

Elucidating the *Fusarium graminearum* species complex on maize in South Africa

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DECLARATION

I declare that the dissertation submitted by me for the degree Masters in Environmental Science at the North-West University (Potchefstroom Campus), Potchefstroom, North-West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

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“Blessed are those who find wisdom, those who gain understanding” - Proverbs 3:13

LIST OF ABBREVIATIONS

°C	degree Celsius
ADON	acetydeoxynivalenol
AFLP	amplified fragment length polymorphism
ANOVA	Analysis of variance
ARC	Agriculture Research Council
ARC – GCI	Agriculture Research Council – Grain Crops Institute
bp	base pair
<i>Bt</i>	<i>Bacillus thuringiensis</i>
cm	centimetre
CTAB	cetyl trimethylammonium bromide
C _q	quantitation cycle
DEB	DNA extraction buffer
DNA	deoxyribonucleic acid
DON	deoxynivalenol
EDTA	ethylene diamine tetraacetic acid
EF	elongation factor
FAO	Food and Agricultural Organisation of the United Nations
FGSC	<i>Fusarium graminearum</i> species complex
FHB	Fusarium head blight
g	gram
GCPSR	genealogical concordance phylogenetic species recognition
GER	Gibberella ear rot
<i>H3</i>	histone 3
ha	hectare
HCl	Hydrochloric acid
HPLC	high performance liquid chromatography
<i>IGS</i>	intergenic spacer
ISSR	inter-simple sequence repeat
<i>ITS</i>	internal transcribed spacer
kg	kilogram
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LSD	least significant difference
M	molar
mg	milligram

min	minutes
ml	millilitre
MLGT	multilocus genotyping
mM	millimolar
N	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NIV	nivalenol
PCR	polymerase chain reaction
PCR – RFLP	polymerase chain reaction - restriction fragment length polymorphism
PDA	potato dextrose agar
pg	picogram
qPCR	quantitative real-time polymerase chain reaction
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
sec	seconds
SDS	sodium dodecyl sulfate
SRAP	sequence related amplified polymorphism
SSCP	single strand conformational polymorphism
SQ	starting quantity
<i>TEF</i> α	translation elongation factor alpha
TCT	trichothecenes
TCT-B	type B trichothecenes
μ l	microlitre
μ M	micromolar
μ g	microgram
ZEA	zearalenone

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PREFACE

This dissertation consists of five chapters. The main objective of this study was to elucidate the presence of *Fusarium graminearum* species complex (FGSC) on maize in South Africa.

Chapter 1 provides an overall literature review of FGSC members associated with Gibberella ear-, root- and stalk rot including host range, sources of inoculum, symptoms and economic impact of these diseases. This chapter also includes the mycotoxins caused by this pathogen and the methods used to detect this species group.

In **chapter 2** the molecular detection, identification and quantification techniques of FGSC members were evaluated. Species-specific polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to identify members of the FGSC collected from maize stems, ears, crowns and roots sampled over South Africa on diseased maize.

Chapter 3 deals with the significance of FGSC members' ability to infect maize ears as well as stems. A glasshouse trial was conducted and ears and stems were inoculated to evaluate pathogenicity of different FGSC members on different maize tissue. Quantitative real-time PCR (qPCR) was used for simultaneous detection and quantification of FGSC deoxyribonucleic acid (DNA) in maize grain and stem samples. The different mycotoxins and concentrations produced by three different artificially inoculated members of the FGSC was also evaluated using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Chapter 4 provides an overview of FGSC members occurring naturally in maize stems as well as the possible correlation with maize stem borers also occurring in the stems. Field trials were conducted in Potchefstroom and in Vaalharts to study the succession of FGSC members and stem borer occurrence over a maize growing season. Real-time PCR was used for qualitative and quantitative analysis to detect FGSC pathogens in the samples.

To conclude the four chapters, **chapter 5** highlights the importance of FGSC members on South African maize.

ABSTRACT

Maize is one of the most important grain crops in South Africa as it serves as the staple diet of millions of people. The increasing populations and demand for food crops are not the only constraint of cereal production, an average percentage of 10-30% is lost due to fungal infections. *Fusarium graminearum* species complex (FGSC) is the most global complex causing diseases on various grain crops worldwide. FGSC causes diseases on maize such as Gibberella ear-, stalk-, root- and crown rot. This species complex can cause yield losses in crops of up to 70%. Recent studies indicated the increasing FGSC levels in South Africa. Biotic constraints on maize such as stem borers can also cause wounds which provides entry points for fungal infection. Furthermore, the infection of FGSC members poses a health threat for humans and animals as this species complex produce secondary toxic metabolites known as mycotoxins. The most common mycotoxins include, zearalenone (ZEA), deoxynivalenol (DON) and nivalenol (NIV). Thus, the importance of monitoring diseases in crops is emphasised and the prediction of disease epidemics lies in the proper identification, management and understanding of genetic diversity and population biology. Fungal identification are fundamental requirements when managing or studying diseases caused by FGSC members. Molecular techniques based on analysis of DNA such as polymerase chain reaction (PCR), species-specific PCR and PCR- restriction fragment length polymorphism (PCR-RFLP) were therefore evaluated to identify FGSC members on diseased maize. Multilocus genotyping (MLGT) were used to evaluate the accuracy of the PCR-based techniques. MLGT were able to identify two new species on maize, *F. lunulosporum* and *F. cerealis*, this is also a first report of these two species on maize. A hybrid species, *F. graminearum* x *F. boothii* was also detected in these samples using the MLGT technique. To study plant part preference by FGSC members, the pathogenicity, colonisation and mycotoxin production of three FGSC members (*F. graminearum*, *F. boothii*, *F. graminearum* x *F. boothii*) collected from diseased maize were tested by the artificial inoculation on maize stems and ears in glasshouse conditions. Fungi are not just able to be pathogenic in maize- ears, roots and crowns, but showed in this study that fungi can also successfully infect maize stems. Different mycotoxins such as zearalenone and nivalenol and was produced in different levels by the isolates in different plant parts. Natural occurring FGSC members in field conditions occurring during the growing season of 2016/17 were evaluated in Vaalharts and Potchefstroom, as well as the possible relationship with natural occurring maize stem borer on maize. A trend of FGSC

members in maize stems for all three cultivars was noted in Vaalharts and Potchefstroom, the most vulnerable stage of maize growth is at 70 days when the plant is busy with grain fill.

Keywords: *Fusarium graminearum* species complex; polymerase chain reaction; pathogenicity; mycotoxins; stem borers

OPSOMMING

Mielies is een van die mees belangrikste graan gewasse in Suid Afrika omdat dit dien as stapel voedsel van miljoene mense. Die groeiende populasie en vraag na voedsel gewasse is nie die enigste risiko's op graan produksie nie, 'n gemiddelde persentasie van 10-30% verlies is toegeken aan swam infeksies. *Fusarium graminearum* spesie kompleks (FGSK) is die mees globale kompleks wat siektes op verkeie graan gewasse wêreldwyd veroorsaak. FGSK veroorsaak siektes soos Gibberella kop-, stam-, wortel- en kroon vrot op mielies wêreldwyd. Opbrengs verliese van tot 70% in gewasse kan deur hierdie kompleks veroorsaak word. Onlangse studies het aangedui dat die FGSK huidiglik besig is om toe te neem. Biotiese beperkinge op mielies soos stam boorders kan ook wonde veroorsaak wat toegang verskaf vir swamme. Verder, hou infeksie deur FGSK lede 'n gesondheidsrisiko in vir mense en diere deur die sekondêre toksiese metaboliete wat hierdie kompleks produseer, beter bekend as mikotoksiene. Die mees algemene mikotoksiene sluit in, zearalenone (ZEA), deoxynivalenol (DON) en nivalenol (NIV). Die belangrikheid om siektes in gewasse te monitor word dus beklemtoon en die voorspelling van siekte epidemies lê in die behoorlike identifikasie, beheer en die verstaan van genetiese diversiteit en populasie biologie. Swam identifikasie is die fundamentele vereiste wanneer siektes wat veroorsaak word deur die FGSK bestuur of bestudeer word. Molekulêre tegnieke gebaseer op analyses van DNA soos polimerase kettingreaksie (PKR), spesie-spesifieke PKR en PKR- restriksie fragment lengte polimorfisme (PKR-RFLP) se akuraatheid was daarom geëvalueer om FGSK lede op geïnfekteerde mielies te identifiseer. Multilokus genotipering (MLGT) was gebruik om die akuraatheid van die PKR-gebaseerde tegnieke te evalueer. MLGT was in staat om twee nuwe spesies op mielies te identifiseer, naamlik; *F. lunulosporum* en *F. cerealis*, hierdie is ook die eerste verslag van hierdie twee spesies op mielies. 'n Hibriede spesie, *F. graminearum* x *F. boothii* was ook opgespoor in die monsters deur die MLGT tegniek. Om plantdeel voorkeur deur FGSK lede te bestudeer is die patogenisiteit, kolonisasie en mikotoksien produksie van drie FGSK lede (*F. graminearum*, *F. boothii*, *F. graminearum* x *F. boothii*), verkry vanaf geïnfekteerde mielies, deur die kunsmatige inokulasie op mielie stamme en koppe in glashuis kondisies te toets. Swamme is nie net in staat om mielie-koppe, wortels en krone te infekteer nie, maar dit is in hierdie studie bewys dat mielie-stamme ook suksesvol besmet kan word. Verskillende mikotoksiene soos zearalenone en nivalenol was geproduseer in verskillende vlakke deur die isolate op verskillende plant dele. Natuurlike voorkoms van FGSK lede in veld kondisies oor die 2016/17 groei seisoen was ook

geëvalueer in Vaalharts en Potchefstroom, met die moontlike invloed van mielie stam boorders. 'n Herhalende tendens van FGSK lede in mielies vir drie kultivars is waargeneem in Vaalharts asook in Potchefstroom, met die mees kwesbaarste stadium op 70 dae genoteer wanneer die plant besig is met graan vul.

Sleutelwoorde: *Fusarium graminearum* spesie kompleks; polimerase kettingreaksie; patogenisiteit; stam boorders

CHAPTER 1 – GENERAL INTRODUCTION

1.1. Introduction

The annual world crop production of cereals is increasing due to the continuous growing population and their demand for food throughout the world (FAOSTAT, 2017; Munkvold, 2003). Maize is one of the most important grain crops in South Africa as it serves as the staple diet for millions of people (Du Plessis, 2003). Maize production is constrained by various biotic and abiotic factors (Nelson *et al.*, 1983). Insect damage, diseases caused by fungi, viruses and bacteria, drought and nutrient deficiency are some of the most important common causes for yield and grain quality reduction. Yield and seed quality can be greatly reduced by plant diseases. Plant pathogens cause symptoms in several ways and each pathogen has evolved a unique mode of causing an infection such as entering a plant through natural openings and wounds (Wise *et al.*, 2016). For a diseases to occur in a plant, three components need to be favourable. A susceptible host, a disease causing pathogen and a favourable environment suitable for disease development are the three elements that needs to be simultaneously present for a disease to exist (Agrios, 2005).

Fusarium graminearum species complex (FGSC) is a major plant pathogen and causes Gibberella root-, crown-, stalk- and ear rot on maize. Previously this pathogen was known as *F. graminearum* sensu lato and was later changed to FGSC (McMullen *et al.*, 2012; Kazan *et al.*, 2012, Rose *et al.*, 2015). The known members of this group infecting maize in South Africa is still limited. Maize diseases can reduce yields, grain and seed quality (Wise *et al.*, 2016). An estimated percentage of between 10-30% of the millions of hectares of annually cultivated harvest is lost due to pest and fungal infections (Eskola, 2002; Munkvold, 2003, FAOSTAT, 2017). The importance of the continuation of monitoring FGSC in cereal grains in South Africa is highlighted since infected grains can be contaminated with mycotoxins such as deoxynivalenol (DON), zearalelone (ZEA) and nivalenol (NIV). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a method used for the quantitative and qualitative detection of mycotoxins (Nordby *et al.*, 2007; Boutigny *et al.*, 2011).

Pathogens can also be transmitted by insects that feed on plants. Being responsible for an average of 10% yield loss on maize in South Africa, *Busseola fusca* (Fuller) (Lepidoptera:

Noctuidae) is the most important and destructive lepidopteran pest on maize which has a big economic impact in many maize growing countries (Mally, 1920; James, 2013). Stalk and ear rot diseases can be influenced by Lepidopteran pests (Smeltzer, 1958; Dowd, 1998). An important mechanism to manage stem borers is to plant transgenic *Bt*-maize hybrids. However, the interactive effect of *B. fusca* and FGSC in *Bt* and non-*Bt* hybrids in maize stems has not been studied in South Africa.

The foundation of disease epidemics prediction and disease management lies in the understanding of genetic diversity and population biology and identification of a disease (Burlakoti *et al.*, 2008). The FGSC consists of a large group of species that are morphologically similar which makes identification challenging to depend on the use of morphological characteristics alone (Leslie and Summerell, 2006). Molecular techniques based on analysis of DNA and RNA such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) have been used to resolve the complex identification of FGSC members to species level (Edwards *et al.*, 2002). PCR provides a fast and specific tool and have been used to investigate FGSC members from a complex pool of DNA, based on amplification of specific DNA fragments (Jurado *et al.*, 2005). Real-time PCR, which is a fast, reliable and convenient method to use, can detect pathogens and also offers an alternative technique for both quantitative and qualitative analysis (Sarlin *et al.*, 2006). Members of the FGSC have also been identified using multilocus genotyping (MLGT) assay, which is a highly accurate, yet expensive method to distinguish between closely related species within the FGSC (Ward *et al.*, 2008).

1.2 Maize overview

Maize (*Zea mays* L.) is a member of the grass family *Poaceae* and is grown under a variety of environmental conditions around the world. Maize is one of the most important grain crops in South Africa as it serves as the staple diet for millions of people (Du Plessis, 2003). Maize, as well as sorghum (*Sorghum bicolor* L.) (Moench), barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) forms part of the top most important cereal crops in the world (Strange and Scott, 2005; Nicolaisen *et al.*, 2009). The forecast report of the annual world crop production of cereals estimated to exceed 3.3 billion tons of harvested cereal crops in 2018 (FAOSTAT, 2017). This figure is increasing due to the continuous growing population and their demand for food throughout the world. An annual estimated percentage

of cultivated harvest that is lost due to fungal infections ranges between 10-30% (Eskola, 2002; Munkvold, 2003; FAOSTAT, 2017).

South Africa is the primary maize producer within the Southern African Development Community (SADC), with millions of small-scale subsistence producers and over 9000 commercial recognized maize producers. Nearly 50% of the required maize supply for the SADC region is sourced from South Africa (Akpalu et al., 2008). South Africa is also listed by the Food and Agriculture Organisation amongst the top 20 maize producing countries worldwide (FAOSTAT, 2017).

Production of maize is supported by advanced cultivation practices, high yields per hectare and adaptability to different agro-ecological zones (Fandohan *et al.*, 2003). Seasonal participation in South Africa fluctuates, thus annual maize yields also vary considerably (Du Toit *et al.*, 2002). Maize production is practiced on over 60% of South Africa's arable areas and contributes to up to 70% of the total grain production. White as well as yellow maize is produced in South Africa, the production of white maize is mainly produced for human consumption, while yellow maize is produced for animal feed. Fifty percent of most maize that are produced in South Africa are used for human consumption and consumed locally, 40% are used for animal feed and 10% for seed and industrial purposes (Maize Market Value Chain, 2010-2011).

Many biotic and abiotic stress factors affect the production of maize in South Africa, of which fungi such as the *Fusarium graminearum* species complex (FGSC) and maize stem borers are a part of the biotic stresses (Nelson *et al.*, 1983; Leslie and Summerell, 2006). This species complex are found as pathogens or secondary invaders (Gilbert and Tekauz, 1999), which causes Gibberella stalk-, root-, crown and ear rot (GER) of maize (Vigier *et al.*, 1997; Zeller *et al.*, 2003; Goswami and Kistler, 2004; Kazan *et al.*, 2012). FGSC linked diseases can be responsible for 30-70% of crop yield loss (Waalwijk *et al.*, 2003).

1.3 The Disease Triangle

An abnormal change in the physical form or function of a plant over a period is attributable to the term plant disease. Plant diseases can reduce yield and seed quality (Wise *et al.*, 2016). Plant pathogens cause symptoms in several ways and each pathogen has evolved a unique mode

of causing an infection such as entering a plant through natural openings and wounds. Symptoms occurring on roots and stalks are unique to one type of tissue, whilst others are observed throughout the plant (Wise *et al.*, 2016). Insects that feed on plants can also transmit pathogens (Wise *et al.*, 2016).

Three components need to be favourable for a disease, such as FGSC, to occur on a plant (Agrios, 2005). A susceptible host, a disease causing pathogen and a favourable environment suitable for disease development are the three elements that need to be simultaneously present for a disease to exist (Wise *et al.*, 2016). Plants and diseases need to interact, and for some time afterwards the environmental conditions need to be favourable for the disease to develop (Agrios, 2005). As one component changes, it affects the disease severity on the targeted plant (Agrios, 2005).

The interactions of these three components can be visualised and represented in a triangle, better known as the disease triangle. Each side of the triangle is the three components that favours the disease, if any of the three components is absent, no disease can occur (Figure 1.1) (Agrios, 2005).

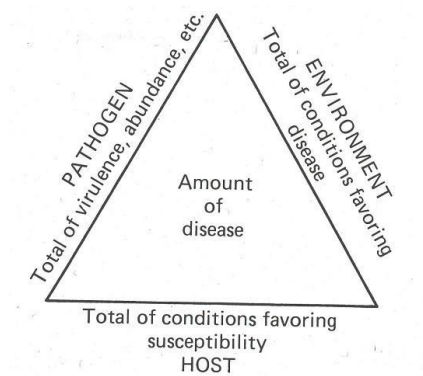


Figure 1.1: The disease triangle represents the three components (environment, host plant and pathogen) that need to be present and favourable for a disease to occur (Agrios, 2005).

The use of management strategies such as cultural, chemical and planting host plant resistant cultivars can eliminate elements of the disease triangle to prevent disease from occurring or to reduce its significance if it does occur. For optimal disease management, more than one strategy is needed to manage diseases (Wise *et al.*, 2016).

1.4 Fungal diseases on maize – *The Fusarium graminearum* species complex (FGSC)

FGSC is a major plant pathogen that is destructive to cereal crops and causes Gibberella root-, crown-, stalk- and ear rot on maize (McMullen *et al.*, 2012; Kazan *et al.*, 2012, Rose *et al.*, 2015). However, the knowledge of the FGSC members associated with maize in South Africa is still limited (Boutigny *et al.*, 2011). Yield, grain-, and seed quality can be reduced by maize diseases (Wise *et al.*, 2016), and are especially significantly reduced by Gibberella ear rot (GER) (Boutigny *et al.*, 2011). These diseases mostly infect crops including wheat, barley and maize (Boutigny *et al.*, 2011).

Since 2000, the FGSC has been divided into 16 new species based on DNA differences. These lineages include *F. austroamericanum* T. Aoki, Kistler, Geiser, O'Donnell (lineage 1), *F. meridionale* T. Aoki, Kistler, Geiser, O'Donnell (lineage 2), *F. boothii* T. Aoki, Kistler, Geiser, O'Donnell (lineage 3), *F. mesoamericanum* T. Aoki, Kistler, Geiser, O'Donnell (lineage 4), *F. acacia-mearnsii* T. Aoki, Kistler, Geiser, O'Donnell (lineage 5), *F. asiaticum* T. Aoki, Kistler, Geiser, O'Donnell (lineage 6), *F. graminearum* sensu stricto Schwabe (lineage 7), *F. cortaderiae* T. Aoki, Kistler, Geiser, O'Donnell (lineage 8), *F. brasilicum* T. Aoki, Kistler, Geiser, O'Donnell (no lineage number), *F. aethiopicum* O'Donnell, Abera, Kistler, Aoki (no lineage number), *F. gerlachii* Aoki, Starkey, Gale, Kistler, O'Donnell (no lineage number), *F. vorosii* Toth, Varga, Starkey, O'Donnell, Suga, Aoki (no lineage number), and *F. ussurianum* Aoki, Gagkaeva, Yli-Mattila, Kistler, O'Donnell (no lineage number) (O'Donnell *et al.*, 2000; 2004; 2008; Starky *et al.*, 2007; Yli-Mattila *et al.*, 2009; Boutigny, *et al.*, 2011; Aoki *et al.*, 2012). According to Sarver *et al.*, (2011) three more members namely, *F. louisianense* Gale, Kistler, O'Donnell, Aoki, *F. nepalense* Aoki, Carter, Nicholson, Kistler, O'Donnell and an as yet unknown *Fusarium* species were added using multilocus genotyping (MLGT). Of the 16 previously described species, only six have been identified on South African grain. This includes *F. graminearum* (maize roots and wheat), *F. boothii* (barley, maize ears and wheat), *F. meridionale* (maize roots, sorghum and wheat), *F. cortaderiae* (wheat and sorghum), *F. acaciae-mearnsii* (wheat and sorghum), and *F. brasilicum* (wheat) (Boutigny *et al.*, 2011; Mavhunga, 2013). According to a study conducted by Lamprecht *et al.* (2011), results proved that only three of these species, namely, *F. graminearum* sensu stricto, *F. meridionale*, and *F. boothii* are currently known to be associated with maize crowns and roots in South Africa. The

importance of proper disease identification is highlighted, in order to manage the disease (Wise *et al.*, 2016).

Disease occurrence can differ from consistent but small losses to sometimes severe losses that are extremely damaging to crops (Wise *et al.*, 2016). The type and severity of diseases are influenced by region, weather conditions, hybrid selection, susceptibility to disease, no-till practices, crop rotation and geographical distribution. Factors that are out of farmers' control include increase in humidity and more frequent and heavy rainfalls, which may increase the likelihood of a pathogen causing a disease (Wise *et al.*, 2016).

A wide variety of plant hosts in temperate and sub-tropical regions are infected by members of the FGSC (Karugia *et al.*, 2009). This species complex is found in maize, wheat, barley, sorghum, rice, oats and rye (*Secale cereal* L.) (Gilbert and Tekauz, 1999; Desjardins *et al.*, 2004). The FGSC are also commonly known as the main agents of Fusarium Head Blight (FHB) of barley and wheat in South Africa (Boutigny, *et al.*, 2011; Lamprecht *et al.*, 2011). The FGSC host range has also developed from cereal- to non-cereal crops such as canola, dry-beans, potatoes and soybeans (Goswami and Kistler, 2004; Burlakoti *et al.*, 2008). FGSC causes Gibberella stalk-, ear- and root rot of maize, as well as stalk rot and seedling blight of sorghum (Zeller *et al.*, 2003; Goswami and Kistler, 2004; McMullen *et al.*, 1997). Diseases caused by FGSC, can cause yield losses in crops of up to 30-70% (Waalwijk *et al.*, 2003). Previous reports showed that FGSC was found in low frequencies and no threat to maize production areas in South Africa (Viljoen, 2003). However, a recent study conducted by Boutigny *et al.* (2011) showed that FGSC are increasing in the maize production areas in South Africa.

