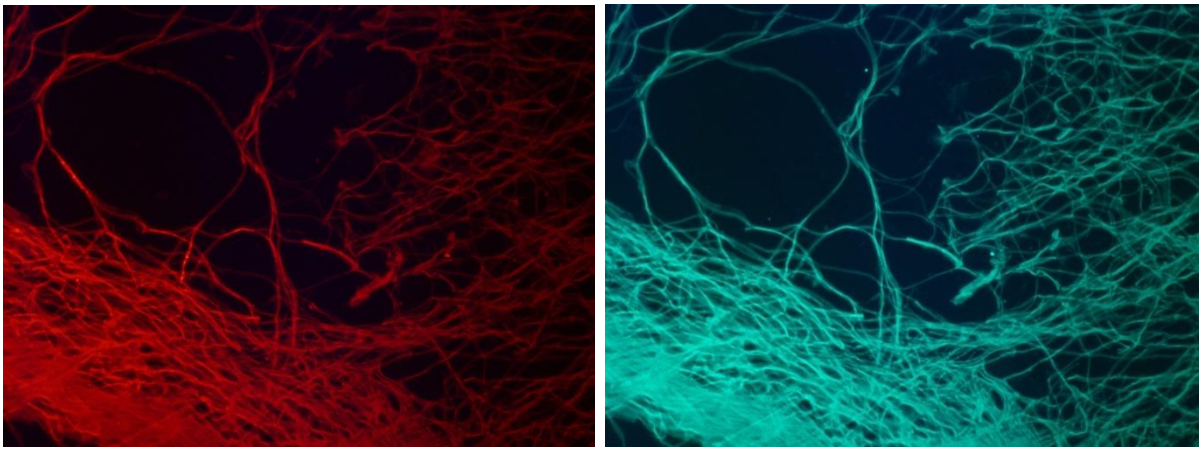

Figure 1. Red (*dsRed*) and green (*eGFP*) fluorescence of the *Fusarium verticillioides* isolate 5-9ss5.

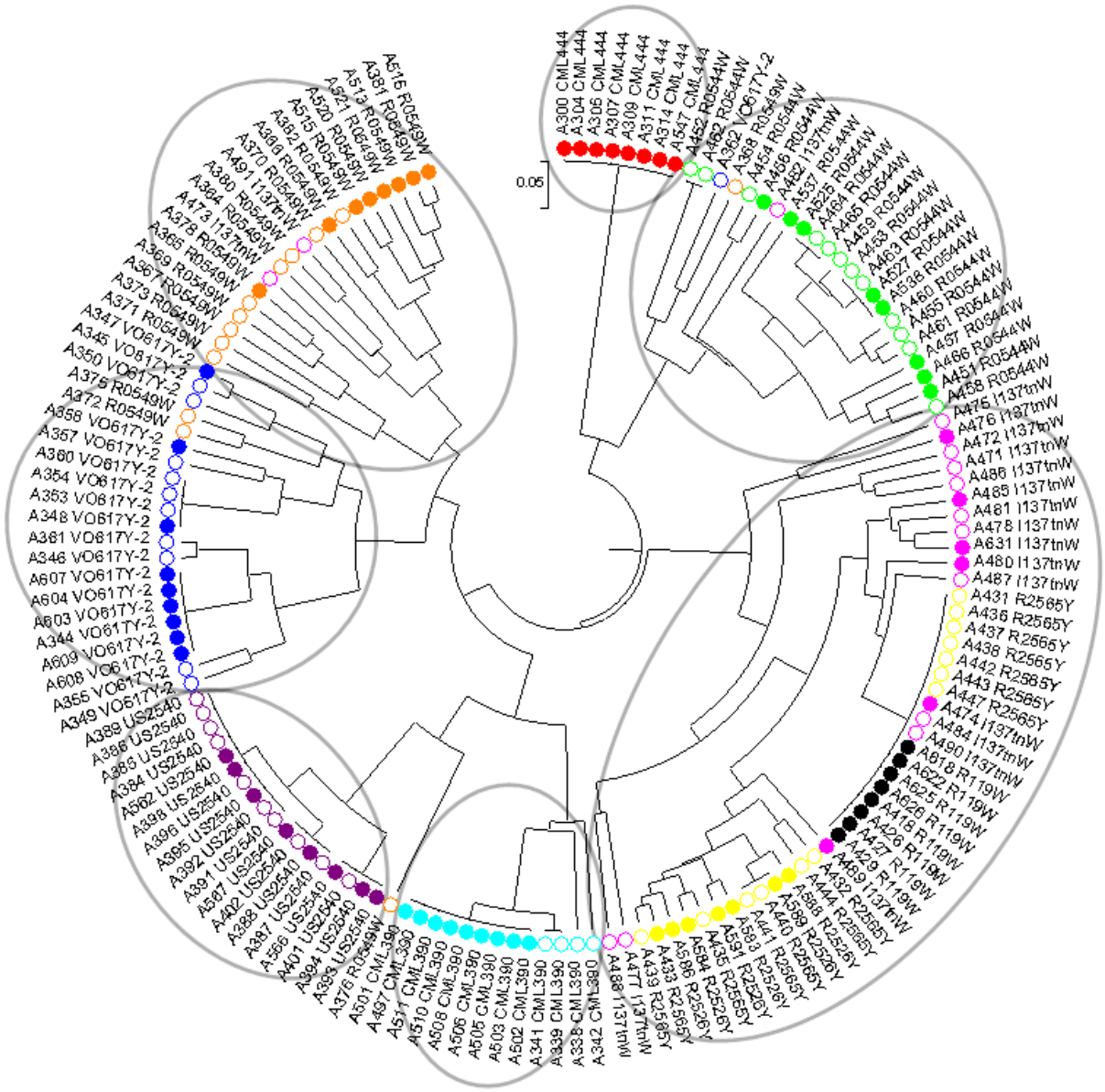

Figure 2. Dendrogram of the individual maize plants representing their inbred lines. The colours correspond to an inbred line while the filled circles represent the individuals from which young maize ears and kernels were sampled. The large ovals notate clusters of individuals falling within similar genetic groups.

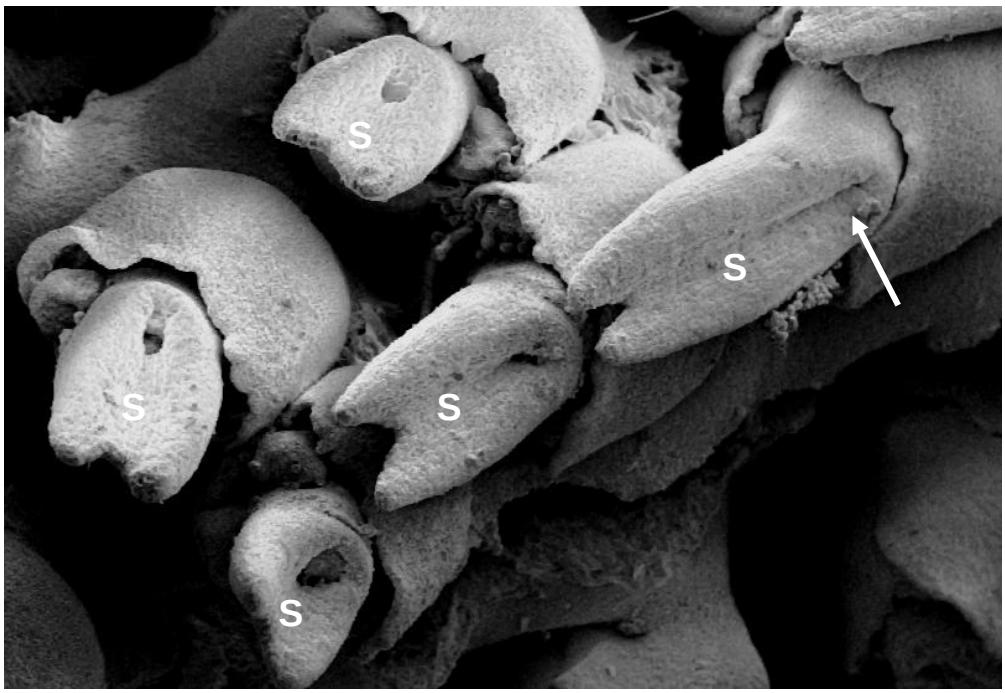

Figure 3. Stylar canals of a young maize ear of the resistant inbred line CML390 sampled before pollination. The stage of development could be indicated by the length of the silks (S) while the arrow shows the closure of a stylar canal.

Figure 4. Open stylar canals of a young maize ear of the intermediately resistant inbred line RO549W sampled before pollination.