The importance of the continuation of monitoring FGSC in cereal grains in South Africa is highlighted since infected grains can be contaminated with mycotoxins such as zearalenone, deoxynivalenol and nivalenol (Nordby *et al.*, 2007; Boutigny *et al.*, 2011). It is also very important to supply high quality healthy crops such as maize, since this crop is mainly produced for human consumption.

The FGSC produces sexual spores (ascospores) and asexual spores (macroconidia) as forms of inoculum (Beyer *et al.*, 2004; Gilbert and Fernando, 2004). Both forms cause substantial infections under favourable conditions and are thus important in disease development (Beyer

et al., 2004). Macroconidia can be distributed by insect vectors or by splash dispersal during rainy seasons (Beyer *et al.*, 2004). According to Xu (2003), ascospores are released into the air and distributed by wind currents.

The primary source of inoculum for infections in field crops are crop residue such as maize-, sorghum- and wheat stubble (Gale *et al.*, 2002; Munkvold, 2003). Wounds caused by birds or insects on host plants make it possible for members of the FGSC to enter host tissue easier (Reid *et al.*, 2002; Wagacha and Munthomi, 2008) however, the main pathway for infections causing ear rot is usually through the silk channel (Reid *et al.*, 2002).

1.4.1 Gibberella ear rot

Gibberella ear rot is a significant problem in many maize areas in the world. Gibberella ear rot occurs sporadically and reduces the quality of the crop (Nordby *et al.*, 2007). Mycotoxins are a major concern and grain infected with *Gibberella zae* may contain zeralenone, deoxynivalenol and nivalenol (Nordby *et al.*, 2007).

Spores are spread by wind and splashing rain which infect the ear through silks. Gibberella ear rot symptoms normally start at the tip of the ear and spread then to the base, although in some cases it may start at the basal end of the ear (Du Toit and Pataky, 1999; Reid *et al.*, 2002; Nordby *et al.*, 2007; Wise *et al.*, 2016). A red or pink mould may develop over a large portion of the ear as the fungus spreads. Early infected ears may be entirely covered by a pinkish mycelium over the ear that causes the husk to tightly adhere to the ear (Payne, 1999). In severe cases, the pink mould is visible on the outside of the husk at the ear tip (Wise *et al.*, 2016). Mycelia colonize the silk channel and grows on developing kernels (Nordby *et al.*, 2007). Cool, wet weather at flowering stage is the most prevalent favourable conditions for Gibberella ear rot to occur. This disease is damaging from the time of silking through to the time of grain fill (Nordby *et al.*, 2007).

Although reduced tillage has many advantages, maize debris remaining on or near the soil can be the primary source of Gibberella ear rot inoculum (Munkvold, 2003). The effects of crop rotation, tillage and nutrients have been studied on the quantity of primary inoculum, and Flett *et al.* (1998) found that ploughing under maize stubble was insignificant to the amount of

Fusarium species inoculum. Reid *et al.* (2001) noted that higher rates of nitrogen (200 kg N ha⁻¹) increased the severity of *Gibberella* infections. According to Reid *et al.* (1992) the best approach to control *Gibberella* ear rot is through the developing of resistant hybrids.

Grain that are mouldy must be stored in such a manner that the impact on grain can be minimized (Wise *et al.*, 2016). Mouldy grain should be stored separately from good quality grain and checked throughout the storage period to ensure that temperature and moisture levels remain constant (Wise *et al.*, 2016). Grain that are stored on the farm should be cooled below 10°C and immediately dried to below 18% moisture. For long term grain storage, the grain should be dried to 15% moisture (Wise *et al.*, 2016).

1.4.2 *Gibberella* stalk rot

Stalk rot is a term that is often used to refer to stalk breakage, stalk lodging and premature death of plants (White, 1999a). Different combinations of several fungal and bacterial species can cause stalk rot diseases on maize. The most common fungi responsible for stalk rots on maize include, FGSC, *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, *Stenocarpella maydis* (*Diplodia*), *Colletotrichum graminicola* and *Macrophomina phaseolina* (Agrios, 2005). FGSC, causing *Gibberella* stalk rot, is one of the most common stalk rots but the similarities of *Fusarium*- and *Gibberella* stalk rot makes it difficult to distinguish (Jackson-Ziems *et al.*, 2014). *Gibberella* stalk rot may also be responsible for ear and root rot (Agrios, 2005; Wise *et al.*, 2016).

Pathogens that occur commonly in the field can infect stressed and injured plants (Jackson-Ziems *et al.*, 2014). Factors contributing to plant stress include soil fertility, drought stress, insect damage and plant density. Plants can become more susceptible to stalk rot when foliar diseases occur because the photosynthesis area of the leaf is reduced and thereby weakening the pith cells. Without leaf blight diseases, conservation tillage has been shown to reduce most stalk rot levels (White, 1999a). Other diseases may occur in maize when multiple stalk rot pathogens infect a single plant. However, as discussed previously, the environmental conditions need to be favourable for each pathogen (Jackson-Ziems *et al.*, 2014).

The fruiting structures produced by the fungus causing *Gibberella* stalk rot are found on the stalk of the maize plant and can easily be scraped off the stalk (Wise *et al.*, 2016). Inside the stalk, the pink discoloration is uniquely evident of a plant infected by *Gibberella* (Reid *et al.*, 2002). These reproductive structures have spores inside which can overwinter on crop residue or in overwintering structures in the soil for many years in the absence of crop hosts, and then act as primary inoculum to infect plants the following season when development are favoured by warm, wet environmental conditions (Jackson-Ziems *et al.*, 2014; Wise *et al.*, 2016). Stalk infections, which usually develop at the basis of the leaf sheaths or around the supporting roots, occur shortly after pollination and when the plant is stressed. It is also possible that the fungus may enter through the roots and grow up into the lower stem (White, 1999a). Plants with rotted stalks almost always have rotted roots (White, 1999a).

Plant wilting is usually one of the first signs of stalk rot. Within days, the ears start to drop, the leaves change from light to dull green, and the outer rind of the lower maize stalk turns brown and straw coloured (Agrios, 2005; White, 1999a). As soon as the outer tissue of the stalk starts turning brown, the lowest internode containing pith tissue is usually rotted and then pulls away from the rind (Agrios, 2005; White, 1999a). After the internal pith disintegrates, the vascular bundle is all that is left (Agrios, 2005, White, 1999a). The structural inner part of the plant changes from a solid rod to a tube-like structure, as the rotting pith tissue pulls away from the rind. Rotted stalks are weak and predisposed to lodging, especially when the rot occurs below the ear (Jackson-Ziems *et al.*, 2014). Plants with prematurely rotted stalks because of the plant's limited access to carbohydrates produce lightweight and poorly filled ears (Jackson-Ziems, *et al.*, 2014).

Rotted stalks can be soft when pinched, and stalk rots may go unnoticed if the only symptom is pith deterioration on the inside of the plant. Like ears, stalks that are infected with FGSC can contribute to mycotoxin contamination of maize that is harvested as silage and used for animal feed (Wise *et al.*, 2016).

Economic and yield losses are caused by *Gibberella* diseases and are distributed worldwide (Agrios, 2005). Losses can be caused by stalk rot in several different ways, including premature plant death, which prevents grain fill, lodged plants, which is a result of stalk rot and cannot be harvested with mechanical equipment. Ear rots can develop through lodged plants touching the soil, and this can result in reduced grain quality and possible dockage when grain is marketed

(White, 1999a). Stalk rot can be responsible for about 10-30% yield loss (Agrios, 2005; Jackson-Ziems *et al.*, 2014). Fields with more than 10% affected plants, should be harvested in an early stage to prevent grain loss from stalk lodging (Wise *et al.*, 2016).

A few control strategies must be put into place to manage stalk rot. Resistance to stalk rot and lodging is a big factor in the marketing of maize hybrids and big consideration is put into selecting hybrids with this resistance (White, 1999a). However, a very important characteristic of a hybrid is yield (White, 1999a). Genotypes with higher yields tend to have larger ears, however when the plant is in stress, such as occurring during foliar diseases which cause loss of the leaf area, the big ears may extricate carbohydrates from the stalk (White, 1999a; Jackson-Ziems *et al.*, 2014). With the added weight of the large ear it will leave the stalk fragile with weakened stalk tissue and more prone to lodging (White, 1999a; Jackson-Ziems *et al.*, 2014). Hybrids with strong stalks are less susceptible to lodging, but may be vulnerable to pith deterioration (Wise *et al.*, 2016). The risk of developing stalk rot may also be reduced by hybrids that are more resistant to foliar diseases, because the stress caused by leaf blight increases susceptibility to stalk rot (Wise *et al.*, 2016).

Therefore, it is important to find a good balance between breeding for resistance to stalk rot and breeding for higher yield, which makes the process very delicate (White, 1999a). Another obstacle making stalk rot breeding so complicated is that there is such a large number of fungi that can cause stalk rots and with the effect of various environmental factors making the plants more susceptible to stalk rot (White, 1999a). Stalk-boring insects can have an influence on stalk rot, thus by controlling these insects it will also be helpful for control of stalk rot (White, 1999a).

Although improvements and control methods have been put into place to control stalk rot, it continues to be widespread and a serious disease of maize. Stalk rot severity and incidences fluctuate from year to year, and some stalk rots occur every year in every field (White, 1999a).

1.4.3 Gibberella root- and crown rot

The least studied and least understood disease of maize among others is root and crown rot (Dodd and White, 1999; Ares *et al.*, 2004). Although root and crown rot, like stalk rot, occurs

on every maize plant in every field each year, the accurate estimation of yield loss is difficult to determine and sometimes not severe enough to cause economic losses (Munkvold and Leslie, 1999). Root rot is seen as a disease complex which involves many different fungi, nematodes, and root-feeding insects. Environmental conditions, genotype, growth stage of the host and the previous planted crop influence the groups of fungi that occur on roots and crowns. Fungi vary in their ability to cause root- and crown rot (Dodd and White, 1999).

Fungi that have been isolated from roots include stalk rot- and seedling blight pathogens as well as secondary invaders (Ares *et al.*, 2004). *Fusarium* species are regularly isolated from maize roots (Munkvold and Leslie, 1999). The following *Fusarium* species tend to be associated with root rots in young plants namely, *F. oxysporum* (Schlentend) emend. Snyder and Hansen and *F. solani* (Sacc). Other pathogens that were retrieved on roots include, FGSC, *F. acuminatum* (Eliis and Everhart), *F. verticillioides* (Sacc), *F. proliferatum* (Matsushima), and *F. subglutinans* (Wollenweber and Reinking) (White, 1999b). Crown rot symptoms includes the slightly discoloured or dark brown and rotted, which can lead to leaf wilting or yellowing (Wise *et al.*, 2016). Severely rotted crowns on plants may suddenly die in warm, sunny weather and can also continue into the growing season leading to stalk rot (Wise *et al.*, 2016). Root rot symptoms range from a light brown-reddish colour to a dark black colour when the roots are completely rotted (Wise *et al.*, 2016). A red or pink colour occurs when the roots are infected with FGSC (Munkvold and Leslie, 1999; Ares *et al.*, 2004). According to Palmer and Kommedahl (1969) and Kommedahl *et al.* (1987) this species complex has been reported to be pathogenic on maize in inoculation tests. A study conducted by Ares *et al.* (2004) showed that out of nine isolates, four produced significant growth reductions on susceptible maize seedlings. Two of them were FGSC isolates which also induced severe root rot symptoms. The study also have shown that FGSC produced the highest seedling growth reductions and that FGSC was the main causal pathogen of root rot.

Root- and crown rot pathogens, like ear and stem rot, survive in soil or crop residue, and are the causal agent of most root rots past the seedling stage. Fungal propagules in the soil can be the start of infection when plant roots come into contact with these propagules (Munkvold and Leslie, 1999). Damaged roots and crowns by insects, nematodes or cultivation can enhance the infection process. Seedborne infections may cause root rot in young plants (Munkvold and Leslie, 1999). Stressed plants, such as inadequate allocation of photoassimilate to the roots and herbicide injury may be more susceptible to root and crown rot (Munkvold and Leslie, 1999).

As the plants become more mature, root and crown rot probability increase as the roots and crowns become more susceptible. *Fusarium* species which infects the roots will most likely initiate crown- and stalk rot when it moves to the base of the maize plant. Root and crown rot caused by *Fusarium* species can occur under a wide range of moisture conditions and temperatures (Munkvold and Leslie, 1999). Plants are often more susceptible to infect under stressful growth conditions, such as wet soils, cold soils, plants with herbicide injuries and fertility problems (Wise *et al.*, 2016).

Breeding genotypes for susceptibility or resistance to *Fusarium* root rots is very challenging (Munkvold and Leslie, 1999). Seeds covered with seed treatments of fungicides can reduce seedling blight, however, the treatment does not provide control for an extended period of time. Tillage has relatively little impact on the control of *Fusarium* root rot; however, root and crown rot is less recurrent when maize is rotated with other crops (Munkvold and Leslie, 1999).

1.5 Stem borer- Fungal interactions

Busseola fusca, which was first named and described by Fuller in 1901, is also known as the African maize stem borer (Harris and Nwanze, 1992). Maize and sorghum are the two preferred host crops for *B. fusca* (Kfir *et al.*, 2002). *Busseola fusca* was first recognised as a pest on maize in South Africa and has become a serious economic impact in many maize growing countries in Africa (Kfir *et al.*, 2002).

Being responsible for an average of 10% yield loss on maize in South Africa (Mally, 1920; James, 2013), *B. fusca* is the most important and destructive lepidopteran pest on maize (Kfir and Bell, 1993; Kfir, 2000; Kfir, 2002). Damage is caused by the larval stage that feeds on the young leaves before larvae tunnel into the whorls. The first indication of infestation can be seen as the leaves unfold, which is also known as “pin-hole” damage (Van Rensburg, 1999). After the 3rd or 4th instar is reached the larvae penetrates the stem resulting in extensive tunnels in the stem, severe tissue damage, destroying the growing point which results in a “dead heart” and results in the inability to form an ear (Harris and Nwanze, 1992). *B. fusca* also causes direct damage to maize ears which has a significant influence on yield (Van Rensburg *et al.*, 1988).

Genetically modified *Bt*-maize containing the *Cry1Ab* protein (MON810) has been grown and deployed to control *B. fusca* since 1998 in South Africa (Kruger *et al.*, 2011). *Bt* genes contain

the crystal protein that are toxic to insects, killing them upon feeding (Munkvold, *et al.*, 1999). The first resistance of the maize stem borer to *Bt*-maize was reported in 2007 against MON810 in South Africa (Van Rensburg, 2007). Monsanto developed a new hybrid in 2011 with two stacked genes, *Cry1A.105* and *Cry2Ab2* (MON89034) to provide effective control to resistant *B. fusca* larvae (Monsanto, 2014).

According to Rutherford *et al.*, (2002) *B. fusca* creates wounds that can allow entry to infection of maize plants with *F. verticillioides*. An experiment conducted by Flett and Van Rensburg (1992) resulted in *B. fusca* showing increased infestation with an increased incidence of Fusarium ear rot (FER) in maize. Infestation with stem borer, *Eldana Saccharina* Walker (Lepidoptera: Pyralidae) on maize, significantly increased the incidence and severity of stem rots (Bosque-Pérez and Mareck, 1991). Lepidopteran pests can influence stalk and ear rot diseases (Dowd, 1998; Smeltzer, 1958). The planting of *Bt* maize hybrids is an important control mechanism to manage maize stem borers (Hellmich *et al.*, 2008). After infestation of stem borer, *Ostrinia nubilalis* Hübner in the United States of America, *Bt* maize hybrids was less susceptible to Fusarium ear rot than non-*Bt* maize (Munkvold, *et al.*, 1999). Hybrids that are resistant to Gibberella stalk rot are not common, but planting hybrids that are resistant to stalk borers may reduce secondary diseases by minimizing the wounds caused by these insect pests (Hellmich *et al.*, 2008; Jackson-Ziems *et al.*, 2014). However, the interactive effect of *B. fusca* and FGSC has not been studied in South Africa.

1.6 Mycotoxins associated with FGSC

Infection of FGSC on grain does not only lead to reduced grain quality and yield, but could also lead to food safety concerns. One or more toxic secondary metabolites are produced in the grain by most *Fusarium* species, commonly known as mycotoxins (Bottalico and Perrone, 2002). Mycotoxins produced by members of the FGSC include the most important zearalenone, an estrogenic mycotoxin and the type B-trichothecenes (TCT-B) (chemotypes), most commonly deoxynivalenol, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) and at a lower frequency, nivalenol (Marasas *et al.*, 1981; Lee *et al.*, 2009; Boutigny *et al.*, 2012; Desjardins and Proctor, 2011; Malbrán *et al.*, 2014). These mycotoxins have a number of health implications and are considered unsafe for human and animal consumption (Rocha *et al.*, 2005, Pestka, 2010). Deoxynivalenol is also known as vomitoxin because of the strong nausea, vomit and diarrhoea effects after ingestion by humans

(Yoshizawa and Morooka, 1974). Deoxynivalenol can lead to vomiting, food refusal and decreased weight when it is consumed by livestock (Young *et al.*, 1983). Tabib and Hamilton (1988) and Hedman *et al.* (1995) reported that slightly more toxigenic nivalenol resulted in decreased liver weights when fed to chickens. However, nivalenol and nivalenol -producing FGSC species have been less frequently associated with grains in South Africa (Boutigny *et al.*, 2011; Boutigny *et al.*, 2012). Zearalenone is the most widely distributed *Fusarium* mycotoxin globally. Zearalenone is biologically very influential and may cause disorders in reproduction, such as abortions in animals, despite its low acute toxicity (Stob *et al.*, 1962; Kuiper-Goodman *et al.*, 1987; Logrieco *et al.*, 2002).

Mycotoxins are produced under various environmental conditions, and the conditions that stimulate grain crops to the production secondary mycotoxins include temperature, moisture and water activity (Eskola, 2002; Munkvold, 2003). Fungi under stress conditions usually produce the production of mycotoxins later in the life cycle of the fungus. Factors such as fungal inoculum, mechanical injury, insect damage, wind, rain, hail damage and susceptibility to the cultivar can also play a role in the development and accumulation of mycotoxins in grain (Eskola, 2002; Munkvold, 2003). Moisture content and temperature remains the most crucial factors affecting fungal growth and mycotoxins (Eskola, 2002).

As previously mentioned, the FGSC consist out of sixteen distinct phylogenetic lineages (O' Donnell *et al.*, 2000, 2004, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009; Sarver *et al.*, 2011; Aoki, *et al.*, 2012). Of the sixteen species, only nine produce one out of the three trichothecene chemotypes while the other seven produce two or three chemotypes (Aoki *et al.*, 2012). According to Wang *et al.* (2011), five chemotypes, namely deoxynivalenol and 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and nivalenol and 4-acetylnivalenol (4-ANIV) have been identified in the FGSC. Limited information about the diversity of FGSC and their trichothecene chemotypes in South Africa cereal grains is available.

When grain is harvested at optimal moisture (<14%), temperature conditions is maintained and insect pests are controlled during storage, the fumonisins and deoxynivalenol levels in grain do not increase significantly (Munkvold and Desjardins, 1997). Removing underdeveloped, mouldy and broken kernels can also significantly reduce mycotoxin levels in cereal grains. The washing of maize with distilled water was found to have reduced deoxinivalenol and zearalenone by 69% and 2% respectively (Trenholm *et al.*, 1992).

In the 2011/2012 season zearalenone has also been found in South African maize with an average of 249 µg/kg, which exceeds the allowed maximum levels of the European Union by 100 µg/kg (Beukes *et al.*, 2017). Commercial farmers take yield into high consideration when deciding on a hybrid to plant and not disease resistance, therefore the high mycotoxin levels in commercial maize (Lamprecht, *et al.*, 2011). Additional yield losses are not only caused by ear rot, but by root, crown and stalk rot (Lamprecht, *et al.*, 2011).

Liquid chromatography-mass spectrometry (LC-MS) have been used to detect quantitative and qualitative mycotoxin levels (Schollenberger *et al.*, 1998). Chromatographic methods measure the compound after sample extraction by separating the compound using liquid chromatography. Using it in combination with High performance liquid chromatography (HPLC) with a range of detectors allows for detection and the separation of practically all mycotoxins (Rahmani *et al.*, 2009). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) offers the concurrent analysis of a wide variety of mycotoxins in a collection of matrices. This method has been used to determine trichothecenes such as deoxynivalenol, nivalenol, fusarenon X, 3- and 15-acetyldeoxynivalenol as well as zearalenone, α - and β -zearalenol in maize (Turner *et al.*, 2009). LC-MS/MS is able to identify multiple mycotoxins in a single run, therefor making it such a valuable method of choice.

1.7 Techniques to identify FGSC members

The FGSC contains of a large group of species that are morphologically comparable (Edwards *et al.*, 2002; O'Donnell *et al.*, 2008), which makes identification more difficult to depend alone on the use of morphological characteristics (Leslie and Summerell, 2006). Although cultural techniques are time consuming and greatly dependent on living propagules, this remains an important technique for identification of fungi (Moss and Thrane, 2004). Morphological characteristics forms the foundation of taxonomic classification and species identification, and are still used when new species are described (Rheeder *et al.*, 1995).

Molecular techniques based on analysis of DNA and RNA have been used to resolve the complex identification of FGSC members to species level with information of the population structure (Edwards *et al.*, 2002). The identification of species using *F. graminearum* sensu lato species specific primers (Schilling *et al.*, 1996; Nicholson *et al.*, 1998) has also included the

quantitative detection of fungal DNA in grain samples (Waalwijk *et al.*, 2004; Nicolaisen *et al.*, 2009) and determination of chemotypes in isolates (Desjardins and Proctor, 2011). The foundation of prediction of disease epidemics and disease management lies in the understanding of genetic diversity and population biology (Burlakoti *et al.*, 2008). Historical identification methods for *Fusarium* research techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), RFLP, single strand conformational polymorphism (SSCP), inter-simple sequence repeat (ISSR) and sequence related amplified polymorphism (SRAP) have all been used to explore the diversity among FGSC populations (Burlakoti *et al.*, 2008). In this study the use of real-time quantitative and conventional PCR with regards to FGSC members will be investigated and discussed.

PCR provides a fast and specific mechanism that are used to detect target DNA molecules within a complex pool of molecules (Jurado *et al.*, 2005). PCR have been used and applied in studies by Nicholson *et al.*, (1998) and Waalwijk *et al.*, (2004), investigating FGSC members based on amplification of specific DNA fragments from a complex pool of DNA (Edwards *et al.*, 2002). PCR have been used to detect plant pathogens in infected seeds and diseased plants, however, this technique is not frequently used for disease identification due to the fact that it is time-consuming and a laborious procedure that also requires the verification of the amplified product (Schaad and Frederick, 2002). A disadvantage of PCR when using it in particular with FGSC members, is that it cannot distinguish between lineages or species within the species complex (O'Donnell *et al.*, 2004; Jurado *et al.*, 2005). Also, the DNA coding regions of eukaryotes subunits are also too preserved to be used for species identification (Seifert and Lévesque, 2004). This may result in the use of many other PCR-based protocols used in *Fusarium* species identification using genomic sequences which encodes for translation elongation factor 1- α (*TEF1*- α), Intergenic spacer (*IGS*), internal transcribed spacer (*ITS*) regions of the rDNA unit (*ITS1* and *ITS2*) which are frequently used when distinguishing between species, and β -tubulin (Edwards *et al.*, 2002; Jurado *et al.*, 2005). Only the translation elongation factor technique will be further examined in this study.

The qPCR offers an alternative tool for both qualitative and quantitative analysis. This method is used for direct quantification of target DNA in complex samples (Sarlin *et al.*, 2006). Sequence-specific probe binding assays and independent detection assays are the two fluorescence methods available. The fluorescence methods include Taqman® (Waalwijk *et al.*, 2004) and SYBR Green (Nicolaisen *et al.*, 2009) to detect the production of PCR amplicons.

qPCR is a safe, fast and convenient method to use to detect pathogens. qPCR data analysis can be used for relative- or absolute quantification (Peirson *et al.*, 2003). A standard curve is constructed for absolute quantification using a sample with a known concentration to determine the concentration of the unknown samples (Kühne and Oschmann, 2002). The relative quantification method measures changes in expression level comparative to another set of experimental samples (Kühne and Oschmann, 2002).

Genealogical concordance phylogenetic species recognition (GCPSR) and multilocus genotyping assay (MLGT) techniques have also been used to identify members of the FGSC (Ward *et al.*, 2008). The MLGT technique is based on the use of probes that target single nucleotide polymorphisms within the genes (Ward *et al.*, 2008) while GCPSR is based on the phylogenetic analysis of several gene regions (O'Donnell *et al.*, 2000). MLGT is a highly accurate, yet expansive method to distinguish between closely related species within the FGSC (Ward *et al.*, 2008).

1.8 Concluding remarks

The rise in the FGSC in maize growing areas across South Africa is a threat to food safety and security. The aim of this study was to elucidate FGSC members occurring on maize. The importance of disease identification is highlighted in Chapter 2 to successfully manage and understand FGSC disease epidemic threats in fields, therefore the aim of this chapter was to determine the identity of FGSC members on maize roots, crowns and stems using species specific primers and PCR-RFLP. Information about the FGSC occurring in South Africa is limited to either the ear or to specific regions in South Africa such as KwaZulu-Natal. In chapter 3 the ability of FGSC members to successfully infect not only ears, but also the stems of maize is revealed. This last mentioned chapter also exposes the production of mycotoxins nivalenol, zearalenone and deoxynivalenol by FGSC members which can seriously affect human and animal health. The aim of chapter 3 was to evaluate the pathogenicity and mycotoxin production of FGSC members in stems and grains. The relationship between stem borer and *F. verticillioides* is well known but FGSC has not been included due to the fact that it was not seen as an important maize pathogen. Clarity regarding the FGSC species occurring in South African maize as well as the possible association with *B. fusca* will be highlighted in Chapter 4. The aim of this chapter was to evaluate the succession of FGSC in maize stems and associated stem borer occurrence over a maize growing season.

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CHAPTER 2 - IDENTIFICATION OF *FUSARIUM GRAMINEARUM* SPECIES COMPLEX MEMBERS ON MAIZE ROOTS, CROWNS AND STEMS IN SOUTH AFRICA

Abstract

Maize serves as a staple food for humans and as feed for animals. The demand of maize will double in the growing population and developing world by 2050. Diseases caused by the mycotoxin producing *Fusarium graminearum* species complex (FGSC) members in particular can cause yield losses in crops between 30-70% and disease incidence increased in maize production areas in South Africa. As each member can produce a different toxin the correct identification is essential. The aim of this study was to evaluate different techniques to identify naturally occurring FGSC members in 331 samples in maize roots, crowns and stems in South Africa. Species-specific polymerase chain reaction (PCR) for *F. graminearum* was used to identify morphologically related FGSC species in South Africa. Polymerase chain reaction-restriction length polymorphism (PCR-RFLP) that produced different banding patterns was further used to evaluate whether it can distinguish between closely related FGSC members. Three out of the six enzymes were able to identify four species with PCR-RFLP technique. Multilocus genotyping (MLGT) method was used to accurately identify 331 unknown samples and was compared to species-specific PCR based technique that was able to group the unknown samples into six groups according to fragment size. Comparing results of species-specific PCR and MLGT, only 27.8% of the samples identified by species-specific PCR corresponded with MLGT identification. This study confirmed the presence of the three FGSC species that were previously found on maize roots and ears. *F. graminearum* and *F. boothii* were isolated from crowns and stems and *F. meridionale* isolated from stems. Another result showed the presence of an interspecific hybrid species, *F. graminearum* x *F. boothii* on maize in South Africa. This study is also the first to report on two *Fusarium* species namely, *F. lunulosporum* and *F. cerealis* which have not been found on maize plants previously.

Keywords: Maize; *Fusarium graminearum* species complex; species-specific polymerase chain reaction; polymerase chain reaction-restriction length polymorphism; multilocus genotyping

2.1 Introduction

The demand of maize will double in the developing world by 2050, and the prediction is that maize will become the crop with the greatest production globally by 2025 (Rosegrant *et al.*, 2012). In the larger part of Africa, maize is the staple food crop of humans (Heisey and Edmeads, 1999). Furthermore, the world population is expected to surpass 9 billion by 2050, which means that with the current levels of production, the supply for future demands will fall far short (Easterling *et al.*, 2007). Diseases caused by *Fusarium graminearum* species complex (FGSC) members in particular can cause yield losses in crops between 30-70% (Waalwijk *et al.*, 2003) and a recent study showed that FGSC are increasing in maize production areas in South Africa (Boutigny *et al.*, 2011). Thus, the importance of monitoring diseases in crops is emphasised, since infected grains can be contaminated with mycotoxins such as zearalenone, deoxynivalenol and nivalenol (Boutigny *et al.*, 2011).

It was first thought that *F. graminearum* represented a single cultivated species based on morphological species recognition (Booth, 1971; Nelson *et al.*, 1983; Leslie and Summerell, 2006), but the phylogenetic species concept indicates that, *F. graminearum* sensu lato now consists of 16 species that are phylogenetically and biogeographically distinct also known as the FGSC (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009; Boutigny *et al.*, 2011; Davari *et al.*, 2012) (Figure 2.1). As is the case with FGSC members, the genus *Fusarium* also contains a large group of species that are morphologically similar (Edwards *et al.*, 2002; O'Donnell *et al.*, 2008). This makes it more challenging to depend alone on the use of morphological characteristics to identify *Fusarium* species (Jurado *et al.*, 2005; Leslie and Summerell, 2006). Pathogen reproduction and gene flow between populations is indicated when gene variation in a population is found in an area. The prediction of disease epidemics and of disease management relies on the fundament of understanding genetic diversity and population biology (Burlakoti *et al.*, 2008).

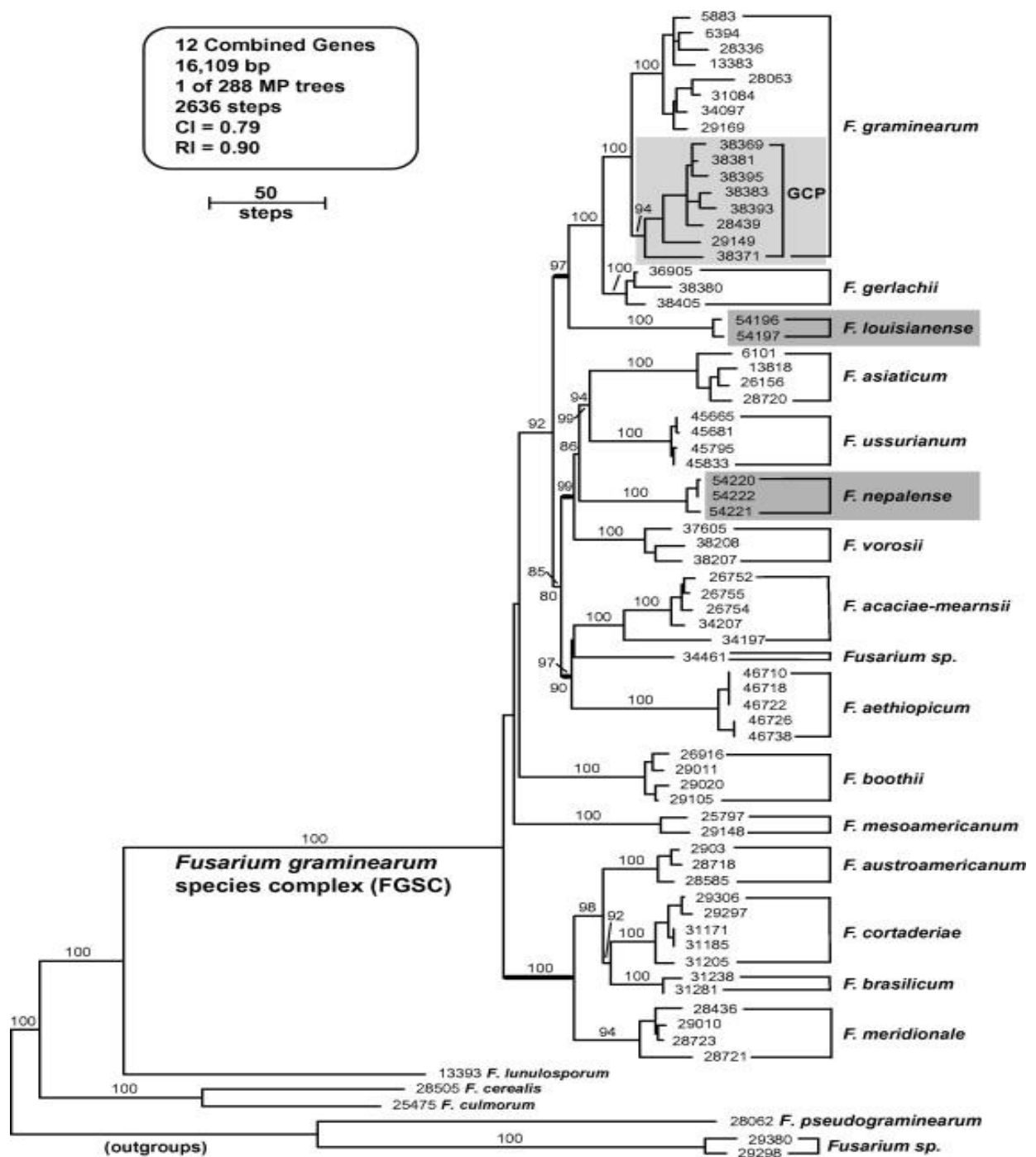


Figure 2.1: Multilocus molecular phylogeny of B-type trichothecene toxin-producing fusaria inferred from portions of 12 genes comprising 16.1 kb of DNA sequence data. The phylogram was rooted on sequences of *F. pseudograminearum* and *Fusarium sp.* NRRL 29298 and 29380 (Saver *et al.*, 2011).

Due to the difficulty in distinguishing between FGSC members (O'Donnell *et al.*, 2000), different identification methods based on molecular and cellular differences, DNA- and polymerase chain reaction (PCR) sequence-based techniques are often used to identify fungi (Hibbett *et al.*, 2007). Molecular techniques based on analysis of DNA or RNA have been used to resolve the complex identification procedures of FGSC isolates to species level and providing information on the population structures (Edwards *et al.*, 2002). FGSC species specific primers has been used for the identification of species (Schilling *et al.*, 1996; Nicholson *et al.*, 1998). Waalwijk *et al.* (2004) and Nicolaisen *et al.* (2009) has incorporated the use of quantitative detection of fungal DNA in grain samples and determination of chemotypes in isolates (Desjardins and Proctor, 2011).

PCR is a specific and fast tool that can be used for detection of target DNA within a complex of molecules (Jurado *et al.*, 2005). PCR is a classic method and has been used for plant pathogens detection in infected seeds and diseased plants, but because it is a quite difficult and time-consuming process which also requires verification of the amplified product, it is not frequently used in diagnosing plant diseases. Another disadvantage of PCR is that when it is used in particular with detection of FGSC members, it cannot be used to differentiate between lineages or species within this species (O'Donnell *et al.*, 2004; Jurado *et al.*, 2005). PCR have been applied by Nicholson *et al.* (1998) and Waalwijk *et al.* (2004) in a study of FGSC members based on amplification of specific DNA fragments from a complex pool of DNA.

PCR- restriction fragment length polymorphism (PCR-RFLP) is a method used to evaluate the presence of natural variation (polymorphisms) or the similarity in different species, races or strains of fungal pathogens within the DNA sequence (Wang *et al.*, 2011). The removal or insertion of DNA sequences may lead to variation in fragment sizes, by using highly repetitive DNA sequences as probes since the indicator is present in numerous copies may enhance the process (Wang *et al.*, 2011). In the history of *Fusarium* research, PCR-RFLP is one of the molecular markers that have been used to research the diversity amongst the FGSC members (Burlakoti *et al.*, 2008). Thus, this technique is more affordable and achievable to study the diversity in morphologically indistinguishable fungi. According to Suga *et al.* (2008) and Kemptner *et al.* (2009) this technique has often been used to investigate the diversity within and among *Fusarium* species.

Species-specific primers have also been designed for identification of FGSC members (Nicholson *et al.*, 1998). Species-specific PCR assays of several *Fusarium* species including *F. acuminatum* Ellis and Everh., *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith), *F. poae* (Peck) Wollenweber and *F. graminearum* Schwabe (Parry and Nicholson, 1996; Schilling *et al.*, 1996; Nicholson *et al.*, 1998; Waalwijk *et al.*, 2003; 2004). The gene region of the *translation elongation factor 1- α* (EF-1 α) has often been explored to develop species-specific primers for *Fusarium* species, as this region contains a high number of polymorphisms (Rahjoo *et al.*, 2008). Primers have been developed by Nicholson *et al.*, (1998) to differentiate between *F. graminearum* and *F. culmorum*. These primers were able to distinguish between four FGSC members namely *F. graminearum*, *F. meridionale* Aoki, Kistler, Geiser and O'Donnell, *F. asiaticum* Aoki, Kistler, Geiser and O'Donnell, and *F. austro-americanum* Aoki, Kistler, Geiser and O'Donnell, based on their PCR product sizes (Waalwijk *et al.*, 2003).

Members of the FGSC have also been identified with the use of genealogical concordance phylogenetic species recognition (GCPSR) and multilocus genotyping (MLGT) assay (Ward *et al.*, 2008). MLGT is based on the use of probes that target single nucleotide polymorphisms within the genes (Ward *et al.*, 2008) while GCPSR is based on the phylogenetic analysis of several gene regions (O'Donnell *et al.*, 2000). Although MLGT is an expensive method, it is a highly accurate method to distinguish between closely related species within the FGSC (Ward *et al.*, 2008).

The six FGSC species identified in South Africa (Table 2.1) produce different mycotoxins in wheat and maize grain. The importance of distinguishing between these species are therefore important. The aim of this study was to evaluate different techniques to identify naturally occurring FGSC members in 331 samples from maize roots, crowns and stems in South Africa. Species-specific PCR was evaluated to identify between morphologically related FGSC species in South Africa using the primer pairs for *F. graminearum* (Nicholson *et al.*, 1998).

Table 2.1: *Fusarium graminearum* species complex (FGSC) members occurring on South African grain and their associated mycotoxins.

Species	Grain host (SA)	References	Mycotoxins produced	References
<i>F. acaciae-mearnsii</i>	Wheat, Sorghum	Boutigny <i>et al.</i> , 2011, Mavhunga, 2013	3-ADON, NIV	Aoki <i>et al.</i> , 2012
<i>F. boothii</i>	Barley, Maize (ears and roots), Wheat	Boutigny <i>et al.</i> , 2011	15-ADON	Aoki <i>et al.</i> , 2012
<i>F. brasilicum</i>	Wheat	Boutigny <i>et al.</i> , 2011	3-ADON, NIV	Aoki <i>et al.</i> , 2012
<i>F. cortaderia</i>	Wheat, Sorghum,	Boutigny <i>et al.</i> , 2011, Mavhunga, 2013	3-ADON, NIV	Aoki <i>et al.</i> , 2012
<i>F. graminearum</i> s.s	Barley, Maize (roots), Wheat	Boutigny <i>et al.</i> , 2011	3-ADON, 15-ADON, NIV	Aoki <i>et al.</i> , 2012
<i>F. meridionale</i>	Maize (roots), Sorghum, Wheat	Boutigny <i>et al.</i> , 2011, Mavhunga, 2013	NIV	Aoki <i>et al.</i> , 2012

3-ADON: 3-acetyldeoxynivalenol; 15-ADON: 15-acetyldeoxynivalenol; NIV: Nivalenol

2.2 Materials and Methods

2.2.1 Collection of FGSC isolate

In the 2013/2014 maize growing season, comprehensive sampling of maize roots, crowns and stems were conducted in 44 fields, on 17 localities and within five provinces (KwaZulu-Natal, North-West, Free State, Northern Cape and Mpumalanga) in South Africa (Figure 2.2). The roots, crowns and stems were washed, cut into 1 cm pieces and plated out on potato dextrose agar (Sigma-Aldrich, Saint Louis, Missouri, USA). The fungal growth that was morphologically identified as FGSC were single-spored and preserved with 15% glycerol at -80°C.

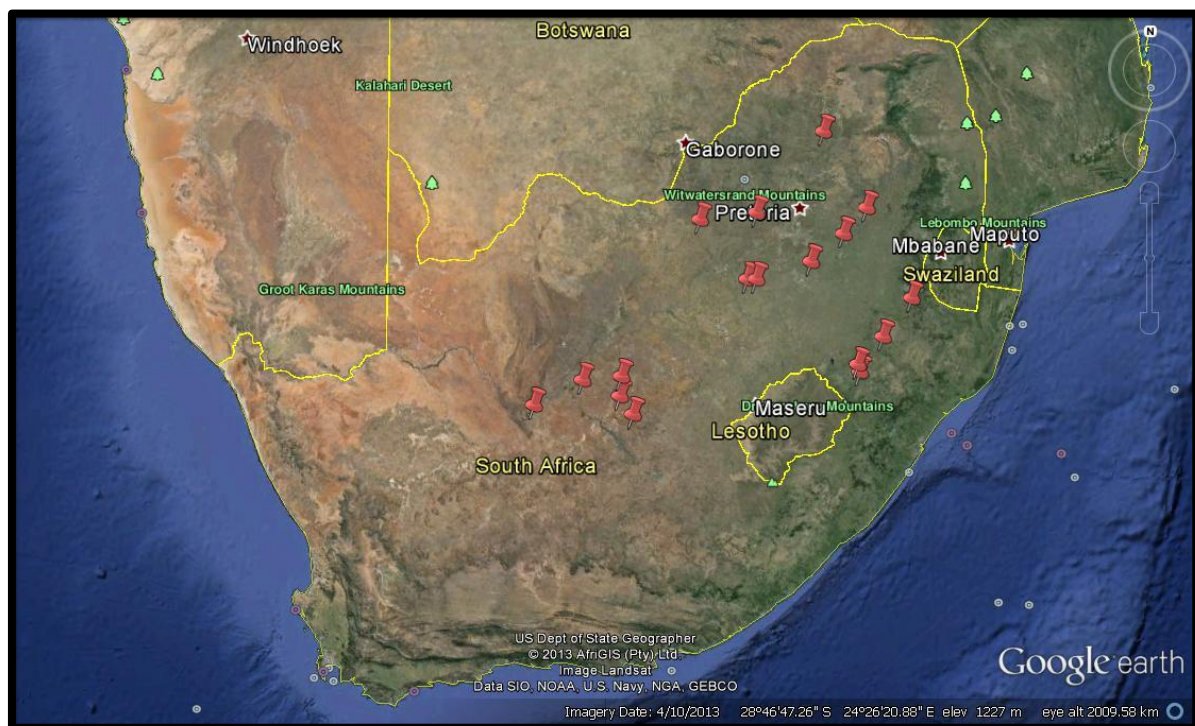


Figure 2.2: Sampling of maize roots, crowns and stems were conducted on 17 localities in five provinces (KwaZulu-Natal, North-West, Free State, Northern Cape, and Mpumalanga) in South Africa.

2.2.2 DNA Extraction

DNA was extracted from a total of 331 FGSC isolates using a modified CTAB (Cetyl Trimethylammonium Bromide) method (Möller *et al.*, 1992) as follows; 0.25 ml of isolated fungi was added to 2 ml centrifuge tubes (Eppendorf, Hamburg, Germany). 1 ml of DNA

extraction buffer (DEB) (0.2 M Tris HCl, 0.75 M NaCl, 0.025 M EDTA (pH 8) 0.025 M SDS) was added to the fungi in the tube and crushed with a sterile pipet tip for about 30 sec. The samples were then frozen in the -80°C freezer for 1 hour. After the samples have been frozen it was placed in boiling water for 5 minutes. 600 µl phenol: chloroform: iso-amylalcohol (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were centrifuged for 20 min at 14 000 rpm. The top aqueous layer was removed and added to a new tube, 200 µl of CTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris (pH 8), 20 mM EDTA, 0.2% β-mercaptoethanol (pH 8)) and 400 µl phenol: chloroform: isoamylalcohol (25:24:1) was added to the new tube and mixed by inversion. The samples were once again centrifuged for 15 min at 14 000 rpm, after which the aqueous layer was again removed and added to a new tube. Sixty µl 3 M Sodium acetate and 800 µl 100% ice cold ethanol were added to the supernatant. After samples have been mixed by inversion it was centrifuged for 10 min at 14 000 rpm. The remaining supernatant was discarded and 500 µl of 70% ethanol was added to wash the DNA pellet. The samples were centrifuged for one last time for 5 min at 14 000 rpm, after which the supernatant was discarded and the tubes were then left open to air dry the pellet in a laminar flowhood for approximately 1 hour. Fifty µl of Low TE buffer (0.1 mM EDTA; 10 mM Tris) (Applichem, Germany) and 2 µl RNase were added to the tube and vortexed until the pellet had dissolved. The samples were quantified and DNA concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, South Africa) and the quality was determined with A260/A280 ratio of between 1.7 - 2.0.