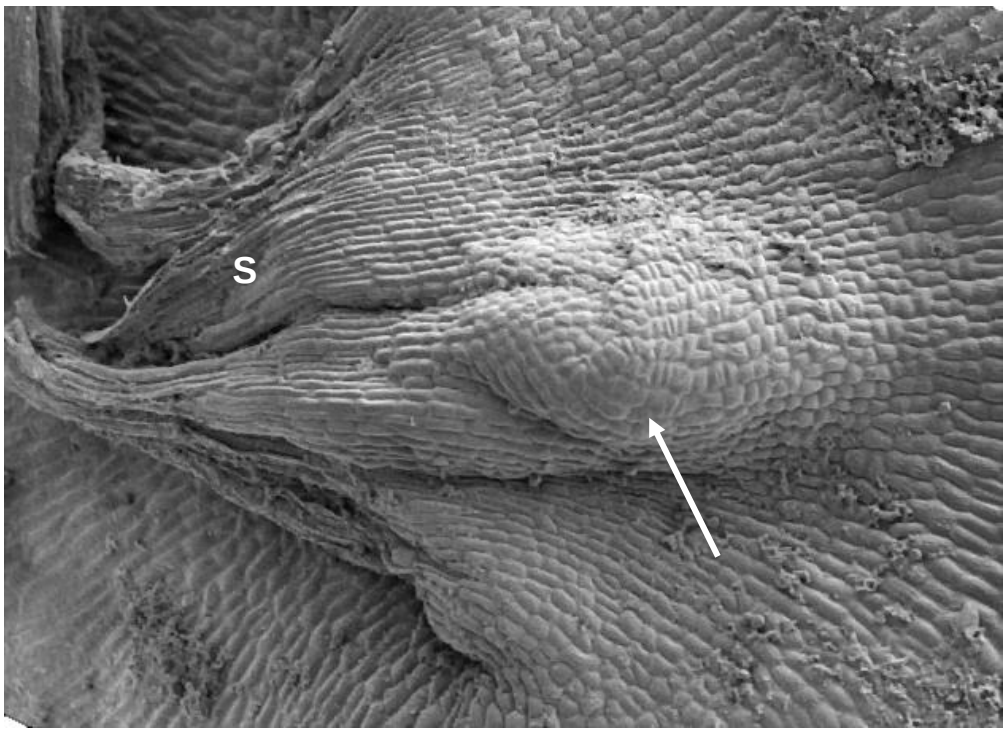

Figure 5. A raised ridge (indicated by the arrow) of the stylar canal region of a susceptible inbred line I137tnW after pollination. The silk (S) is still visible on the developing maize kernel.

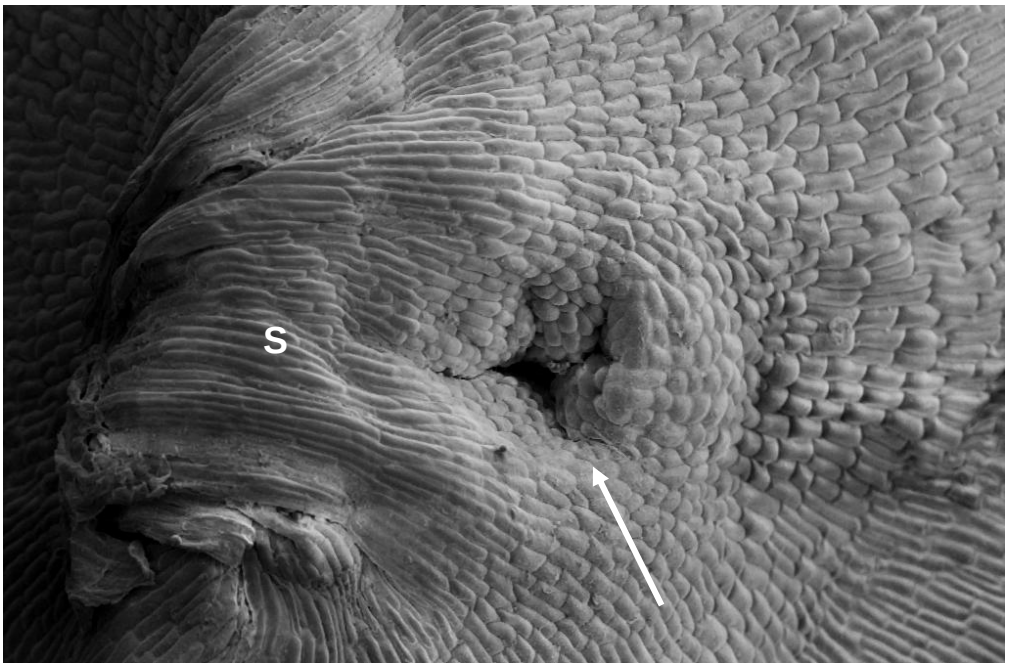

Figure 6. An opening in the stylar canal region (indicated by the arrow), near the silk (S), observed in the resistant inbred line CML444 after pollination.

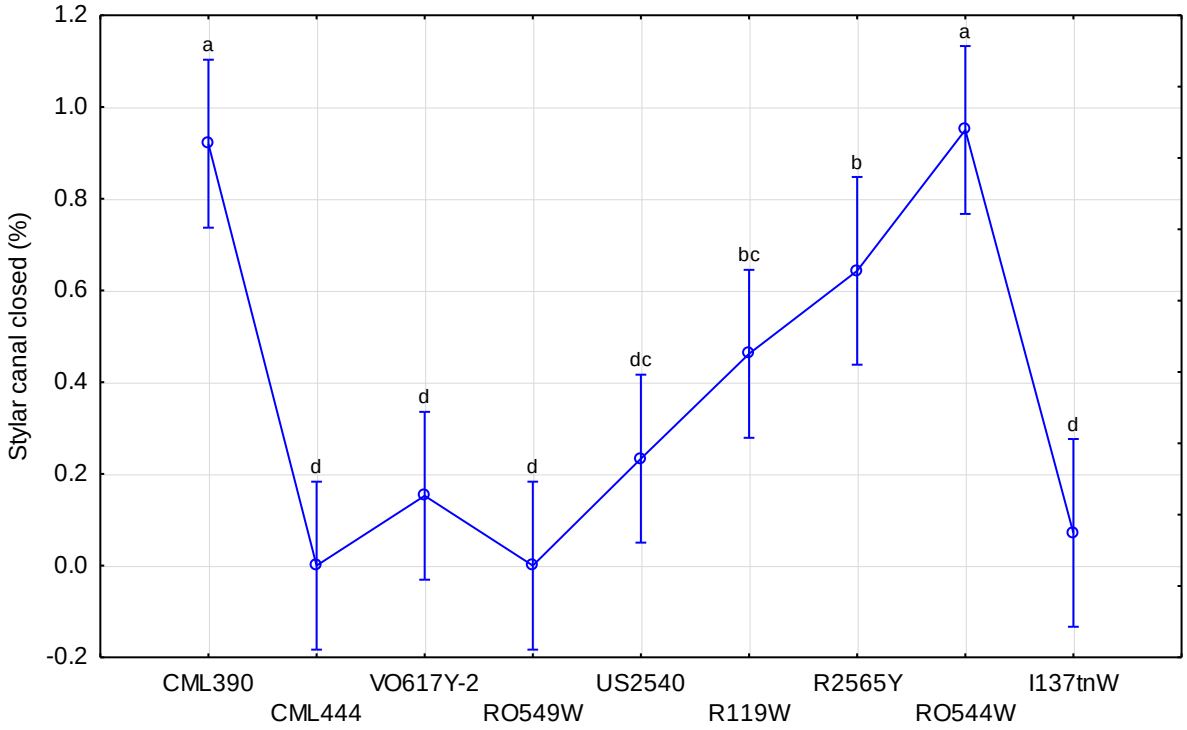

Figure 7. A graph representing the percentage of samples that exhibited closed stylar canals in nine maize inbred lines prior to pollination. The letters represent the significant difference of stylar canal architecture between inbred lines and the bars denote the confidence intervals ($P < 0.05$).

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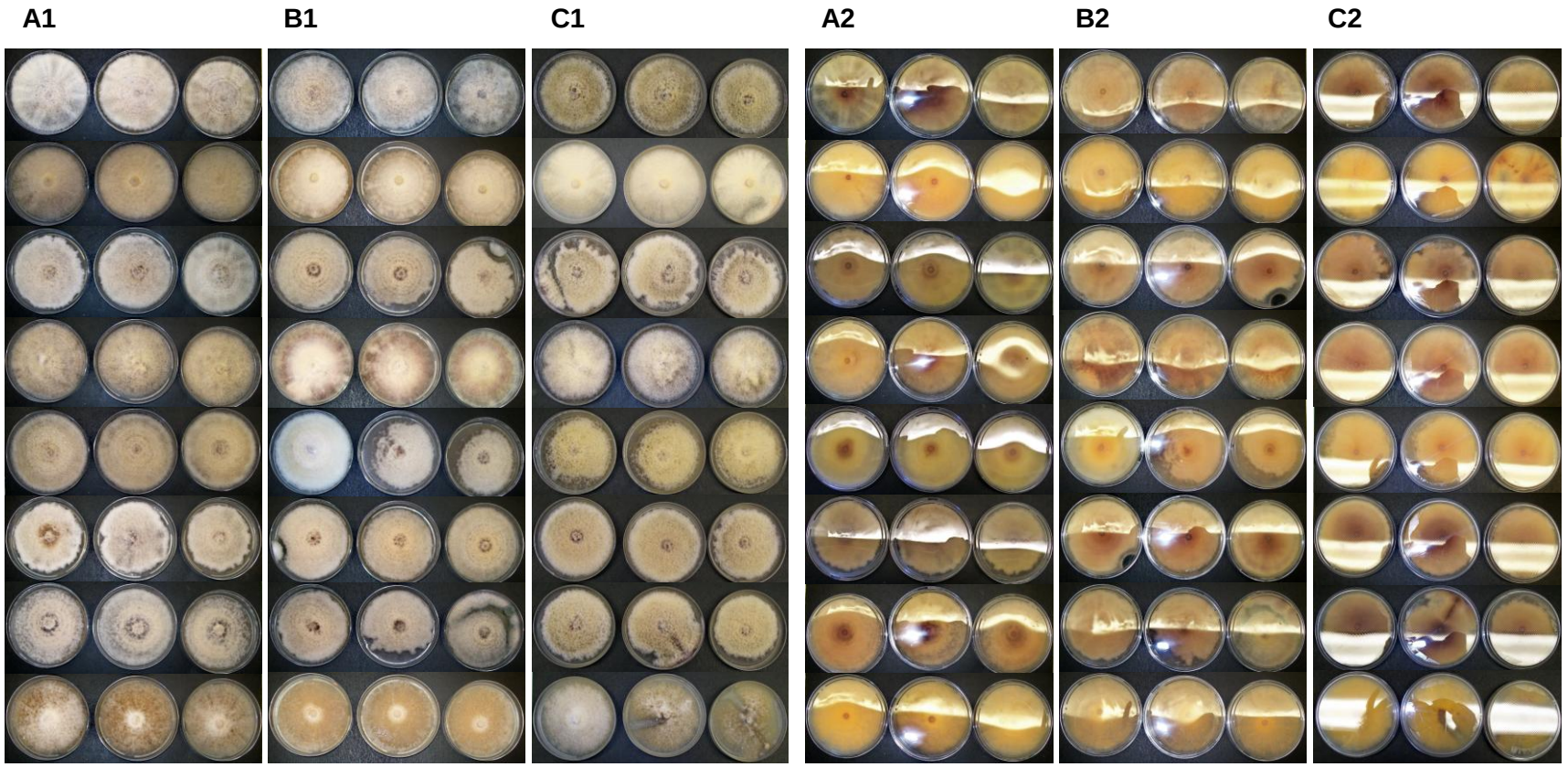

Figure 8. The colour of wild-type and genetically engineered *Fusarium verticillioides* isolates. Viewed from above (**A1-C1)** and below (**A2-C2**). Isolates M-3120, M-3125, TEF Red1, M3125-G6-1, 5-7ss4, 5-8ss4, 5-9ss5 and MRC 826 were placed in the respective order from top to bottom.