2.2.3 Species-specific polymerase chain reaction (PCR)

Reference isolates representing five out of six FGSC species that are found on maize in South Africa namely; *F. graminearum* (13.08), *F. boothii* (M0100), *F. cortaderiae* (2.551), *F. acacia-mearnsii* (2.887) and *F. meridionale* (2.855) with an extra *F. graminearum* (2.570) and *F. boothii* (2.881) (Table 2.2) were supplied by the University of Stellenbosch and were used to determine the unique banding pattern of each FGSC member. This pattern was then compared to the 331 isolates.

Table 2.2: Reference isolates representing five out of six species within the *Fusarium graminearum* species complex, previously reported on South African grain and provided and identified by Stellenbosch University using MLGT.

Species identity	Isolate number
<i>F. acacia-meransii</i>	2.887
<i>F. boothii</i>	M0100; 2.881
<i>F. brasilicum</i>	2.940
<i>F. cortaderidae</i>	2.551
<i>F. graminearum</i>	13.08; 2.570
<i>F. meridionale</i>	2.853

Amplification was conducted using *F. graminearum* s.l species-specific primers (Table 2.3) from Nicholson *et al.* (1998) and the reaction mixture consisted of 1.6 ng/ μ l DNA, 12.5 μ l 2x MasterMix (Promega Corporation, Madison, USA) and 0.2 μ M of each primer. Nuclease-free water was used to make up the reaction volume to 25 μ l. Amplification was performed on the Bio-Rad C1000 Touch Thermal Cycler (Biorad, Hercules, USA) and the conditions were as follows: denaturation for 2 min at 94°C followed by 35 cycles of 94°C, 30 sec at 58°C, 45 sec at 72°C, with a final extension step of 5 min at 72°C (Nicholson *et al.*, 1998). The PCR products (containing 6x DNA loading Dye) (Fermentas, Hanover, USA) stained with GelRed (Biotum, Hayward, USA) were separated on a 1% agarose gel and electrophoresed for 20 min at 120 V and visualised using the Geldoc system (Biorad). The molecular size of each fragment was determined using the 100-bp GeneRuler DNA ladder (Fermentas, Hanover, USA) and the FGSC isolates were grouped according to amplicon sizes of the five known FGSC species that occur in South Africa which served as positive controls.

2.2.4 Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP)

Amplification of the translation elongation factor EF-1 α (TEF1) and histone 3 (H3) gene regions:

The *TEF1* gene region was amplified with EF1 and EF2 primers (O'Donnell *et al.*, 2004; Geiser *et al.*, 2004), and *H3* region with H3dStyl (Suga *et al.*, 2008) and H3R1 primers (O'Donnell *et*

al., 2004) (Table 2.3). The *TEF1* and *H3* amplification was performed using the same reaction set-up as above. Thermal settings for amplification of *EF-1 α* gene region consisted of 5 min at 94°C; followed by 35 cycles for 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C; before a final step for 7 min at 72°C. The thermal cycle conditions for *H3* consists of an initial step for 2 min at 95°C; followed by 35 cycles for 30 sec of 95°C, 45 sec at 56°C and 45 sec at 72°C; with a final step for 2 min at 72°C. The PCR products were visualised as previously mentioned.

Restriction digests:

For PCR-RFLP analysis of the *TEF1* and *H3* amplicons, 10 µl of the PCR product was mixed in a 20 µl reaction volume with the restriction enzymes *BsaHI*, *BfaI*, *EaeI*, *MseI*, *SpeI* and *DraI* (New England Biolabs, United Kingdom) respectively, according to manufacturer's recommendations (Table 2.4). The PCR products were visualised as described previously but a 3% agarose gel and electrophoresis at 100 V for 45 min was used.

2.2.5 Multilocus genotyping (MLGT)

The 331 unknown samples that were collected throughout South Africa were subjected to a MLGT assay for simultaneous determination and species identity prediction of trichothecene chemotype by Professor Todd Ward (USDA ARS, Peoria, Illinois, USA) (Ward *et al.*, 2008). The results received from the MLGT were compared to the results obtained by the PCR technique.

Table 2.3: Gene regions, primer names and sequences used for the molecular identification of *Fusarium graminearum* species complex.

Target	Primer name	Primer sequence	References
<i>F. graminearum</i>	EF1	ATGGGTAAGGARGACAAGAC	O'Donnell <i>et al.</i> , (1998); Geiser <i>et al.</i> , (2004)
	EF2	GGARGTACCAGTSATCATGTT	O'Donnell <i>et al.</i> , (1998); Geiser <i>et al.</i> , (2004)
	H3dStyl	AGCATCACCYGAACATCGCATCATCCCATG	Suga <i>et al.</i> , (2004)
	H3R1	TTGGACTGGATRGTAACACGC	O'Donnell, <i>et al.</i> , (2004)
	Fg16F	CTCCGGATATGTTGCGTCAA	Nicholson <i>et al.</i> , (1998)
	Fg16R	GGTAGGTATCCGACATGGCAA	Nicholson <i>et al.</i> , (1998)

Table 2.4: Restriction enzymes used for the identification of *Fusarium graminearum* species complex by means of PCR-restriction fragment length polymorphism (Enzymes supplied by New England Biolabs)

Target gene region	Enzyme name	Restriction recognition site
EF-1 α	<i>Bfa</i> I	C^TAG
EF-1 α	<i>Bsa</i> HI	GR^CGYC
EF-1 α	<i>Ea</i> rI	CTCTTC (1/4)
EF-1 α	<i>Dra</i> I	TTT^AAA
EF-1 α	<i>Spe</i> I	A^CTAGT
EF-1 α	<i>Mse</i> I	T^TAA
H3	<i>Bfa</i> I	C^TAG
H3	<i>Bsa</i> HI	GR^CGYC
H3	<i>Ea</i> rI	CTCTTC (1/4)
H3	<i>Dra</i> I	TTT^AAA
H3	<i>Spe</i> I	A^CTAGT
H3	<i>Mse</i> I	T^TAA

2.3 Results

2.3.1 Species-specific polymerase chain reaction (PCR)

Different FGSC members produced different size amplicons. A total of 331 FGSC samples were isolated. An amplicon was generated by most of the isolated FGSC isolates and the size of the different amplicons were compared to the reference FGSC isolates' amplicon sizes (Figure 2.3). As previously reported by (Nicholson *et al.*, 1998) *F. graminearum* sensu stricto isolates produced a single 400 bp product. Species previously reported as *F. boothii* (2.881) produced a multiple banding pattern; no banding pattern was found for *F. boothii* (M0100) and *F. acacia-mearnsii* (2.887); *F. brasiliicum* (2.940) produced a single 450 bp band and *F. meridionale* (2.853) and *F. cortaderiae* (2.551) produced a 500 bp band (Figure 2.3). Amplicon sizes that were not comparable to the control FGSC isolates were also generated. Based on these results the 331 FGSC isolates were divided into six groups (Table 2.5). *F. boothii* (2.881), *F. meridionale* (2.855) and *F. cortaderia* (2.551) were mostly found in internode 3, while most *F. graminearum* (13.08) and *F. graminearum* (2.570) was found in internode 2 (Table 2.6). The most species were found in the Northern Cape Province in the species groupings of *F. boothii* (M0100) or *F. acacia-mearnsii* (2.587) and *F. meridionale* (2.855) or *F. cortaderiae* (2.551) (Table 2.7).

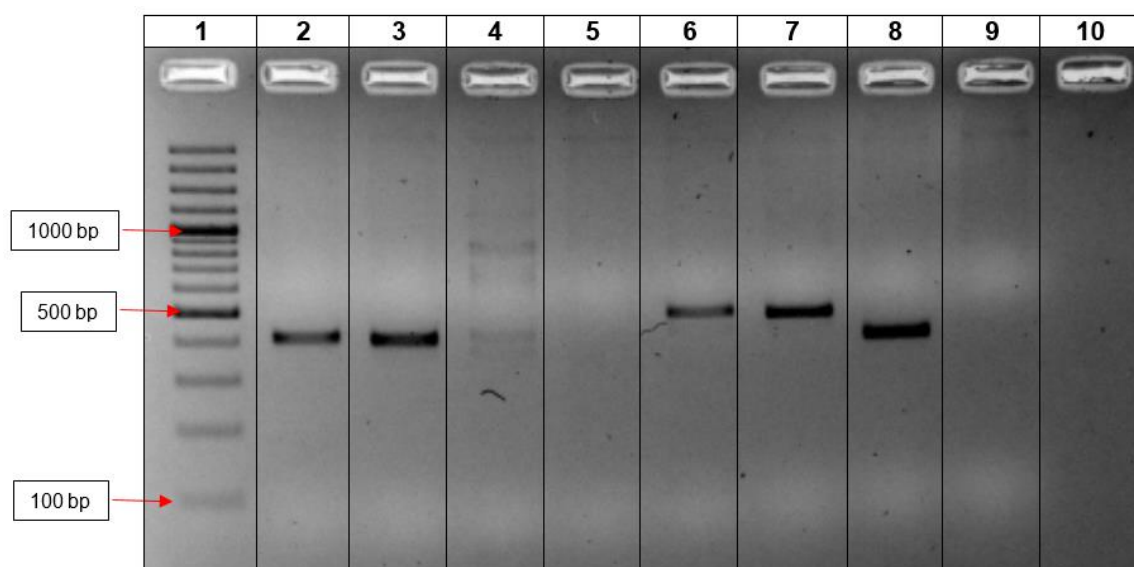


Figure 2.3: Amplicons generated with species –specific PCR. 1- DNA Ladder, 2- *F. graminearum* (13.08), 3- *F. graminearum* (2.570), 4- *F. boothii* (2.881), 5- *F. boothii* (M0100), 6- *F. meridionale* (2.855), 7- *F. cortaderiae* (2.551), 8- *F. brasiliicum* (2.940), 9- *F. acacia-mearnsii* (2.887), 10- Non-template control (NTC).

Table 2.5: Results of 331 isolates divided into seven groupings according to species-specific PCR product size, according to geographical area and plant part. The distribution is expressed as percentage of the 331 isolates.

Geographical area	Species	Total	Internode 1	Internode 2	Internode 3	Internode 4	Crowns	Roots
KwaZulu-Natal	<i>F. boothii</i> (2.881)	11	27.3	0	63.3	0	0	9.1
	<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	36	11.1	27.8	47.2	2.8	11.1	0
	<i>F. brasiliicum</i> (2.940)	13	7.7	38.5	53.8	0	0	0
	<i>F. graminearum</i> 913.08) / <i>F. graminearum</i> (2.570)	20	30	20	30	15	5	0
	<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	11	18.2	0	63.6	9.1	9.1	0
	Unknown	1	100	0	0	0	0	0
	Failed DNA	8	50	12.5	25	12.5	0	0
Northern Cape	<i>F. boothii</i> (2.881)	2.3	66.7	0	0	0	33.3	0
	<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	28.6	31.6	26.3	18.4	7.9	10.5	5.3
	<i>F. brasiliicum</i> (2.940)	24.1	25	3.13	53.13	0	18.75	0
	<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	32.3	13.2	23.7	23.7	7.9	31.6	0
	<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	4.5	16.7	50	33.3	0	0	0
	Failed DNA	8.3	0	36.4	36.4	18.2	9.1	0
Free State	<i>F. boothii</i> (2.881)	0	0	0	0	0	0	0
	<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	37.7	5	40	10	15	30	0
	<i>F. brasiliicum</i> (2.940)	22.6	16.7	25	8.3	16.7	33.3	0
	<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	18.9	10	40	20	10	20	0
	<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	9.4	0	60	0	20	20	0
	Failed DNA	11.3	16.7	16.7	16.7	33.3	16.7	0
Mpumalanga	<i>F. boothii</i> (2.881)	5.4	0	50	50	0	0	0
	<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	21.6	0	50	50	0	0	0
	<i>F. brasiliicum</i> (2.940)	24.3	0	55.6	22.2	22.2	0	0
	<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	27	30	50	10	0	10	0
	<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	8.1	0	33.3	66.7	0	0	0
	Failed DNA	13.5	20	0	40	40	0	0
North West	<i>F. boothii</i> (2.881)	0	0	0	0	0	0	0
	<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	12.5	0	0	0	0	100	0
	<i>F. brasiliicum</i> (2.940)	0	0	0	0	0	0	0
	<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	12.5	0	0	0	0	100	0
	<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	12.5	100	0	0	0	0	0
	Failed DNA	62.5	60	0	0	0	40	0

Table 2.6: Tentative groupings of 331 isolates according to FGSC reference genes to determine the tissue specificity of these members and percentage in South Africa. The distribution is expressed as a percentage of the 330 isolates.

	Amplicon size (bp)	Total	Internode 1	Internode 2	Internode 3	Internode 4	Crowns	Roots
<i>F. boothii</i> 2.881	Multiple bands	4,8	1,5	0,3	2,4	0	0,3	0,3
<i>F. boothii</i> M0100 / <i>F. acaciae-mearnsii</i> 2.587	No bands	31,1	5,1	9,7	9,1	2,1	4,5	0,6
<i>F. brasiliicum</i> 2.940	450	19,9	3,3	4,2	8,2	1,2	3	0
<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	400	24,8	4,2	7,6	5,4	2,1	5,4	0
<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	500	7,9	1,5	2,1	3,3	0,6	0,3	0
Unknown	600	0,3	0,3	0	0	0	0	0
Failed DNA	-	11,2	3,3	1,8	2,7	2,1	1,2	0

Table 2.7: Tentative groupings of 331 isolates according to FGSC reference isolates to determine the distribution of these members across the five provinces in all tissue types. The distribution is expressed as a percentage of the 330 isolates.

	Northern Cape	Free State	KwaZulu-Natal	Mpumalanga	North West
<i>F. boothii</i> (2.881)	0.9	0	3.3	0.6	0
<i>F. boothii</i> (M0100) / <i>F. acaciae-mearnsii</i> (2.587)	11.5	6	10.9	2.4	0.3
<i>F. brasiliicum</i> (2.940)	1.8	3.6	3.9	2.7	0
<i>F. graminearum</i> (13.08) / <i>F. graminearum</i> (2.570)	11.2	3	6	3	0.3
<i>F. meridionale</i> (2.855) / <i>F. cortaderiae</i> (2.551)	11.5	1.5	3.3	0.9	0.3
Unknown	0	0	0.3	0	0
Failed DNA	3.3	1.8	2.4	1.5	1.5

2.3.2 Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP)

Six restriction recognition sites were identified within the *EF-1 α* and *H3* gene regions for FGSC species (Gokul *et al.*, 2015). The restriction enzymes included, *Bsa*HI, *Bfa*HI, *Eae*I, *Spe*I, *Dra*I and *Mse*I (Table 2.4). Digestion of *EF-1 α* with *Bsa*HI produced a double restriction fragment at 250 bp and 350 bp for two FGSC species, namely *F. graminearum* (13.08; 2.570) and *F. brasiliicum* (2.940) that distinguish them from the other (Figure 2.4). Only *F. meridionale* could be identified with the *Bfa*I enzyme (Figure 2.5), as this species showed no restriction patterns. Digestion of *EF-1 α* with *Eae*I produced unique restriction fragment patterns for *F. brasiliicum* (2.940) with the only species with one band at 600 bp and *F. meridionale* (2.855) with no fragment patterns (Figure 2.6). Enzymes *Spe*I, *Dra*I and *Mse*I showed no restriction patterns with the *EF-1 α* gene.

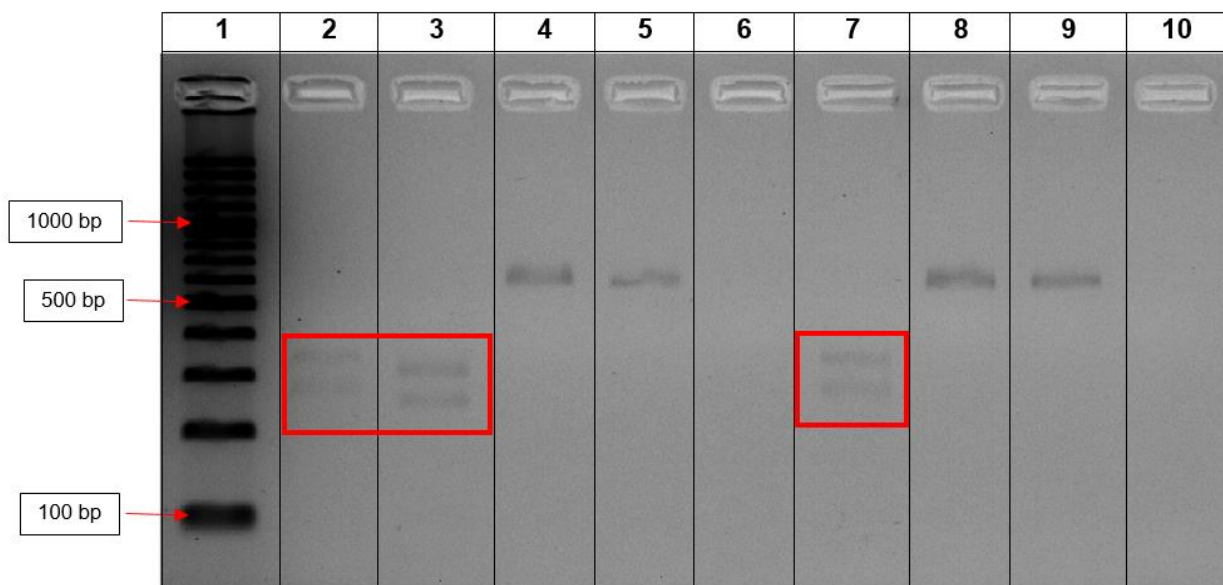


Figure 2.4: Translocation elongation factor α -1 (*EF-1 α*) region of *Fusarium* species and *Bsa*HI. 1- DNA Ladder, 2- *F. graminearum* (13.08), 3- *F. graminearum* (2.570), 4- *F. boothii* (2.881), 5- *F. boothii* (M0100), 6- *F. meridionale* (2.855), 7- *F. brasiliicum* (2.940), 8- *F. cortaderiae* (2.551), 9- *F. acacia-mearnsii* (2.887), 10- NTC.

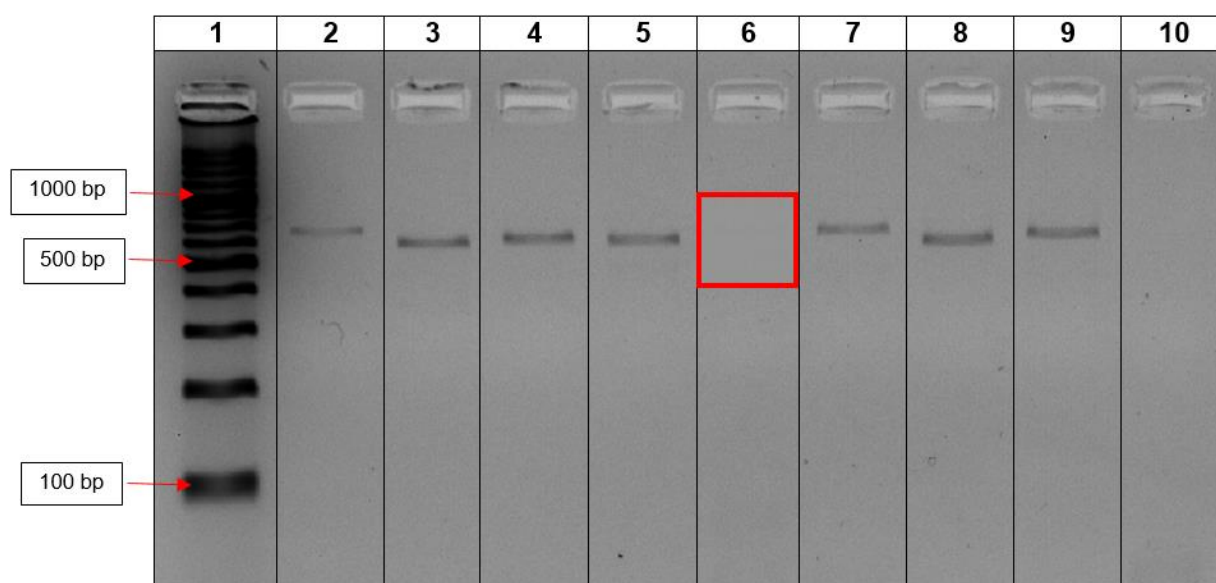


Figure 2.5: Translocation elongation factor α -1 (EF-1 α) region of *Fusarium* species and *Bfa*I. 1- DNA Ladder, 2- *F. graminearum* (13.08), 3- *F. graminearum* (2.570), 4- *F. boothii* (2.881), 5- *F. boothii* (M0100), 6- *F. meridionale* (2.855), 7- *F. brasiliicum* (2.940), 8- *F. cortaderiae* (2.551), 9- *F. acacia-mearnsii* (2.887), 10- NTC.

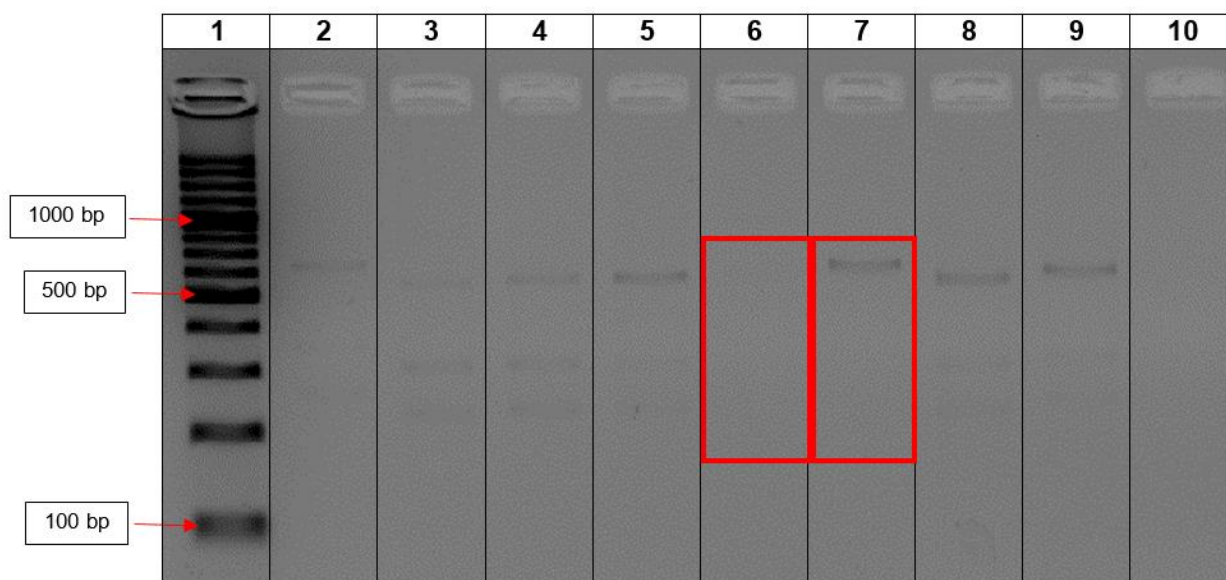


Figure 2.6: Translocation elongation factor α -1 (EF-1 α) region of *Fusarium* species and *Ear*I. 1- DNA Ladder, 2- *F. graminearum* (13.08), 3- *F. graminearum* (2.570), 4- *F. boothii* (2.881), 5- *F. boothii* (M0100), 6- *F. meridionale* (2.855), 7- *F. brasiliicum* (2.940), 8- *F. cortaderiae* (2.551), 9- *F. acacia-mearnsii* (2.887), 10- NTC.

Digestion of the *H3* gene with enzymes *SpeI* and *DraI* produced similar singular banding patterns and no unique pattern was produced to distinguish between FGSC species. However, the enzyme *MseI* was the only enzyme to produce unique banding patterns, and *F. acacia-mearnsii* could be distinguished from the other isolates with a double banding pattern at 150 bp and 200 bp (Figure 2.7). Digestion of the *H3* gene with the remaining three enzymes (*BsaHI*, *BfaI*, *EaeI*) was not able to produce any unique restriction patterns to distinguish between FGSC members.

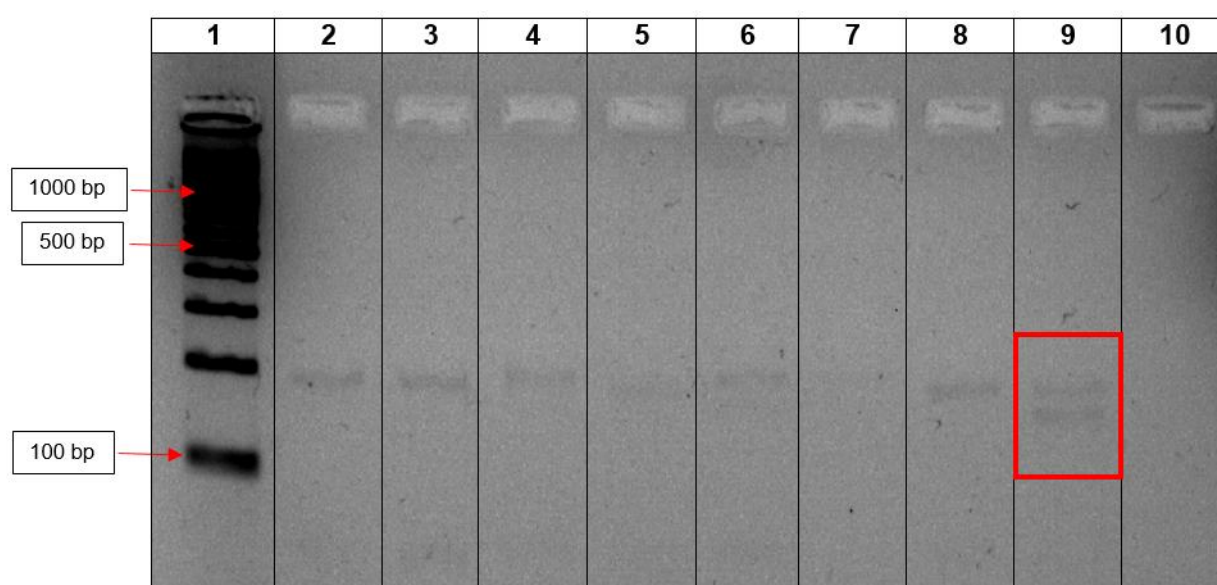


Figure 2.7: Histone gene (*H3*) region of *Fusarium* species and *MseI*. 1- DNA Ladder, 2- *F. graminearum* (13.08), 3- *F. graminearum* (2.570), 4- *F. boothii* (2.881), 5- *F. boothii* (M0100), 6- *F. meridionale* (2.855), 7- *F. brasilicum* (2.940), 8- *F. cortaderiae* (2.551), 9- *F. acacia-mearnsii* (2.887), 10- NTC.

2.3.3 Multilocus genotyping (MLGT)

Results from MLGT technique confirmed the finding of *F. boothii*, *F. graminearum* s.s and *F. meridionale* in the 331 unknown samples. A hybrid species, *F. graminearum* x *F. boothii* as well as a first identification of *F. cerealis* (Cooke) Sacc. (synonym *F. crookwellense* L.W. Burgess and, P.E Nelson et Tousson) and *F. lunulosporum* Gerlach were also found in the maize samples (Table 2.8). The hybrid species was found in maize stems in Northern Cape and Mpumalanga while in KwaZulu-Natal it was found in roots (Table 2.8). *F. cerealis* and *F. lunulosporum* was both found in Northern Cape Province in maize stems (Table 2.8).

The most abundant species in each province was *F. boothii* in KwaZulu-Natal, Free State, Mpumalanga and North West and *F. graminearum* being the most abundant species found in Northern Cape (Table 2.9).

In combination of all the provinces, *F. boothii* was the most abundant, responsible for 38.4% of the isolates, *F. boothii* was mostly found in internode 3 of the maize plant throughout South Africa (Table 2.10). *F. graminearum* was second most found in maize plants responsible for 14.8% of the isolates. Different mycotoxins such as 15-acetyldeoxynivalenol and nivalenol were also produced by the different species found (Table 2.11).

Table 2.8: Results of 331 isolates divided into groups according to species identified by the MLGT technique, according to geographical area and plant part. The distribution is expressed as percentage of the 331 isolates.

Geographical area	Species	Total	Internode 1	Internode 2	Internode 3	Internode 4	Crowns	Roots
KwaZulu-Natal	<i>F. boothii</i>	56	9	11	31	3	2	0
	<i>F. graminearum</i>	5	2	1	1	1	0	0
	<i>F. graminearum</i> x <i>F. boothii</i>	1	0	0	0	0	0	1
	<i>F. meridionale</i>	2	1	0	0	1	0	0
	<i>F. cerealis</i>	0	0	0	0	0	0	0
	<i>F. lunulosporum</i>	0	0	0	0	0	0	0
	Failed DNA	36	9	9	14	1	3	0
Northern Cape	<i>F. boothii</i>	20.3	6	1.5	7.5	1.5	3.8	0
	<i>F. graminearum</i>	30.8	4.5	9.8	4.5	1.5	10.5	0
	<i>F. graminearum</i> x <i>F. boothii</i>	6	0.8	0	5.3	0	0	0
	<i>F. meridionale</i>	0	0	0	0	0	0	0
	<i>F. cerealis</i>	3	1.5	0.8	0.8	0	0	0
	<i>F. lunulosporum</i>	1.5	1.5	0	0	0	0	0
	Failed DNA	38.4	8.3	10.5	11.3	3	3.8	1.5
Free State	<i>F. boothii</i>	43.4	1.9	15.1	1.9	11.3	13.2	0
	<i>F. graminearum</i>	3.8	0	1.9	0	0	1.9	0
	<i>F. graminearum</i> x <i>F. boothii</i>	0	0	0	0	0	0	0
	<i>F. meridionale</i>	0	0	0	0	0	0	0
	<i>F. cerealis</i>	0	0	0	0	0	0	0
	<i>F. lunulosporum</i>	0	0	0	0	0	0	0
	Failed DNA	52.8	7.5	18.9	9.4	5.7	11.3	0
Mpumalanga	<i>F. boothii</i>	51.4	5.4	21.6	18.9	5.4	0	0
	<i>F. graminearum</i>	2.7	0	2.7	0	0	0	0
	<i>F. graminearum</i> x <i>F. boothii</i>	2.7	2.7	0	0	0	0	0
	<i>F. meridionale</i>	0	0	0	0	0	0	0
	<i>F. cerealis</i>	0	0	0	0	0	0	0
	<i>F. lunulosporum</i>	0	0	0	0	0	0	0
	Failed DNA	43.2	2.7	18.9	13.5	5.4	2.7	0
North West	<i>F. boothii</i>	25	12.5	0	0	0	12.5	0
	<i>F. graminearum</i>	0	0	0	0	0	0	0
	<i>F. graminearum</i> x <i>F. boothii</i>	0	0	0	0	0	0	0
	<i>F. meridionale</i>	0	0	0	0	0	0	0
	<i>F. cerealis</i>	0	0	0	0	0	0	0
	<i>F. lunulosporum</i>	0	0	0	0	0	0	0
	Failed DNA	75	37.5	0	0	0	37.5	0

Table 2.9: Tentative groupings of 331 isolates according to MLGT identification technique to determine the distribution of these members across the five provinces in all tissue types. The distribution is expressed as a percentage of the 331 isolates.

	Northern Cape	Free State	KwaZulu-Natal	Mpumalanga	North West
<i>F. boothii</i>	8,5	6,9	16,9	5,7	0,6
<i>F. graminearum</i>	12,4	0,6	1,5	0,3	0
<i>F. graminearum</i> x <i>F. boothii</i>	2,4	0	0,3	0,3	0
<i>F. meridionale</i>	0	0	0,6	0	0
<i>F. cerealis</i>	1,2	0	0	0	0
<i>F. lunulosporum</i>	0,6	0	0	0	0
Failed DNA	15,4	8,5	10,9	4,8	1,8

Table 2.10: Tentative groupings of 331 isolates according to MLGT identification technique to determine the tissue specificity of these members and percentage in South Africa. The distribution is expressed as a percentage of the 331 isolates.

SOUTH AFRICA	(%)	Internode 1	Internode 2	Internode 3	Internode 4	Crowns	Roots
<i>F. boothii</i>	38,4	6,4	8,8	14,8	3,9	4,5	0
<i>F. graminearum</i>	14,8	2,4	4,8	2,1	0,9	4,5	0
<i>F. graminearum</i> x <i>F. boothii</i>	3	0,6	0	2,1	0	0	0,3
<i>F. meridionale</i>	0,6	0,3	0	0	0,3	0	0
<i>F. cerealis</i>	1,2	0,6	0,3	0,3	0	0	0
<i>F. lunulosporum</i>	0,6	0,6	0	0	0	0	0
Failed DNA	41,4	8,5	12,1	11,8	3	5,4	0,6

Table 2.11: Mycotoxigenic *Fusarium* species identified in comprehensive sampling in South African maize and their associated toxins.

Specie	Mycotoxins produced
<i>F. boothii</i>	15-ADON
<i>F. graminearum</i>	15-ADON
<i>F. graminearum</i> x <i>F. boothii</i>	15-ADON
<i>F. meridionale</i>	NIV
<i>F. cerealis</i>	NIV
<i>F. lunulosporum</i>	NIV

15-ADON: 15-acetyldeoxynivalenol; NIV: Nivalenol

2.3.4 Comparing PCR based- and MLGT techniques

The results obtained from the MLGT technique and the results from species-specific PCR were compared. From the 331 samples the species-specific PCR technique showed that 4.8% are *F. boothii* (2.881) and 31.1% could be *F. boothii* (M0100) or *F. acacia-mearnsii* (2.587), seeing that these two last mentioned species had the same banding pattern. The MLGT technique accurately identified 38.4% of the samples to be *F. boothii*. The species-specific PCR technique and MLGT technique corresponded over 11.8% of the samples to be *F. boothii*.

The species-specific PCR technique identified 24.8% as *F. graminearum* and the MLGT identified 14.8% to be *F. graminearum*, with a correspondence of 10.9% of the samples. The species-specific PCR technique identified 7.9% of the samples to be *F. meridionale* or *F. cortaderiae* of which 0.6% was identified by the MLGT technique to be *F. meridionale*. The species-specific PCR failed to produce any banding patterns for 10.6% of the samples and could not be identified, the MLGT technique also showed 41.4% of the samples that failed identification because of failed DNA.

From the remaining 4.8% of the samples, 0.6% was identified as *F. lunulosporum*, 1.2% as *F. cerealis* and 3% as *F. graminearum* x *F. boothii* (hybrid), which couldn't be identified with the species-specific PCR technique because there was no reference gene for these species.

2.4 Discussion

This study is the first to determine the FGSC composition in maize roots and stems occurring throughout South Africa. Information on the distribution of FGSC species in South Africa, as well as the toxins they produce may be helpful for the future development of resistant maize cultivars and sustainable disease management practices. To date, the MLGT assay has been the only technique to accurately identify FGSC species (Ward *et al.*, 2008).

The *F. graminearum* species-specific primer pair developed by Nicholson *et al.* (1998), was able to divide 331 isolates in seven groupings according to amplification product. *F. graminearum* s.s isolates produced a single 400 bp product, as was previously reported by Nicholson *et al.* (1998). Although *F. boothii* (M0100) and *F. acacia-mearnsii* (2.587) as well as *F. meridionale* (2.855) and *F. cortaderiae* (2.551) produced the same banding patterns respectively, *F. boothii* (2.881), *F. brasiliicum* (2.940) and *F. graminearum* (13.08; 2.570) all produced unique banding patterns respectively to distinguish all from one another. Waalwijk *et al.* (2003) also reported the identification of *F. graminearum* s.s. and *F. meridionale* through the amplification of their DNA with this primer pair. It is not expected that the primer pair will be able to differentiate between all the species occurring in the FGSC, but could be of value to detect existing FGSC species occurring on South African grain crops.

PCR-RFLP of the *EF-1 α* and *H3* gene regions was useful to distinguish *F. meridionale* (2.855), *F. brasiliicum* (2.940) and *F. acacia-mearnsii* (2.587) from the other isolates. While this is a relatively easy and affordable method that can identify a large quantity of samples and does not require advanced instruments for identification of closely related species, it is still relatively time-consuming (González Jaén *et al.*, 2004; Wang *et al.* 2011). More gene regions and restriction enzymes will have to be evaluated and tested before it could be used to identify species within the FGSC (González Jaén *et al.*, 2004). The use of this PCR based technique also requires reference isolates in order to compare isolates for identification. Although methods from Gokul *et al.* (2015) have been used, the only correspondence found in this study was the single 400 bp product produced by the *F. graminearum* (13.08; 2.570) isolate with the species-specific PCR that was also reported by Nicholson *et al.*, (1998). Further, no other amplicon sizes corresponded to those found in this study, and no other resemblance could be seen in the results retrieved in this study. The value of this technique needs to be investigated

with all 16 FGSC species. In addition, future studies could include more isolates representing the FGSC species on South African grain.

In contrast with the RFLP technique, the MLGT method used for accurate identification of FGSC members were able to not only identify members in the FGSC and a hybrid species, but also two *Fusarium* species that haven't been found on maize before, namely, *F. cerealis* and *F. lunulosporum*. *F. lunulosporum* has only been reported to be found on grapefruit before in South Africa in 1976 (Gerlach, 1977). Thus, this would be the first finding of *F. lunulosporum* on maize. However, *F. cerealis* have been reported to be found in wheat crowns (Van Coller *et al.*, 2011) and in wheat heads (Van Coller *et al.*, 2012) in South Africa as well as in wheat grains in Argentina (Palacios *et al.*, 2017) and Canada (Amarasinghe *et al.*, 2015). *F. cerealis* has also been found in barley seeds in Argentina (Castañares *et al.*, 2013) thus, also a first finding of this species on maize.

A naturally occurring hybrid species and interaction between *F. graminearum* and *F. boothii* has also been identified by the MLGT technique. Boutigny *et al.* (2011) documented the second only natural-occurring hybrid between FGSC species, *F. graminearum* and *F. boothii* on maize ears, which was confirmed by DNA sequence analysis, and first to be detected by MLGT analysis. In a previous demonstration by O'Donnell *et al.* (2000) a strain from Nepal was the product of interspecific hybridization between *F. asiaticum* and *F. meridionale*. The hybrid species found on maize in this study, however, was found on stems in Northern Cape and Mpumalanga and on roots in KwaZulu-Natal.

Phylogenetic analysis based on numerous independent loci have proved that the occurrence of such hybridization occasions has been insufficient to combat isolation by genetic drift (O'Donnell *et al.*, 2000; 2004; 2008; Starkey *et al.*, 2007). The species specific stability and variation targeted by the MLGT assay is consistent with this conclusion (O'Donnell *et al.*, 2008; Ward *et al.*, 2008; Yli-Mattilla *et al.*, 2009; Gale *et al.*, 2011;).

Although six FGSC species are known to infect grain crops in South Africa namely, *F. acacia-mearnsii*, (wheat and sorghum) (Boutigny *et al.*, 2011; Mavhunga, 2013), *F. boothii* (maize ears and roots, barley and wheat) (Boutigny *et al.*, 2011), *F. brasiliicum* (wheat) (Boutigny *et al.*, 2011), *F. cortaderiae* (wheat and sorghum) (Boutigny *et al.*, 2011; Mavhunga, 2013), *F. graminearum* s.s. (maize roots and wheat) (Boutigny *et al.*, 2011), and *F. meridionale* (maize

roots, sorghum and wheat) (Boutigny *et al.*, 2011; Mavhunga, 2013), only three of the above mentioned are known to infect maize roots (*F. graminearum*, *F. boothii* and *F. meridionale*). However, in this study, not only was *F. graminearum* and *F. boothii* found in maize stems and crowns and *F. meridionale* on stems but an interspecific FGSC hybrid species of *F. graminearum* and *F. boothii* was also identified on maize stems and crowns. Two Fusarium species, *F. lunulosporum* and *F. cerealis* were also identified amongst the samples on maize stems.

Members of the FGSC produce type B trichothecenes (Goswami and Kistler, 2004; O'Donnell *et al.*, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009), which includes 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as nivalenol (NIV). *F. cerealis* and *F. lunulosporum* have also been reported to produce nivalenol chemotype (O'Donnell *et al.*, 2008). Results from this study also confirmed the 15-acetyldeoxynivalenol chemotypes that were found on *F. boothii*, *F. graminearum* and *F. graminearum* x *F. boothii* and nivalenol chemotypes on *F. meridionale*, *F. cerealis* and *F. lunulosporum*.

2.5 Conclusion

Comparing results of species-specific PCR and MLGT, it is clear that species-specific PCR could not deliver to accurately identify the isolates, seeing that only 27.8% of the isolates identified by PCR corresponded with MLGT identification.

Three out of the six enzymes were able to identify four species with the PCR-RFLP technique. Before considering the use of PCR-RFLP as an alternative method for distinguishing between closely related FGSC members, all 16 FGSC members must be tested in order to ensure accuracy and more gene regions and restriction enzymes will have to be evaluated and tested before it could be used to identify species within the FGSC. Because the use of this PCR based technique also requires reference isolates in order to compare isolates for identification, future studies could include more isolates representing the FGSC species on South African grain.

Although methods from Gokul *et al.* (2015) were used, the only correspondence found in this study and the mentioned study was the single 400 bp product produced by the *F. graminearum* (13.08; 2.570) isolate with the species-specific PCR that was also reported by Nicholson *et al.*

(1998). Further, no other amplicon sizes corresponded to those found in this study, and no other resemblance could be seen in the results retrieved in this study.

This study not only confirmed the presence of three FGSC species that were previously found on maize roots and ears (Boutigny *et al.*, 2011; Mavhunga *et al.*, 2013), but also confirmed the presence of *F. graminearum* and *F. boothii* on crowns and stems and *F. meridionale* on stems by using the MLGT technique. This study also confirmed the presence of an interspecific hybrid species on maize in South Africa, as well as two *Fusarium* species which have not been found on maize plants previously.

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CHAPTER 3 - EVALUATION OF PATHOGENICITY AND MYCOTOXIN PRODUCTION OF *FUSARIUM GRAMINEARUM* SPECIES COMPLEX MEMBERS IN STEM AND GRAIN ISOLATES

Abstract

Fusarium graminearum species complex (FGSC) is the causal agent of ear, root-, crown- and stem rot in maize. Previously, only three of the FGSC species have been identified on maize in South Africa namely; *F. graminearum* sensu stricto (maize roots and ears), *F. boothii* (maize ears) and *F. meridionale* (maize roots). Grains that are infected with FGSC members may result in low kernel weight and reduced seed quality and another concern is that these fungi are able to produce toxic secondary metabolites known as mycotoxins. Mycotoxins such as deoxynivalenol (DON), zearalenone (ZEA) and nivalenol (NIV) are known to be produced by FGSC on maize grain. Very little information about the diversity of FGSC and their trichothecene chemotypes in South Africa cereal grains is known. Although FGSC host preferences have been studied before, the preference of FGSC members on specific maize plant tissue has not been studied adequately and the information whether FGSC members are plant part specific coupled with possible pathogenicity and mycotoxin contamination is lacking. Therefore, the aim of this study was to artificially inoculate three species of the FGSC; *F. graminearum* s.s., *F. boothii* and a hybrid species *F. graminearum* x *F. boothii*, into maize stems and ears to see whether each species were only pathogenic on specific plant parts and if mycotoxins could be detected. Results from this study showed that all three the FGSC members were pathogenic to all inoculated maize plant parts. Successful infection of stems and ears were observed. Even though *F. boothii* was only previously found on maize ears it showed to be a very pathogenic pathogen on stems. The hybrid species of *F. graminearum* x *F. boothii* was also virulent to maize ears and stems. The FGSC isolates are not just able to be pathogenic in ears, roots and crowns, but also in stems, and are in some cases able to be more virulent in stems than in the ears. FGSC members are pathogenic on maize and thus all members can cause diseases. This highlighted the need for resistant cultivars against stem and ear rots. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) results showed levels of zearalenone in maize ears inoculated with *F. graminearum* and *F. boothii* collected from different provinces in South Africa. Nivalenol concentrations were found in maize stems where *F. graminearum* and *F. boothii* were inoculated and the mycotoxin potential was proven by the hybrid species

F. graminearum x *F. boothii* with different nivalenol-levels from different provinces in South Africa. No deoxynivalenol levels could be detected in the inoculated ears and stems. Mycotoxin concentrations are a big concern and poses health risks for both humans and animals.

Keywords: *Fusarium graminearum* species complex; pathogenicity; stem rot; ear rot; first report

3.1 Introduction

Fusarium species are often found as pathogens of cereal crops, including maize worldwide. This includes members of the *Fusarium graminearum* species complex (FGSC) which are the main agents of seedling blight, Gibberella ear, root-, crown- and stem rot on maize (Booth, 1971; Carter *et al.*, 2002). Cereal grains that are infected with FGSC members may result in low kernel weight and reduced seed quality (Parry *et al.*, 1995; Dexter *et al.*, 1997), but it also raises concerns as this fungus is able to produce secondary toxic metabolites known as mycotoxins. Zearalenone (ZEA), an estrogenic mycotoxin and the type B-trichothecenes (TCT-B) (chemotypes), most commonly deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) and at a lower frequency, nivalenol (NIV) are the two groups of mycotoxins that are frequently associated with *F. graminearum* (Marasas *et al.*, 1981; Lee *et al.*, 2009; Desjardins and Proctor, 2011; Boutigny *et al.*, 2012; Malbrán *et al.*, 2014).