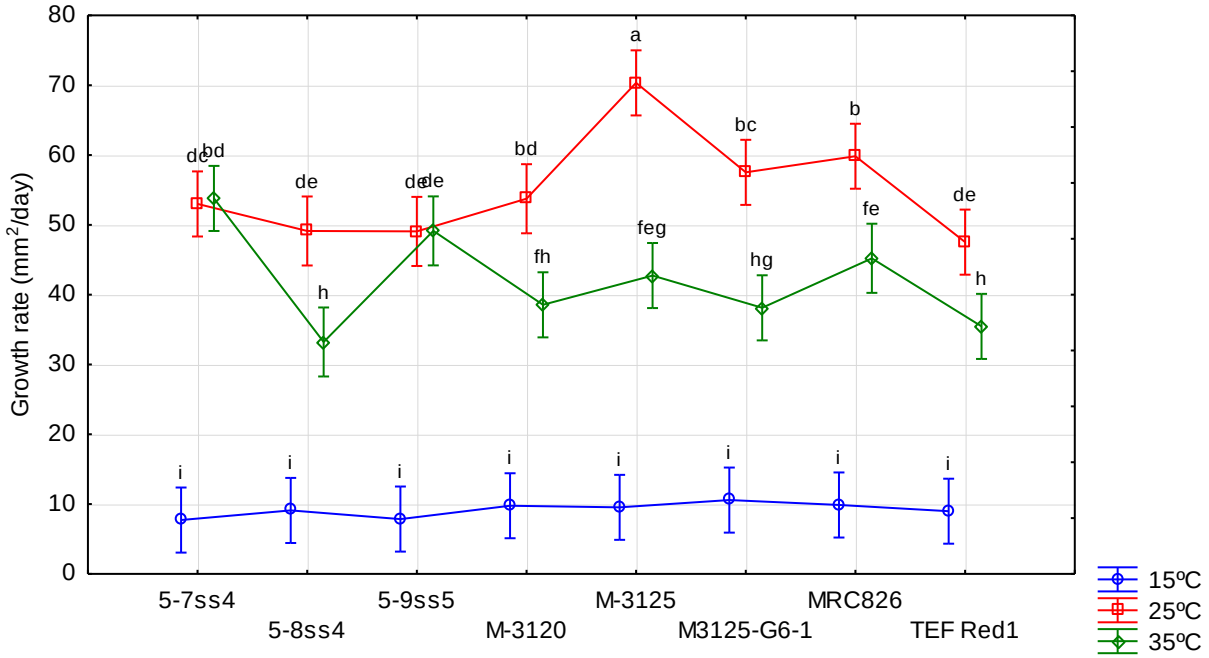

Figure 9. Growth-rates of the wild-type and genetically engineered *Fusarium verticillioides* isolates at three temperatures. Significant differences between isolates are indicated by letters while the bars denote the confidence intervals (P < 0.05).

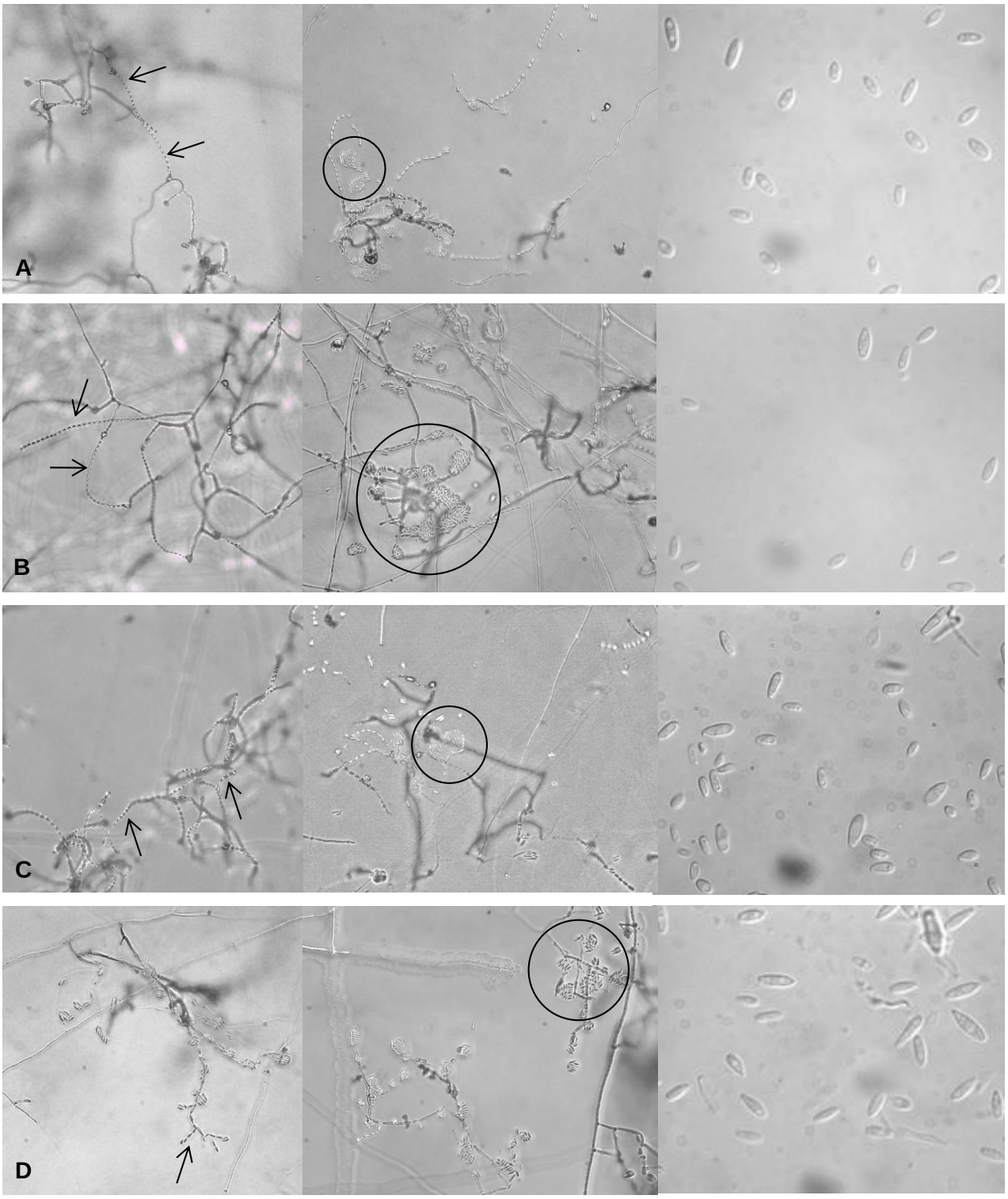

Figure 10. Conidial chains, false-heads and microconidia formations of *Fusarium verticillioides.* The chains (showed by arrows) and false-heads (encircled) are viewed at 400x magnification while the spores (pictures on the far right) are captured at 1000x magnification for **(A)** M-3120, **(B)** M-3125, **(C)** 5-9ss5 and **(H)** MRC 826.

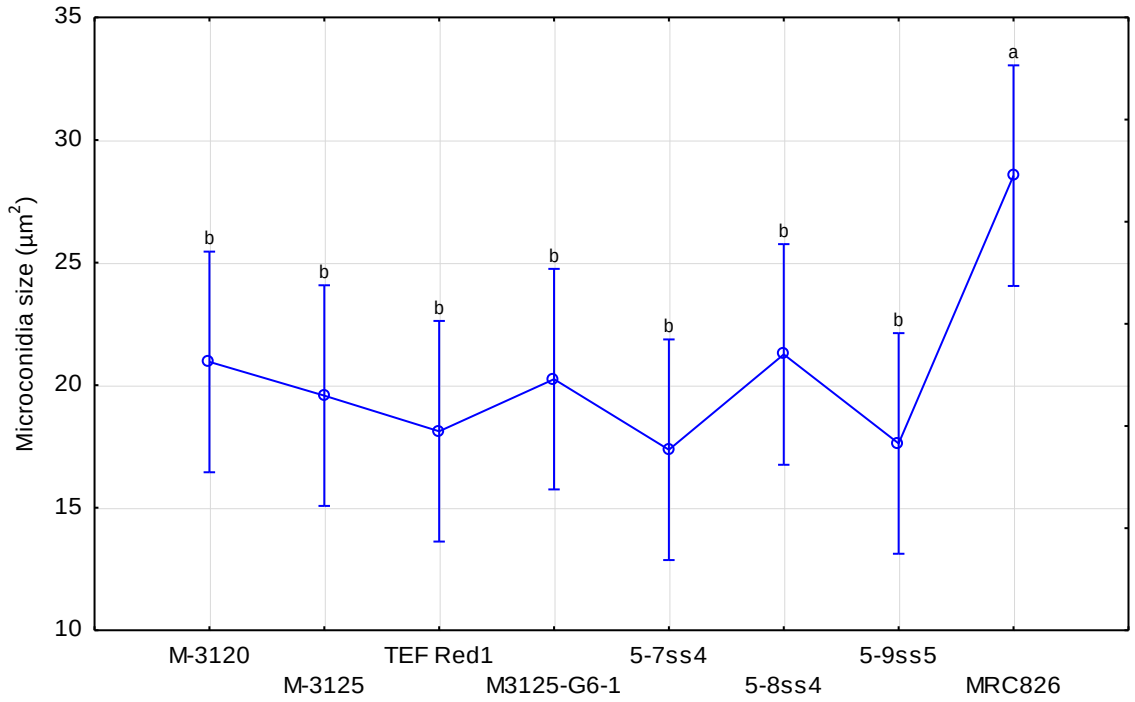

Figure 11. Microconidial sizes of the wild-type and genetically engineered *Fusarium verticillioides* isolates. Significant difference among the isolates with regard to spore size is shown by the letters while the bars indicate the confidence intervals (P < 0.05).