Reduced TCT-B production does not have an impact on the capacity of *F. graminearum* to infect maize, but the infection does develop slower due to a decrease in virulence (Proctor *et al.*, 1995). Although deoxynivalenol is more toxic to plants (Desjardins, 2006), the production of nivalenol is more toxic to animals and humans (Ryu *et al.*, 1988). The production of zearalenone by *F. graminearum* increases the development of perithecia which improves survival on plant residue and this would increase the infection rates the following season (Wolf and Mirocha, 1973).

Sixteen distinct phylogenetic lineages within the *F. graminearum* morphospecies have been described and collectively known as the FGSC (O' Donnell *et al.*, 2000, 2004, 2008; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009; Sarver *et al.*, 2011; Aoki, *et al.*, 2012). Nine out of the sixteen species only produce one out of the three chemotypes while the other seven produce two or three chemotypes (Aoki *et al.*, 2012). According to Wang *et al.* (2011), three chemotypes, namely deoxynivalenol and 3-acetyldeoxynivalenol, deoxynivalenol and 15-acetyldeoxynivalenol and nivalenol and 4-acetylnivalenol (4-ANIV) have been identified within the FGSC. Very limit information about the diversity of FGSC and their trichothecene chemotypes in South Africa cereal grains is available. In a recent study conducted by Boutigny *et al.* (2011), it provided an understanding of the geographic distribution and host range as well as the prevalence of FGSC species occurring in South Africa. Results proved that at least six

members of the FGSC producing nivalenol and 15-acetyldeoxynivalenol occurs on grain in South Africa. Significant differences in the toxicity of these trichothecenes have been noted, thus the importance of understanding the diversity and nature of the maize infected FGSC in South Africa is highlighted, as these toxins pose a health hazard for humans and animals (Boutigny *et al.*, 2011).

The mycotoxin, deoxynivalenol, is also known as vomitoxin because of the strong vomit effects after consumption. Deoxynivalenol can also lead to vomiting, food refusal, decreased weight gain and less effective feed utilisation if consumed by livestock (Young *et al.*, 1983). Consumption of deoxynivalenol -contaminated food by humans can lead to nausea, vomiting and diarrhoea (Yoshizawa and Morooka, 1974). Nivalenol, which is more toxigenic than deoxynivalenol, has resulted in lower feed conversion, reduced feed consumption and decreased liver weights when ingested by chickens (Hedman *et al.*, 1995). However, nivalenol-producing FGSC members have been less frequently associated with grain in South Africa (Boutigny *et al.*, 2011; 2012). Zearalenone is the most widely distributed *Fusarium* mycotoxin globally. Zearalenone is biologically very potent, despite its low acute toxicity (Stob *et al.*, 1962; Kuiper-Goodman *et al.*, 1987; Logrieco *et al.*, 2002).

Of the 16 FGSC members, only six have been identified on South African grain, this includes; *F. graminearum* sensu stricto (maize roots and wheat) (Boutigny *et al.*, 2011), *F. boothii* (maize ears, barley and wheat) (Boutigny *et al.*, 2011), *F. meridionale* (maize roots, sorghum and wheat) (Boutigny *et al.*, 2011; Mavhunga, 2013), *F. cortaderiae* (wheat and sorghum) (Boutigny *et al.*, 2011; Mavhunga, 2013), *F. acaciae-mearnsii* (wheat and sorghum) (Boutigny *et al.*, 2011; Mavhunga, 2013), and *F. brasilicum* (wheat) (Boutigny *et al.*, 2011). According to a study conducted by Lamprecht *et al.* (2011) in KwaZulu-Natal, results proved that only three of these species, namely, *F. graminearum* s.s., *F. meridionale*, and *F. boothii* are currently known to be associated with maize crowns and roots in South Africa. Due to only *F. boothii* being confirmed as a pathogen on maize ears by Boutigny *et al.* (2011), the question is raised if the different members of the FGSC differ in pathogenicity on the different plant parts of maize. Thus, there is a possibility that *F. boothii* is more virulent on maize ears than on maize stems or that *F. graminearum* is more virulent on stems. Furthermore, what the effect of the presence of mycotoxins are and do certain mycotoxins aid better in pathogenicity? In this study three species of the FGSC; *F. graminearum* s.s.; *F. boothii* and a hybrid species *F. graminearum* x *F. boothii* were isolated from diseased maize from different provinces and were

artificially inoculated into maize stems and ears, respectively, in order to investigate pathogenicity and mycotoxin production.

The mycotoxins produced by maize ears contaminated with *F. graminearum* have previously been studied (Harris *et al.*, 1999) and a few studies have shown the mycotoxin accumulation in maize stems in Switzerland (Dorn *et al.*, 2011; Eckard *et al.*, 2011). However, no experiment has been performed to determine whether this is also the case for FGSC species that were previously found on South African maize stems. The interaction of *F. graminearum* with wheat has also been studied more intensively than the interaction with maize, thus the information about mycotoxin production, accumulation and possible correlation with maize stems is lacking (Trial, 2009).

FGSC chemotypes have been reported to differ in geographic distribution (Ji *et al.*, 2007). The mycotoxin contamination was also tested in this study through artificial inoculation of the previously mentioned three FGSC members on maize ears and stems collected from infected maize from different provinces within South Africa (Chapter 2). Although FGSC host preference has been studied by Boutigny *et al.* (2011), the preference of FGSC members on specific maize plant tissue has not been studied previously. Therefore, the aim of this study was to understand the difference in pathogenicity on specific plant tissue and mycotoxin production of FGSC isolates in maize stems and grain.

3.2 Materials and methods

3.2.1 Glasshouse trials

Trial layout

A white maize cultivar PAN 6479 was planted and grown under greenhouse conditions with natural day/night hours at an average of 25°C through the day and night at the Agriculture Research Council – Grain Crops Institute in Potchefstroom, South Africa. The intra-row spacing were 90 cm and inter-row spacing were 30 cm. Two maize kernels were planted every 30 cm in a row and the weaker seedling was removed two weeks after plant emergence. The trial layout consisted of 18 plants that were planted in a complete randomised block design for

ear inoculation in 3 rows, with 6 plants in a row equally divided to be inoculated with 3 species fungal isolates respectively (2 plants per inoculated isolate), derived from different provinces throughout South Africa. This layout was repeated 3 times, thus a total of 18 plants per inoculated fungal isolate on ears. This trial layout was repeated for stem inoculation. In addition, 108 plants were also used for water inoculation for an equal amount stems and ears respectively which served as controls. The plants in the glasshouse were watered twice a week.

FGSC isolates and inoculum preparation.

Three isolates, namely, *F. graminearum*, *F. boothii* and a hybrid species, *F. graminearum* x *F. boothii* which was obtained from diseased maize plants, collected from four different locations (KwaZulu-Natal, Free State, Northern Cape and Mpumalanga) from trials mentioned in Chapter 2, were used to inoculate plants. The isolates were previously identified and chemotyped by multilocus genotyping (MLGT) by Professor Todd Ward (USDA ARS, Peoria, Illinois, USA) (Ward *et al.*, 2008) (Chapter 2).

Fungal mycelia suspensions for maize inoculations were prepared by cultivating the FGSC isolates in 100 ml Armstrong Fusarium medium (Booth, 1977) in 500 ml Erlenmeyer flasks at 25°C on a rotary shaker at 130 rpm. After $\pm$ 7 days the fungal growth was collected by filtration through sterile cheesecloth. The suspension was centrifuged at 4000 rpm for 5 min at 4°C, the supernatant was discarded and the mycelia were washed twice with deionised, autoclaved water and adjusted to a final concentration of 1×10^6 spores/ml. Control isolates consisted of distilled water only.

Artificial inoculation

Maize ears were inoculated six days after silk emergence (Reid *et al.*, 1999; 2002) by using disposable, sterile syringes fitted with 18 G 1.5" needles (Small *et al.*, 2012). Two ml of the liquid mycelia was injected down the silk channel of primary ears (Afolabi *et al.*, 2007). Inoculation with sterile de-ionised water served as controls.

Maize stems were inoculated at the time the ears started grain fill, when the plant takes up the most water and nutrients (Ritchie *et al.*, 1986). Using a sterile scalpel to strip the sheath tissue from the plant in the area between the soil line and the first internode above the secondary

roots, one ml of mycelium which was filtrated through a sterile cheesecloth and washed twice with deionised, autoclaved water and was placed over the open wound and covered with parafilm to maintain a moist environment.

This was replicated for three ears and three stems per inoculated isolate that served as reps respectively, and each rep was repeated three times. For each inoculated ear or stem there was also an ear and stem that served as the control plants. The inoculated maize ears and stems were harvested at 14% kernel moisture.

Disease assessment and plant tissue processing

At harvest, maize ears were handpicked, de-husked and inspected for visual *Gibberella* ear rot disease symptoms (Figure 3.1). Visual disease severity was positively or negatively rated of each ear surface showing *Gibberella* ear rot symptoms and qPCR's were further used to determine disease severity. The three reps with three replicates were pooled to only three singular reps (three ears per rep). The maize grain was hand-shelled. The kernels were ground to a fine powder.

Maize stems were cut open and inspected for positive or negative visual *Gibberella* stem rot (Figure 3.2) and qPCR's were used to determine disease severity. Stems were also pooled from nine stems to three stems representing three reps. Stems were also ground to a finer form. Subsamples of maize kernels and stems of 5 g of each sample were weighed into 50 ml falcon tubes for mycotoxin analysis and 250 mg into 2 ml centrifuge tubes for DNA extractions and stored at -20°C.

qPCR assay has been developed to not only detect pathogens, but also reveal the quantification levels of the pathogens and is also the most sensitive and rapid assay to detect pathogens (Law *et al.*, 2015). Pathogenicity of pathogens has been noted to have a direct correlation to the qPCR levels (Wang *et al.*, 2011).



Figure 3.1: Positive visual *Gibberella* ear rot disease symptoms on artificially inoculated maize ears.



Figure 3.2: Positive visual *Gibberella* stem rot disease symptoms on artificially inoculated maize stems.

3.2.2 DNA extraction

DNA was extracted from FGSC samples using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Möller *et al.*, 1992) as follows: after 0.25 ml of inoculated ground plant material was added to 2 ml centrifuge tubes (Eppendorf, Hamburg, Germany), 1 ml of DNA extraction buffer (DEB) (0.2 M Tris HCl, 0.75 M NaCl, 0.025 M EDTA (pH 8) 0.025 M SDS) was added in the tube and crushed with a sterile pipet tip for about 30 sec. The samples were then frozen in the -80°C freezer for 1 hour. After the samples have been frozen it was placed in boiling water for 5 minutes. 600 µl phenol: chloroform: iso-amylalcohol (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were centrifuged for 20 min at 14 000 rpm. The top aqueous layer was removed and added to a new tube, where 200 µl of CTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris (pH 8), 20 mM EDTA, 0.2% β-mercaptoethanol (pH 8)) and 400 µl phenol: chloroform: isoamylalcohol (25:24:1) were added to the new tube and mixed by inversion. The samples were once again centrifuged for 15 min at 14 000 rpm, after which the aqueous layer was again removed and added to a new tube. Sixty µl 3 M Sodium acetate and 800 µl 100% ice cold ethanol was added to the supernatant. After samples have been mixed by inversion it was centrifuged for 10 min at 14 000 rpm. The remaining supernatant was discarded and 500 µl of 70% ethanol was added to wash the DNA pellet. The samples were centrifuged for one last time for 5 min at 14 000 rpm, after which the supernatant was discarded and the tubes were then left open to allow the pellet to air dry in a laminar flowhood for approximately 1 hour. Fifty µl of Low TE buffer (0.1 mM EDTA; 10 mM Tris) (Applichem, Germany) and 2 µl RNase were added to the tube with the pellet and vortexed until the pellet had dissolved. The samples were quantified and DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, South Africa) and the quality was determined with A260/A280 ratio of between 1.7-2.0.

3.2.3 Quantification of FGSC members using quantitative polymerase chain reaction (qPCR)

After the DNA was extracted from the samples, the samples were individually diluted to 10 ng/µl. qPCR was performed with Bio-Rad CFX 96 (United States, Hercules, USA) using a SYBR[®] green protocol and the fungal mass was quantified as pg/µl for *F. graminearum*. The

SYBR® Green protocol was done under the following conditions and reaction mix (10 µl Bio-Rad iTaq Universal SYBR® Green Supermix (Hercules, USA), 11.75 µl H₂O, 2 µl DNA (10 ng) and 0.625 µl of the forward primer (10 µM) (Fgram379-fwd (5'-CCATTCCCTGGGCGCT-3') and reverse primer (10 µM) (FgramB411-rev (5'-CCTATTGACAGGTGGTTAGTGACTGG-3')) (Nicolaisen *et al.*, 2009), designed to amplify the translation elongation factor (EF-1 α) gene of *F. graminearum* sensu lato. The qPCR cycles consisted of an initial step at 94°C for 10 min followed by 40 cycles of 94°C for 30 sec; 60°C for 30 sec and 72°C for 30 sec before two final extension steps at 95°C for 30 sec and 40°C for 30 seconds.

The C_q value of the standard curve was used to determine fungal DNA concentration for the samples. To ensure that the samples could be used, the efficiency was between the optimal range of 90-110%, the R² < 0.95 and the slope at -3.32 (Figure 3.3 and 3.4).

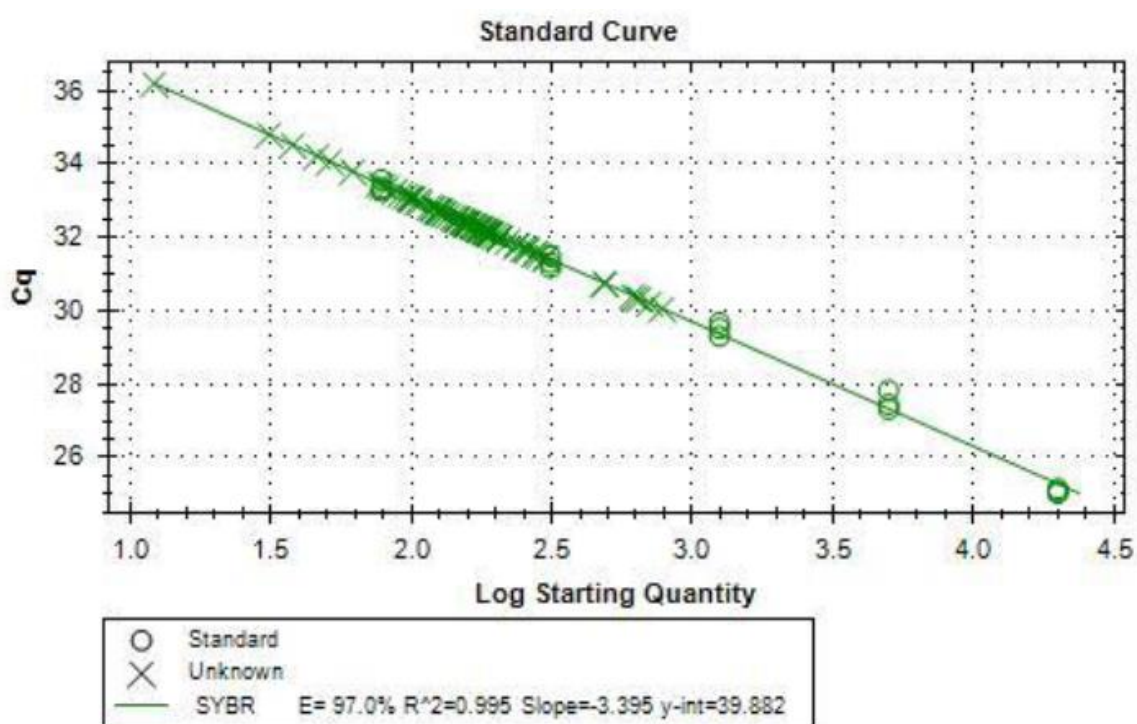


Figure 3.3: Standard curve analysis demonstrating the analytical specificity of the quantitative real-time PCR assay performed with FgramB470-fwd/ FgramB411-rev (Nicolaisen *et al.*, 2009) primers representing the FGSC members in maize stems.

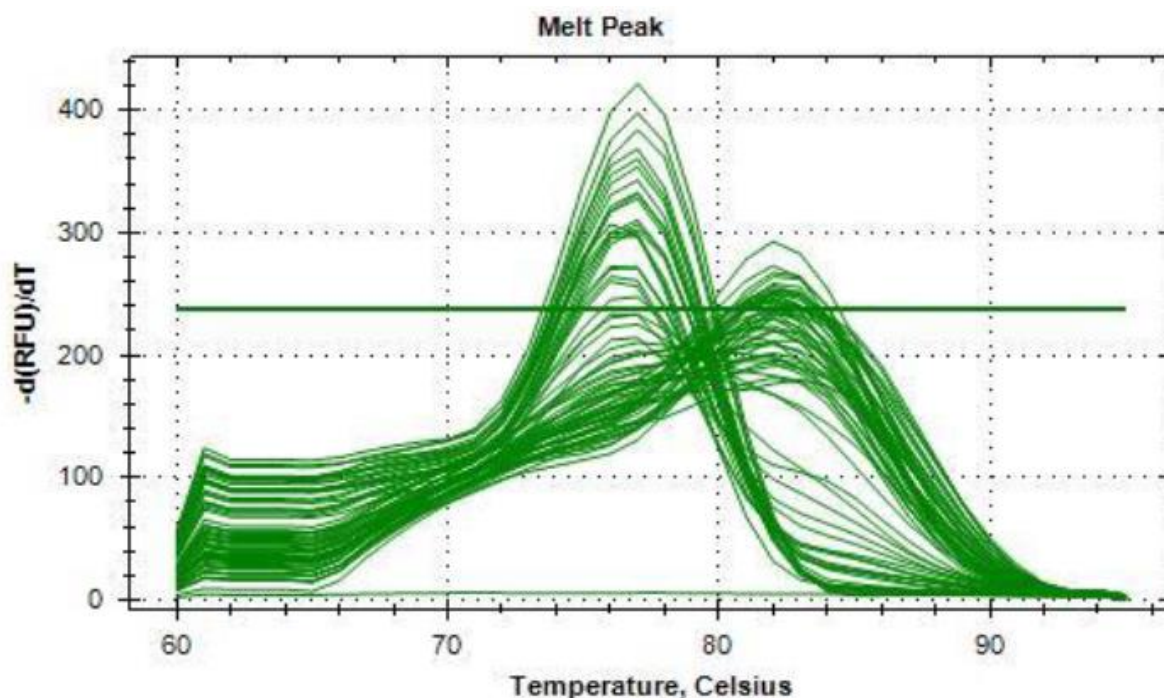


Figure 3.4: Melt curve analysis demonstrating the analytical specificity of the quantitative real-time PCR assay performed with FgramB470-fwd/ FgramB411-rev (Nicolaisen *et al.*, 2009) primers representing the FGSC members in maize stems.

3.2.4 Mycotoxin extraction

Mycotoxins were extracted by adding 20 ml extraction buffer (70% methanol and 30% HPLC water) to each of the 5 g ground samples. After the extraction buffer was added the samples were mixed on the shaker for 30 min at 200 rpm at 25°C after which the samples were centrifuged at 4°C for 10 min at 500 rcf. A syringe was then used to extract 2 ml supernatant from the centrifuged samples, the liquid was filtered through 0.25 µm filters into a 2 ml Eppendorf tube. The samples were placed in a fridge overnight. The samples were centrifuged again at 14 000 rpm and 900 µl supernatant was extracted into a 2 ml centrifuge tube after which 900 µl HPLC water was added. The samples were vortexed and sent to Stellenbosch University for LC-MS/MS analysis.

3.2.5 Statistical analysis

The qPCR assay was used to evaluate fungal colonisation of the inoculated maize grain and stems. A reference isolate of *F. graminearum* (13.08) was provided by the University of Stellenbosch and was used to produce DNA standards for the quantification of the inoculated FGSC isolates in maize grain and stems. The standard curve of the reference isolate was created by the dilution of the isolate's DNA in water 4x, 16x, 64x, 465x and 1024x to 20 000 pg/μl, 5000 pg/μl, 1250 pg/μl, 312.5 pg/μl and 78.125 pg/μl respectively. Each sample was replicated three times. The C_q value of the standard curve was used as DNA concentration for the samples. To ensure that the samples could be used, the efficiency was between the optimal range of 90-110%, the R² value <0.95 and the slope at -3.32.

The qPCR values of the Starting Quantity (SQ) were used in a general Analysis of Variance (ANOVA) to compare the mean of three reps of three FGSC isolates (*F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii*) inoculated on ears and stems respectively and was also used to compare the influence of four different provinces (Free State, KwaZulu-Natal, Northern Cape, and Mpumalanga). Means of significant differences were compared using Fisher's protected t-test and least significant differences (LSD) at a 5% significance level (Snedecor and Cochran, 1967). All the statistical procedures were performed using GenStats 15th Edition SP 1 (64 bit).

3.3 Results

3.3.1 Fungal colonisation – quantitative polymerase chain reaction (qPCR) concentrations

Although no significant differences (P<0.05) were found for *F. boothii* from the different provinces, statistical analysis showed significant differences between the combined provinces of ears and stems that were inoculated with *F. boothii* (Figure 3.5). qPCR concentration results showed a significant overall higher colonisation and better pathogenicity of *F. boothii* in the stems than in the ears (Figure 3.5). There was a significant difference for *F. graminearum* with ears and stems as well as between provinces (Figure 3.6). Isolates from KwaZulu-Natal and

Northern Cape showed a higher pathogenicity and abundance of *F. graminearum*, however, Free State showed significantly higher accumulation and pathogenicity of this inoculated isolate in the ears (Figure 3.6). The hybrid species, *F. graminearum* x *F. boothii* also showed significant differences between the inoculated ears and the stems with combined provinces, seeing that no significant difference was found comparing provinces, also, as with *F. boothii*, the stems had a higher pathogenicity than the ears (Figure 3.7).