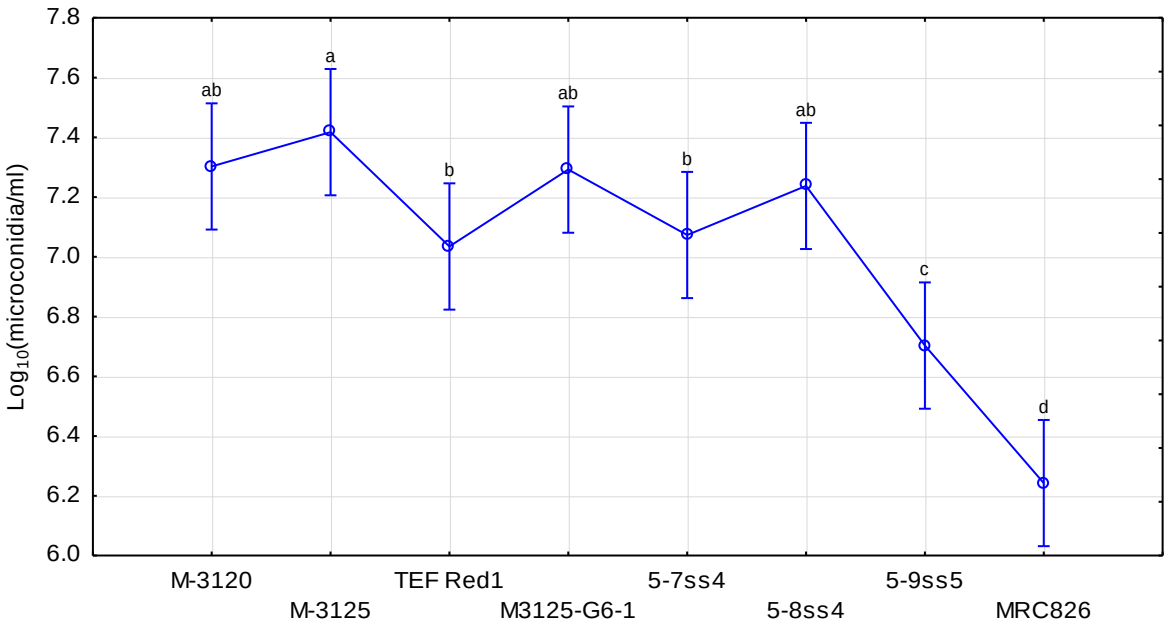

Figure 12. Spore producing potential of the wild-type and genetically engineered *Fusarium verticillioides* isolates. Significance is denoted by the letters and the confidence intervals are shown by the bars (P < 0.05).