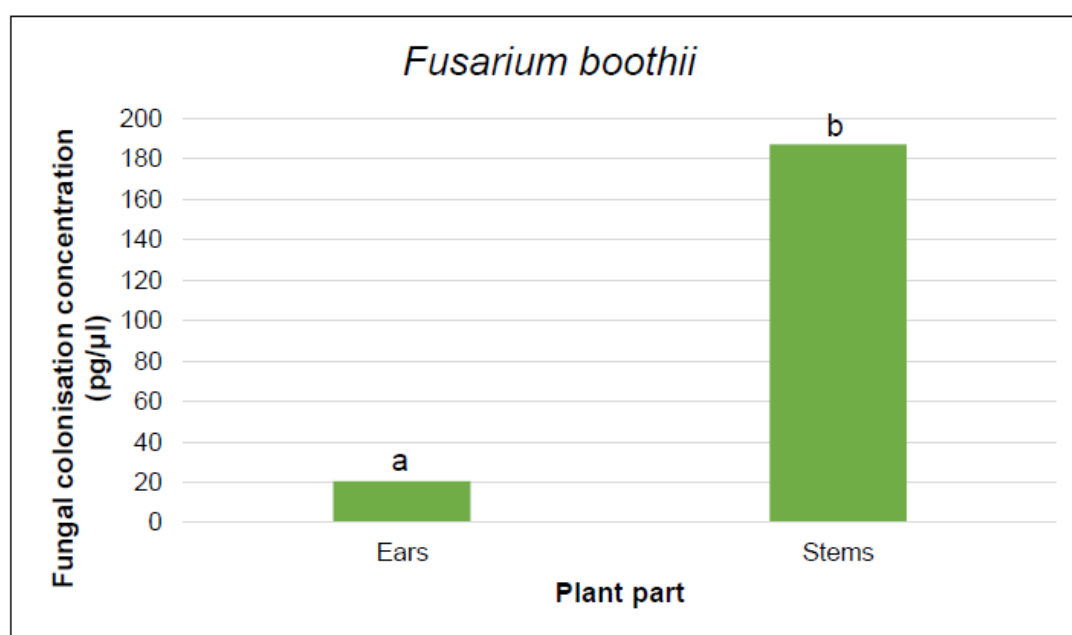


Figure 3.5: Significant differences ($P < 0.05$) between ears and stems of plants inoculated with *F. boothii* in combined localities.

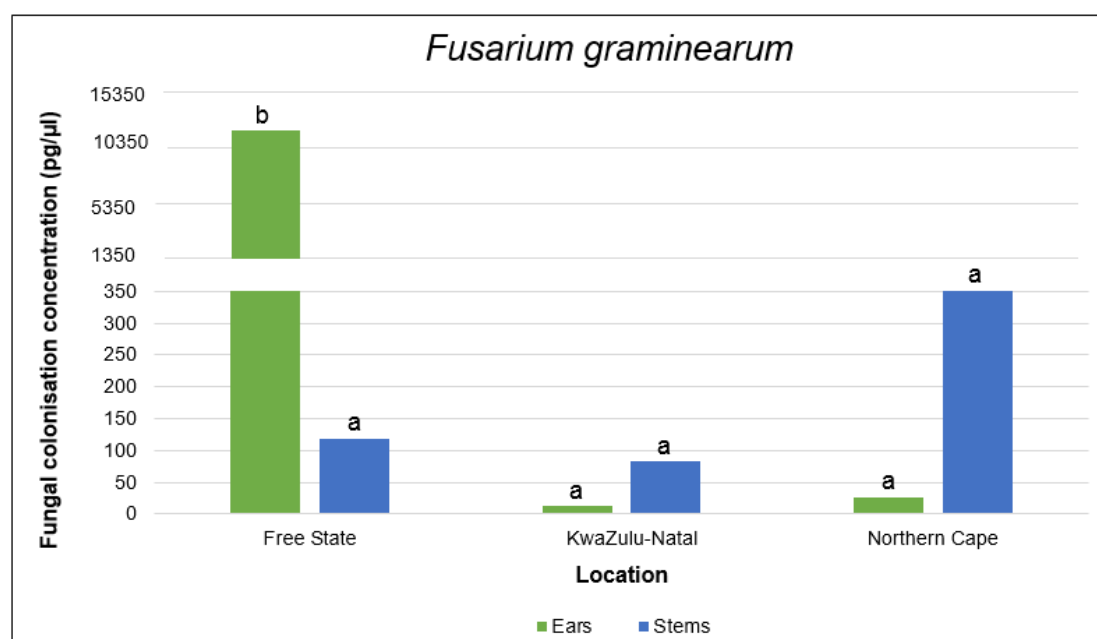


Figure 3.6: Significant differences ($P < 0.05$) between ears and stems of plants inoculated with *F. graminearum* isolated from different localities.

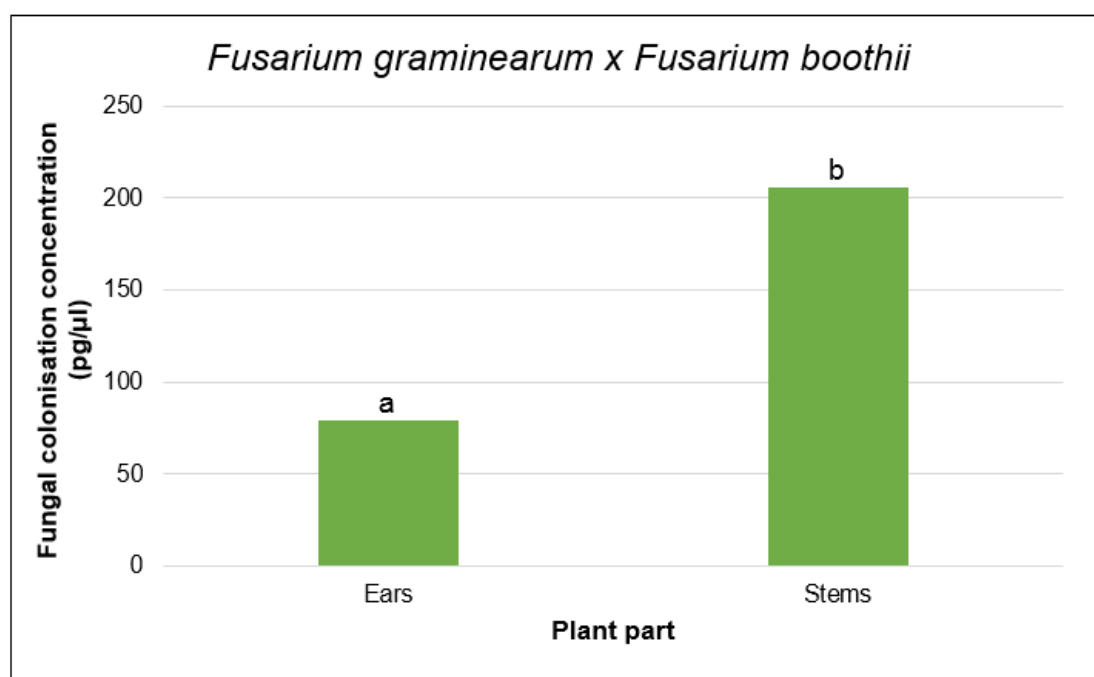


Figure 3.7: Significant differences ($P<0.05$) between ears and stems of plants inoculated with *F. graminearum* x *F. boothii* isolated from combined localities.

There was a significant difference between inoculated isolates and plant tissue from isolates from the Free State. The Free State province showed an outlier in the inoculated *F. graminearum* ears, with a much higher pathogen accumulation with a significant difference to the inoculated stems (Figure 3.8). Analysis also showed no significant difference between *F. boothii* ears and stems from the Free State and didn't differ significantly to *F. graminearum* stems (Figure 3.8). There was a significant difference between ears and stems, and again showing more pathogenicity and more pathogen accumulation in stems from KwaZulu-Natal isolates (Figure 3.9). Figure 3.10 shows significant differences between KwaZulu-Natal isolates of *F. boothii*, *F. graminearum* and *F. graminearum* x *F. boothii*, with last mentioned differing significantly from the other two isolates, having a higher combined ears and stems pathogen accumulation in this province. The FGSC isolates from Mpumalanga also showed a higher pathogen accumulation and pathogenicity of stems than in the ears of combined isolates hence the no significant difference between isolates (Figure 3.11). Significant differences of plant tissue from the Northern Cape is indicated in figure 3.12. This figure also shows that the FGSC isolates on stems were more pathogenic for the combined isolates than in the ears, again there were no indication of significant differences between separate isolates from this province.

An overall analysis with significant differences is shown in figure 3.13, with *F. graminearum* from the Free State inoculated on maize ears being an obvious outlier.

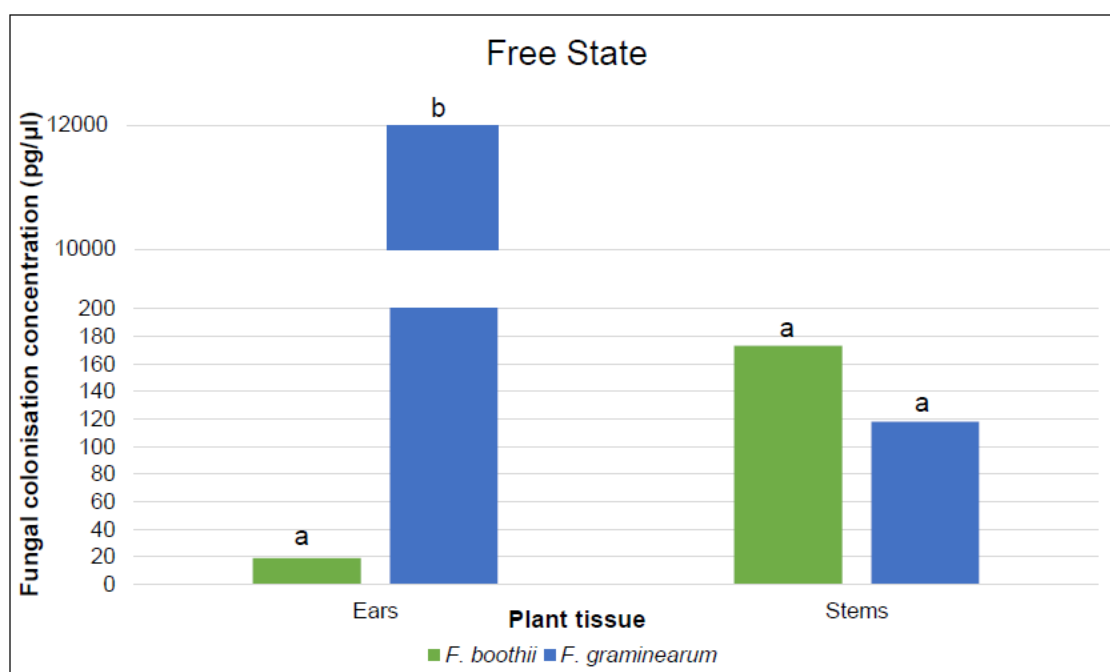


Figure 3.8: Significant differences ($P < 0.05$) between ears and stems of plants inoculated with *F. boothii* and *F. graminearum* isolated from Free State.

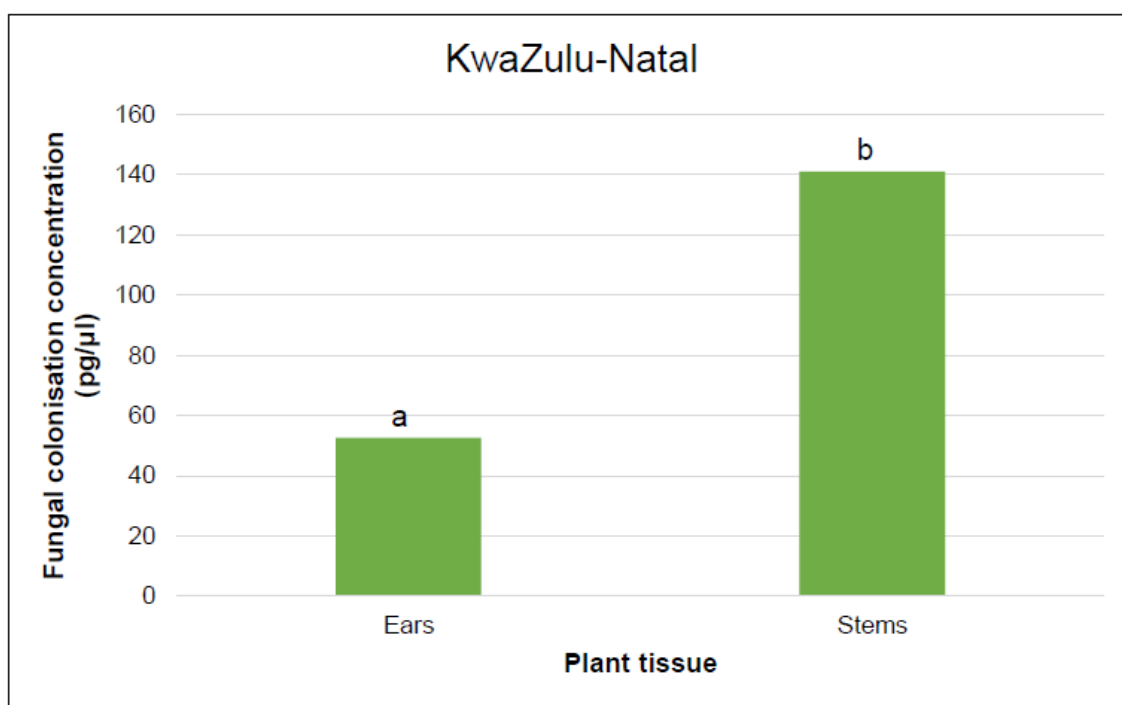


Figure 3.9: Significant differences ($P < 0.05$) between ears and stems of plants with combined isolates (*F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii*) from KwaZulu-Natal.

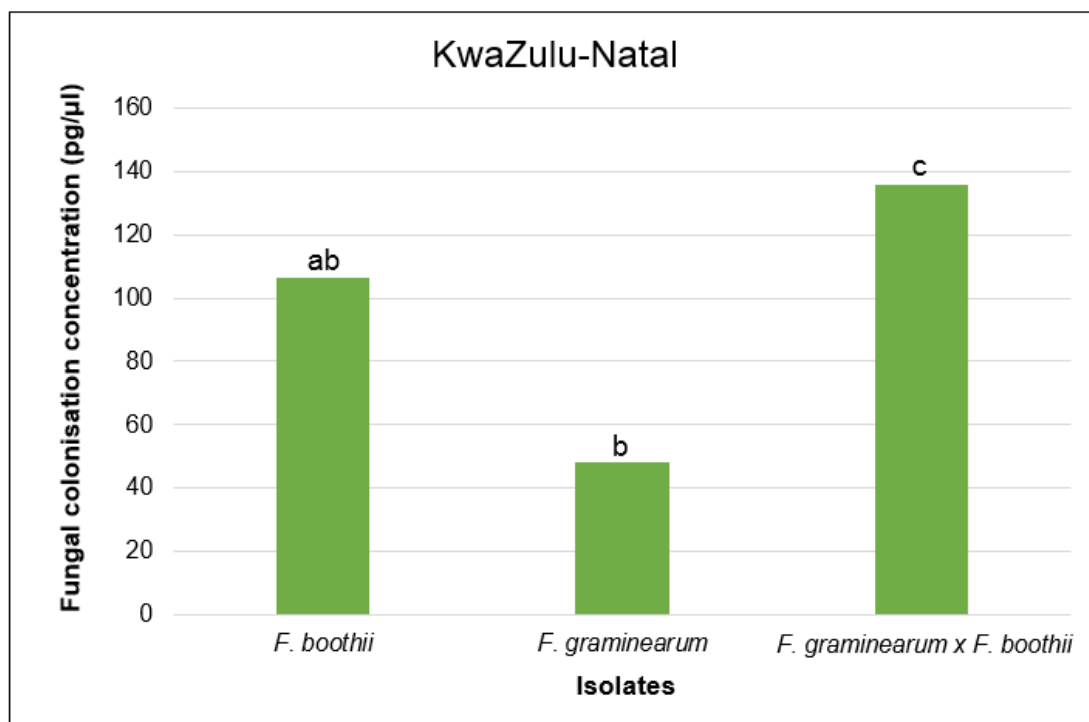


Figure 3.10: Significant differences ($P < 0.05$) between combined plant tissue of plants with isolates from KwaZulu-Natal.

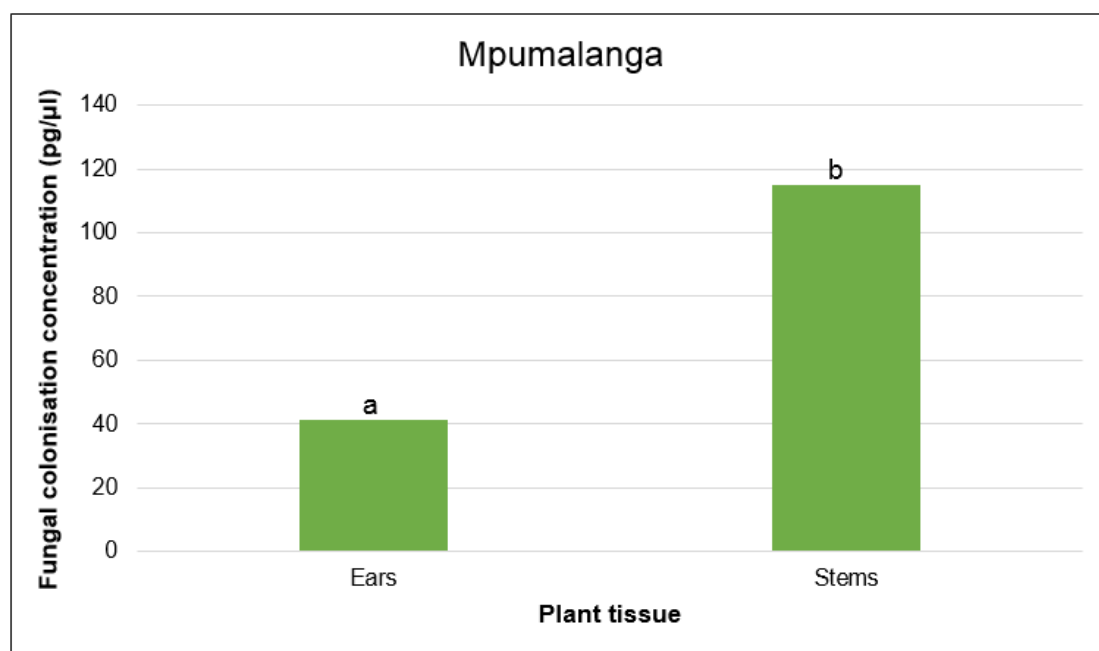


Figure 3.11: Significant differences ($P < 0.05$) between ears and stems of plants inoculated with combined isolates (*F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii*) from Mpumalanga.

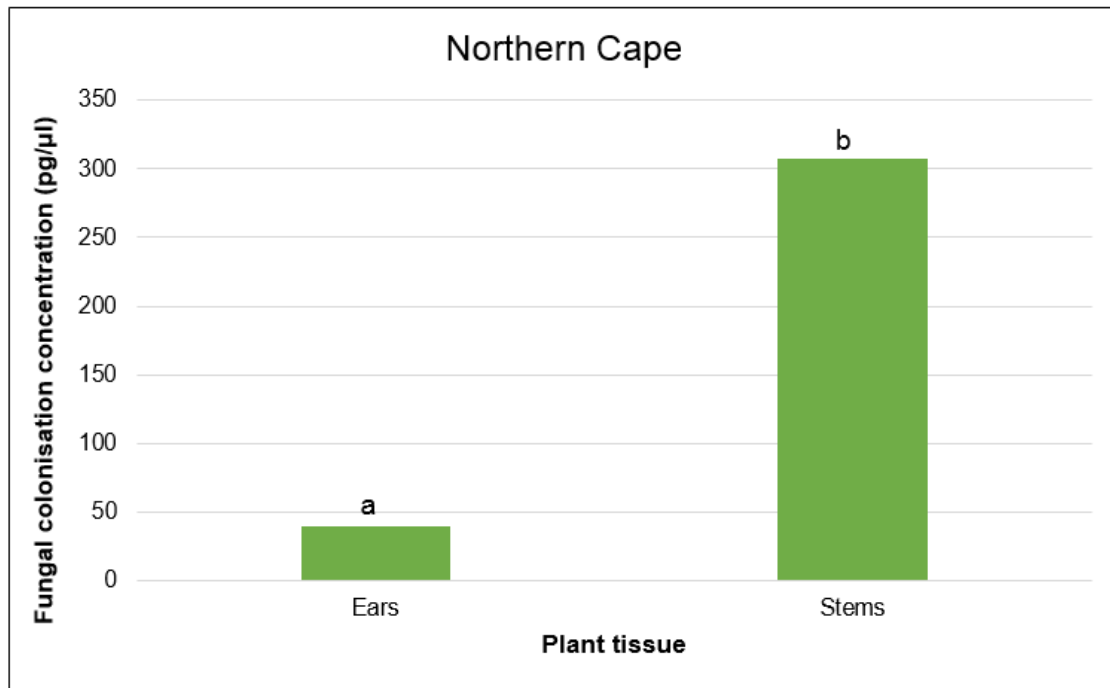


Figure 3.12: Significant differences ($P<0.05$) between combined ears and stems of plants inoculated with combined isolates (*F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii*) from Northern Cape.

The control maize ears and stems inoculated with water also showed significant differences to the maize that were inoculated with isolates and none to very little pathogens could be detected in these samples. These results, however were not included.