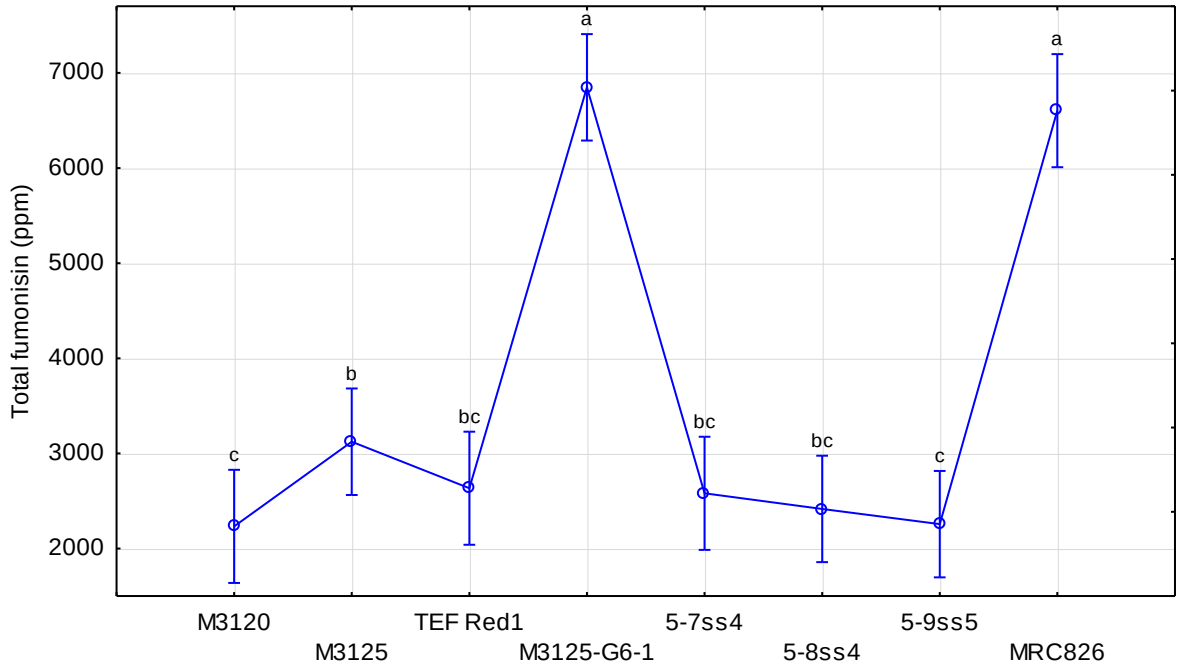

Figure 13. Fumonisin producing potential of the wild-type and genetically engineered *Fusarium verticillioides* isolates. Letters indicate the significant difference while bars denote the confidence intervals ($P < 0.05$).

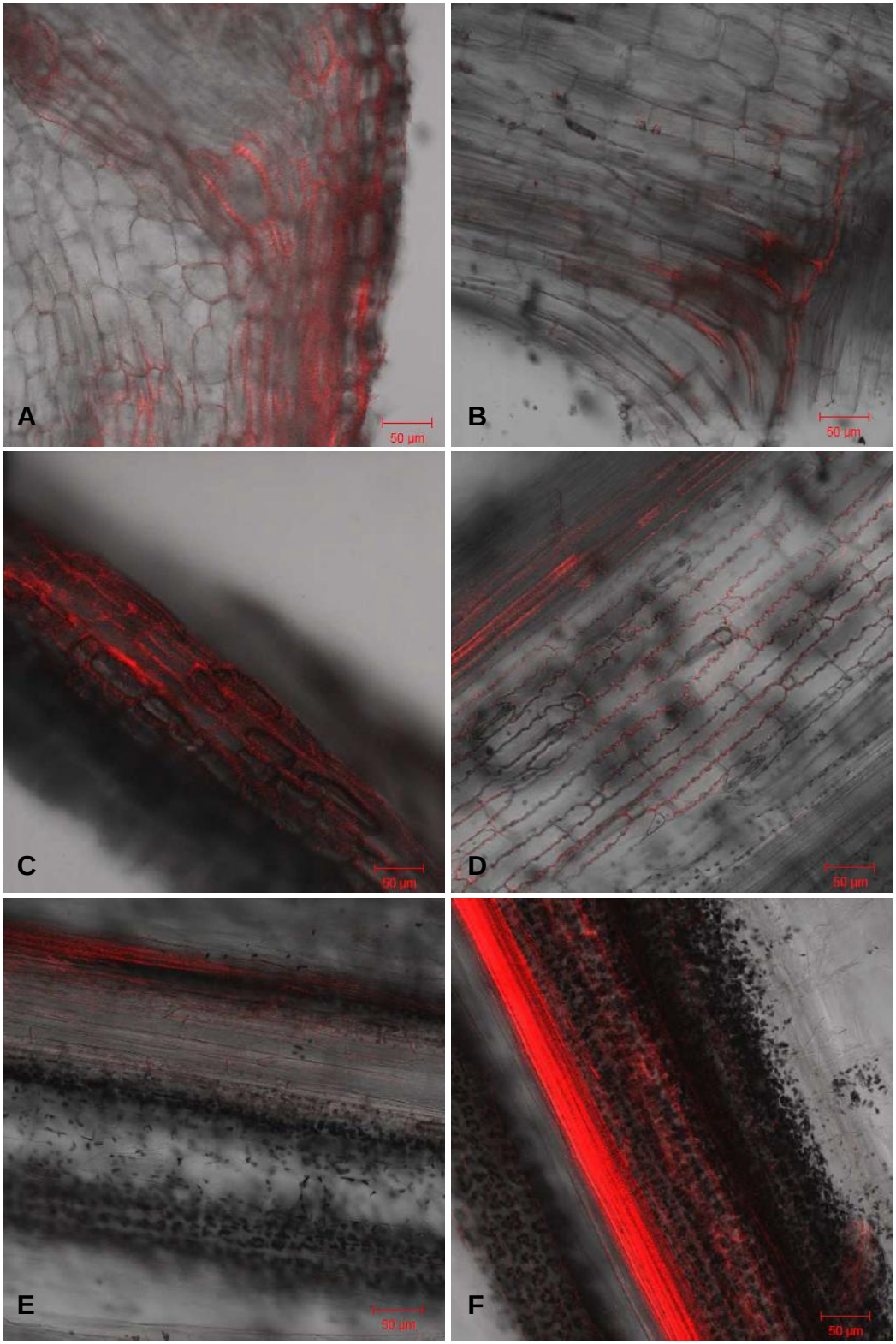

Figure 14. The *Fusarium verticillioides* isolate 5-9ss5 colonised the roots, stems and leaves of maize seedlings of both resistant (CML390) and susceptible (R2565Y) inbred lines. The fungal growth is represented by the red signal in the root **(A** and **B)**, stem **(C** and **D)** and leaf **(E** and **F)** tissues of inbred lines CML390 **(A**, **C** and **E)** and R2565Y **(B**, **D** and **F)**.