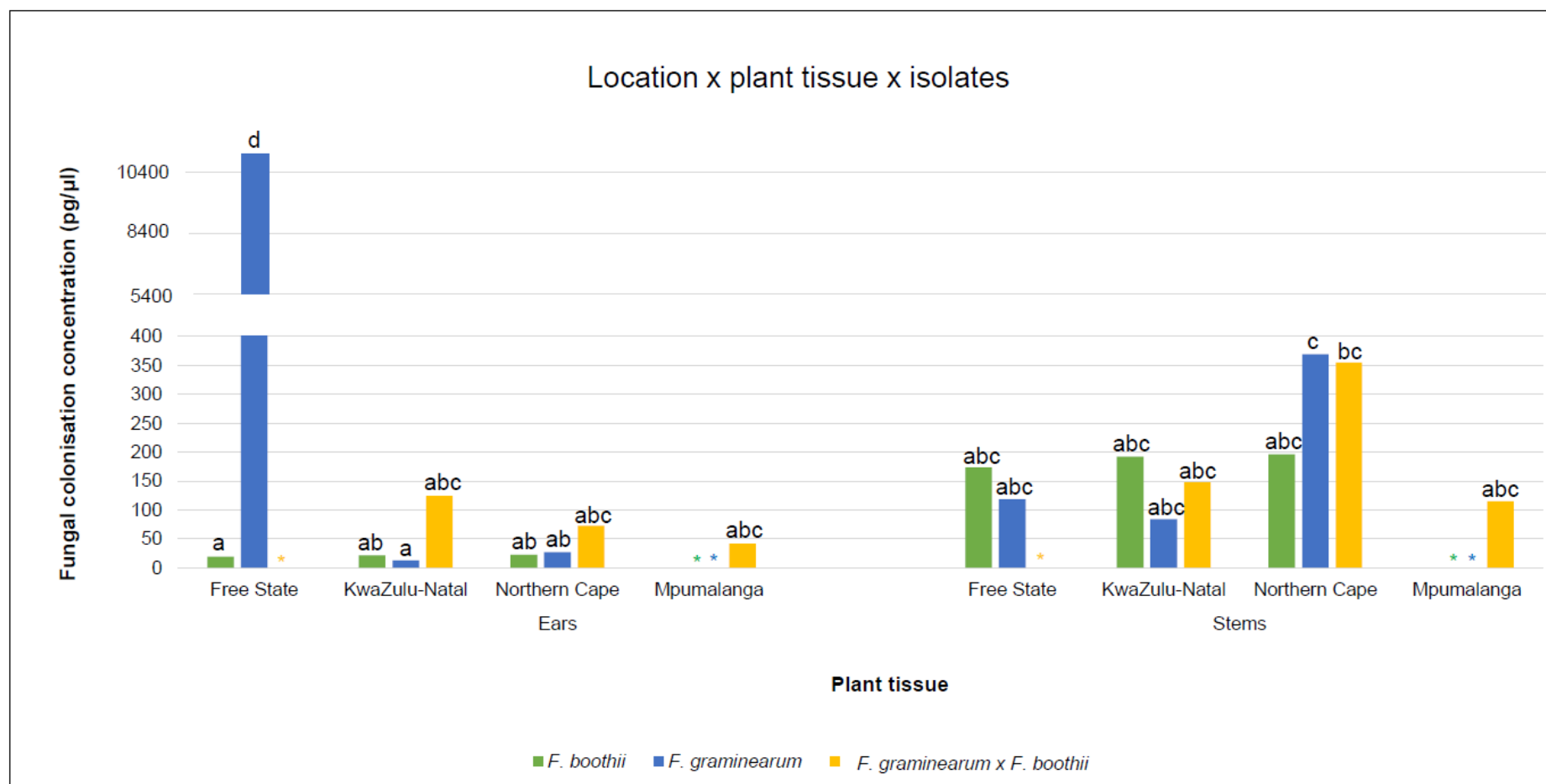


Figure 3.13: Significant differences ($P < 0.05$) between localities, plant tissue and isolates.

*No samples were available

3.3.2 Mycotoxin analysis - Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

No nivalenol results could be retrieved from the inoculated ears, however nivalenol was found in stems that were inoculated with *F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii* from provinces such as Free State, KwaZulu-Natal, Northern Cape and Mpumalanga respectively. The highest nivalenol concentration was found in *F. graminearum* x *F. boothii* from KwaZulu-Natal with a concentration of 2.2 µg/g (Table 3.1). *F. graminearum* produced 0.59 µg/g nivalenol from the Free State and from Northern Cape but failed to produce any traceable concentrations isolated from KwaZulu-Natal (Table 3.1). Maize stems inoculated with *F. boothii* produced nivalenol in the Free State (0.93 µg/g) and KwaZulu-Natal (0.45 µg/g) but yet failed to produce any concentration from the Northern Cape. The hybrid isolate *F. graminearum* x *F. boothii* produced mean concentrations of nivalenol in all inoculated provinces of 2.22 µg/g, 0.33 µg/g and 0.44 µg/g in maize stems from KwaZulu-Natal, Northern Cape and Mpumalanga respectively (Table 3.1).

No zearalenone could be found in maize stems, but was found that *F. graminearum* and *F. boothii* had produced zearalenone in the ears from isolates from Free State, KwaZulu-Natal and Northern Cape (Table 3.2). *F. graminearum* produced the highest concentration in the ears from the isolates from the Free State with a final concentration of 9.35 µg/g (Table 3.2). In this study, certain isolates from certain provinces were more aggressive with regards to mycotoxin production than in other provinces, for example *F. graminearum* produced a mean concentration of 9.35 µg/g zearalenone in maize ears from the Free State province but the concentration of the same isolate remained zero for the other provinces (Table 3.2). *F. boothii* also produced 3.11 µg/g isolated from KwaZulu-Natal and 0.71 µg/g in maize ears from Northern Cape but yet the concentration remained zero with the same species collected from the Free State (Table 3.2). None of the isolates were able to produce deoxynivalenol in the maize ears or stems.

Table 3.1: The mean mycotoxin levels ($\mu\text{g/g}$) of nivalenol measured in maize ears and stems inoculated with three isolates representing members of the FGSC of four different provinces.

NIVALENOL				
		<i>F. graminearum</i>	<i>F. boothii</i>	<i>F. graminearum</i> x <i>F. boothii</i>
EARS	Free State	0	0	*
	KwaZulu-Natal	0	0	0
	Northern Cape	0	0	0
	Mpumalanga	*	*	0
	H ₂ O (Control)	0	0	0
STEMS	Free State	0.59	0.93	*
	KwaZulu-Natal	0	0.45	2.22
	Northern Cape	0.29	0	0.33
	Mpumalanga	*	*	0.44
	H ₂ O (Control)	0	0	0

* No samples

Table 3.2: The mean mycotoxin levels ($\mu\text{g/g}$) of zearalenone measured in maize ears and stems inoculated with three isolates representing members of the FGSC of four different provinces.

ZEARALENONE				
		<i>F. graminearum</i>	<i>F. boothii</i>	<i>F. graminearum</i> x <i>F. boothii</i>
EARS	Free State	9.35	0	*
	KwaZulu-Natal	0	3.11	0
	Northern Cape	0	0.71	0
	Mpumalanga	*	*	0
	H ₂ O (Control)	0	0	0
STEMS	Free State	0	0	*
	KwaZulu-Natal	0	0	0
	Northern Cape	0	0	0
	Mpumalanga	*	*	0
	H ₂ O (Control)	0	0	0

* No samples

3.4 Discussion

In this study FGSC members who were previously only isolated from either stem or ears were evaluated for pathogenicity on other maize plant parts. Also, insight into the influence of geography was evaluated and found that FGSC members from certain provinces in South Africa were better colonisers of stems than ears and vice versa. Very few studies worldwide have investigated the diversity of FGSC members recovered from maize and lack the information of whether members are plant part specific as well as evidence about the pathogenicity of species within the FGSC and the occurrence of mycotoxin accumulation in the different plant parts with a focus especially on maize stems and grains.

A study conducted by Boutigny *et al.* (2011), indicated the abundance of *F. boothii* in maize ears, while this was also the only FGSC pathogen retrieved from maize ears in South Africa. However, *F. graminearum*, *F. boothii* and *F. meridionale* was found on maize roots. Furthermore, all the isolates from maize roots were found in KwaZulu-Natal and differed significantly from the FGSC composition found on maize ears (Boutigny *et al.*, 2011). The results in a study of Boutigny *et al.* (2011) indicated that *F. boothii* was the dominant species responsible for Gibberella ear rot, while *F. graminearum* were the responsible pathogen causing Fusarium head blight on wheat. In addition Boutigny *et al.* (2011) stated that *F. graminearum* has a reduced ability and may not be the predominant FGSC pathogen to cause Gibberella ear rot on maize worldwide, with a concluding hypothesis stating that *F. graminearum* may be at a selective disadvantage on maize ears relative to other members of the FGSC.

Dorn *et al.* (2009) found in a study conducted in Switzerland that *F. graminearum* was one of the dominant species found on naturally infected maize kernels as well as in maize stems. This study also found that the overall infection of *Fusarium* species was higher in maize stems than in the kernels, but *F. graminearum* specifically was higher in kernels (31.3%) than in stems (9.5%).

Overall, from the results retrieved in this study, the inoculated stems had a much higher incidence of pathogen accumulation for *F. graminearum* x *F. boothii* and *F. boothii* in all four the provinces. *F. graminearum* from the Free State had a significantly higher pathogenic effect

on maize ears than on stems. The statement and results from Boutigny *et al.* (2011) is in contrast with the results found in this study, seeing that *F. graminearum* from the Free State was the most abundant and pathogenic isolate found on maize ears and also significantly higher than any other isolate found on ears and stems. The hypothesis of Boutigny *et al.* (2011) that states that *F. graminearum* sensu stricto is not a predominant Gibberella ear rot species contradicts with results retrieved in this study. These results, however, corresponds to the findings of naturally occurring *F. graminearum* in ears and stems of maize of Dorn *et al.* (2009).

According to Malihipour *et al.* (2012), difference in pathogen virulence among geographical zones may be attributed to the presence of different FGSC species in different locations. The FGSC isolates from the Free State showed a significant higher *F. graminearum* accumulation in the ears and *F. boothii* showed a higher abundance in the stems. FGSC members from KwaZulu-Natal showed a higher accumulation of pathogens in the stems of all three isolates combined. *F. graminearum* x *F. boothii* were more pathogenic between the three isolates of this province. Interestingly, the FGSC members from Mpumalanga and Northern Cape had no significant difference between different isolates and both locations resulted in a higher pathogen accumulation and pathogenicity in the stems.

According to Palmer and Kommedahl (1969) and Kommedahl *et al.* (1987) *F. graminearum* has been reported to be pathogenic on maize in inoculation tests. A study conducted by Ares *et al.* (2004) also showed that *F. graminearum* was the most pathogenic fungus on root rots and also produced the highest seedling growth reduction.

As previously mentioned, three of the FGSC species have been identified on specific maize tissue namely; *F. graminearum* sensu stricto (maize roots) (Boutigny *et al.* 2011), *Fusarium boothii* (maize ears and roots) (Boutigny *et al.*, 2011) and *Fusarium meridionale* (maize roots) (Boutigny *et al.*, 2011; Mavhunga, 2013).

Results from this study showed that *F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii* are not only associated with stems, but *F. boothii* and *F. graminearum* x *F. boothii* appeared to be more pathogenic in stems than in ears. However, *F. graminearum* was more pathogenic in the ears and overall the most virulent in the ears as well as in the stems. These species that usually occur on maize ears and roots are able to grow and successfully be pathogenic in maize stems. Overall in all four the provinces *F. boothii* and *F. graminearum* x

F. boothii proved that they can infect stems more easily than ears seeing that the biotic factors such as temperature and inoculated isolate for both ears and stems were the same. *F. graminearum* proved to be much more pathogenic in the ears, especially isolates from the Free State province, than the stems.

Nivalenol, deoxynivalenol and zearalenone have been reported to be found in different geographical locations (Lee *et al.*, 2002). Although deoxynivalenol is the most widespread of the trichothecenes worldwide (Sarlin *et al.*, 2006), none of the inoculated isolates produced deoxynivalenol in ears and stems. Nivalenol has been reported to be found in Africa, Asia and Europe (Ji *et al.*, 2007), however nivalenol occurs less frequently in South African grains (Boutigny *et al.*, 2011, 2012). As previously mentioned, *F. graminearum* produces mycotoxins in maize grain, however, only a few studies have reported mycotoxin accumulation in maize stalks (Dorn *et al.*, 2011; Eckard *et al.*, 2011). This study showed the production of nivalenol in maize stems for all three inoculated isolates in all four provinces from which isolates were collected. Inoculated ears failed to produce any nivalenol for any of the inoculated isolates. The opposite is true for zearalenone where stems failed to produce any of this toxin, but inoculated ears produced zearalenone for *F. graminearum* from the Free State and *F. boothii* from KwaZulu-Natal and Northern Cape.

According to the Act of foodstuffs, cosmetics and disinfectants (54 of 1972), amended in 2016 for regulations governing tolerances for fungus-produced toxins in foodstuffs, only 2000 µg/kg (2 µg/g) of deoxynivalenol is allowed in unprocessed grains. Unfortunately, nivalenol and zearalenone have not yet been legally restricted in South Africa. This imposes big health risks as nivalenol is often overshadowed by the well-studied deoxynivalenol while nivalenol is believed to be almost ten times more toxic than deoxynivalenol (Yoshida and Nakajima, 2010). Zearalenone has a potent estrogenic activity and therefore the European Union has implemented a set maximum of tolerated levels for zearalenone in food commodities between 20 and 100 µg/kg (2 and 10 µg/g) (Santini *et al.*, 2008).

It was found in this study that not only *F. graminearum* and *F. boothii* can produce mycotoxins but also the first report of *F. graminearum* x *F. boothii* hybrid to produce nivalenol in vivo in South Africa. Boutigny *et al.* (2014) have found that *F. graminearum* x *F. boothii* produced 15-acetyldeoxynivalenol chemotype on maize in France. It seems that the statement of Lee *et al.* (2002) could be true with regards to different mycotoxins found in different geographical

locations as results from study showed that different isolates produced different concentrations at different locations over South Africa.

A possible correlation can be drawn between the concentrations of pathogen and mycotoxin occurrence as *F. graminearum* in the Free State showed a definite outlier with a mean pathogen concentration of 11930 pg/μl and also showed the highest mycotoxin concentration of 9.35 μg/g which is just below the tolerated levels of zearalenone in food commodities of the European Union.

Aoki *et al.* (2012) have found that *F. graminearum* s.s produces 3- acetyldeoxynivalenol, 15-acetyldeoxynivalenol and nivalenol whereas *F. boothii* only produce 15-acetyldeoxynivalenol. Results from chemotyped isolates used in this chapter showed in chapter 2 that *F. boothii*, *F. graminearum* as well as the hybrid species *F. graminearum* x *F. boothii* produced 15-acetyldeoxynivalenol. The results retrieved in this chapter, however, is in contrast with the findings of Aoki *et al.* (2012) that showed that *F. boothii* produced 15-acetyldeoxynivalenol, but instead produced nivalenol and zearalenone. In an analyses done in this chapter, *F. graminearum* showed the same results as found by Aoki *et al.* (2012) when nivalenol was produced, but contrasted with the results found in chapter 2 where *F. graminearum* produced 15-acetyldeoxynivalenol. Interestingly *F. graminearum* x *F. boothii* also resulted to produce 15-acetyldeoxynivalenol in chapter 2, but results contrasted in this chapter when nivalenol was produced. The findings should be further investigated in order to better understand the discrepancies between chemotype and measured mycotoxins in FGSC members found in this study.

3.5 Conclusion

F. graminearum and *F. boothii* not only confirmed to be pathogenic on maize but *F. boothii* also confirmed its pathogenicity on ears and also resulted in its association with stems with a higher pathogen count than what was found on ears. *F. graminearum* x *F. boothii* was also associated with maize stems and grain for the first time and proved that it can infect and cause disease on maize ears and stems. Fungi are not just able to be pathogenic in ears, roots and crowns, but also in stems, and are in some cases able to be more virulent in stems than in the ears. The fact that these fungi are able to successfully be pathogenic in maize stems raises concerns knowing that there are no resistant cultivars available against stem rot.

Information on the distribution of different *Fusarium* species and their given geographical area is valuable data for predicting mycotoxin contamination risks. Not only was *F. graminearum* and *F. boothii* found to produce mycotoxins but also a first report of *F. graminearum* x *F. boothii* hybrid species that proved to be a mycotoxin-producer in all inoculated provinces especially on stems. In the results from the previous chapter *F. graminearum*, *F. boothii* and *F. graminearum* x *F. boothii* resulted in the production of the chemotype 15-acetyldeoxynivalenol. In the results retrieved in this chapter *F. graminearum* produced the normal, previously reported nivalenol (Aoki *et al.*, 2012) on stems but also produced zearalenone on ears. *F. boothii* were able to produce nivalenol on stems as well as zearalenone on ears. The mycotoxin potential of the hybrid species, *F. graminearum* x *F. boothii* was proven when nivalenol in stems were produced, but no mycotoxin level could be found in the ears for this isolate. Not only does the mycotoxin concentration levels raise concerns for humans, but infected grain and stems are also a big health risk and concern for animals.

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CHAPTER 4 - THE SUCCESSION OF THE *FUSARIUM* *GRAMINEARUM* SPECIES COMPLEX MEMBERS IN MAIZE STEMS ASSOCIATED WITH STEM BORER OCCURRENCE

Abstract

Maize (*Zea mays* L.) is the most important crop cultivated for human consumption and as feed for livestock in South Africa. However, the production of maize in the country is at risk with various biotic and abiotic stresses affecting optimal maize growth of which maize stem borers and fungi are a part of the biotic constraints. Maize plants can become infected by fungi through contaminated seed, lateral root infection by soil borne spores or through insects that causes wounds which also provides an important entry point for the fungus. Previous reports suggested that *Fusarium graminearum* species complex (FGSC) members were a minor threat to maize production in South Africa, but it was shown in a recent study that infections by the FGSC increased in the South African maize production areas. The continuation of monitoring FGSC in cereal grains within South Africa is thus necessary. The interaction of FGSC with maize has not been intensively studied with regards to succession of the fungus and its possible correlation with stalk rot as well as stem borers throughout a maize growing season. The aim of this study was to improve the understanding of how the FGSC changes during a maize growing season and also investigate the possible interaction between FGSC members and stem borers throughout the season. Field trials were planted in Potchefstroom and in Vaalharts under irrigation and dryland conditions respectively to evaluate naturally occurring FGSC and stem borers during the 2016/17 season. Maize plants were collected during the season at different growth stages and the presence of FGSC was evaluated with quantitative Polymerase chain reaction (qPCR). The maize fields were also scouted for the presence of stem borers. A repeated trend of FGSC members in maize stems for all three cultivars was noted in Vaalharts and Potchefstroom. The most vulnerable stage of maize growth is at 70 days- when the plant is busy with grain fill and showed the highest accumulation of FGSC in the season for both localities. Stem borers (*Busseola fusca* (Fuller) (Lepidoptera: Noctuidae)) were found at increasing levels throughout the season, however could not be directly correlated to FGSC infection but regression graphs showed a possible correlation between specific cultivar, infection by FGSC and stem borer occurrence. The fungal accumulation pattern found in this study is also in coherence with the plants' needs for nutrients in the soil, which suggests that

the plants is infected through the fungal infested soil and not by direct stem borer damage. This is the first study to investigate FGSC succession using qPCR technology and to elucidate a possible relationship with the stem borer damage.

Keywords: *Fusarium graminearum* species complex succession; maize stems; stem borers

4.1 Introduction

Maize (*Zea mays* L.) is the most important cultivated crop for human consumption and as feed for livestock in South Africa. An annual average of 10.8 million tons of maize was produced by this country in the years 2010-2016 (CEC, 2016). The maize production, however, is affected by abiotic and biotic stresses, of which maize stem borers and fungal pathogens are a part of these biotic constraints (Nelson *et al.*, 1983; Leslie and Summerell, 2006). Infected maize plants can occur from contaminated seed (Munkvold and Carlton, 1997), lateral root infection by soil borne spores (Oren *et al.*, 2003) or through insects that causes wounds which also provides easy access for the fungus (Flett and Van Rensburg, 1992; Munkvold and Carlton, 1997; Robertson *et al.*, 2011). Members of the *Fusarium graminearum* species complex (FGSC) infects a wide range of plant hosts (Karugia *et al.*, 2009) including maize, which is of economic importance in South Africa. This complex is found as pathogens or secondary invaders (Gilbert and Tekauz, 1999), which causes Gibberella stalk- and ear rot (GER) of maize (Vigier *et al.*, 1997; Zeller *et al.*, 2003; Goswami and Kistler, 2004; Kazan *et al.*, 2012). FGSC linked diseases can be responsible for 30-70% of crop yield loss (Waalwijk *et al.*, 2003). Previous reports suggested that FGSC members had no threats to maize and sorghum production in South Africa (Viljoen, 2003), but Boutigny *et al.* (2011) has shown in a more recent study that FGSC are increasing in the South African maize production areas. The continuation of monitoring FGSC in cereal grains within South Africa is thus necessary, since infected grains can be contaminated by mycotoxins. FGSC can be managed by reducing inoculum through crop rotation, burial of crop residue and controlling insects that causes wounds that could aid in pathogen entry and infection (Quesada-Ocampo *et al.*, 2016). The interaction of FGSC with maize with regards to accumulation of the fungus and its possible correlation with stalk rot has not been intensively studied (Trial, 2009).

In South Africa, the African maize stem borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) is considered the most destructive pest of maize (Van Rensburg and Flett, 2010). An estimated annual loss of 10-60% of total maize production under favourable conditions can be caused by *B. fusca* (Kfir *et al.*, 2002; Van Rensburg *et al.*, 1987). *Busseola fusca* can cause extensive tunnelling and damage to internal stem tissue, starting in the whorl of the plant (Van Rensburg *et al.*, 1989).

Damage caused by this maize stem borer can be reduced by crop residue management (Kfir *et al.*, 2002), application of insecticides (Meissle *et al.*, 2010; Beyene *et al.*, 2011), and biological control with parasitoids (Kfir *et al.*, 2002). The most effective control strategy is, however, through planting genetically modified maize, modified with *Bacillus thuringiensis* (*Bt*) genes that encode for δ -endotoxin crystal proteins that are toxic and can be fatal for Lepidopteran insects (Hellmich *et al.*, 2008; Ranum *et al.*, 2014). *Bt* maize comprising the *Cry1Ab* protein has been widely cultivated in South Africa to reduce stem borer damage, however, the first report of *B. fusca* resistance against maize hybrids cultivated with this protein, was in 2007 (Van Rensburg, 2007). As a result, maize companies released and introduced a new *Bt* hybrid containing stacked genes of *Cry1A.105* and *Cry2Ab2* proteins, which has the ability to control resistant *B. fusca* and contribute to an effective insect resistant management tool (CERA, 2015). Maize with genetically modified maize consisting of *Bt* insect resistance, herbicide tolerance and combined (stacked) traits have been shown by statistics to occupy 70% of maize that is planted in South Africa. These hybrids have the confidence of yield protection and the reduction of insecticide use (James, 2012).

Maize plants with wounds caused by *B. fusca* can enable infection with *F. verticillioides* (Rutherford *et al.*, 2002). An experiment conducted by Flett and Van Rensburg (1992) resulted in *B. fusca* showing increased infestation with an increase incidence of Fusarium ear rot (FER) in maize. Infestation with the African Sugarcane borer, *Eldana Saccharina* Walker (Lepidoptera: Pyralidae) on maize, significantly increased the incidence and severity of stem rots (Bosque-Pérez and Mareck, 1991). According to Dowd (1998) and Smeltzer (1958), stalk and ear rot diseases can be influenced by Lepidopteran pests. The planting of *Bt* maize hybrids is an important control mechanism to manage maize stem borers (Hellmich *et al.*, 2008). *Bt* maize hybrids have been found to be less susceptible to Fusarium ear rot than non-*Bt* maize after infestation of stem borer, *Ostrinia nubilalis* Hübner, in the United States of America (Munkvold *et al.*, 1999). Hybrids resistant to Gibberella stalk rot are not common, but planting hybrids that are resistant to stalk borers may reduce secondary diseases by minimizing the wounds caused by these insect pests (Jackson-Ziems *et al.*, 2014).

Some stages of plant growth may or may not be more susceptible to pathogens. Maize plant development has both vegetative stages as well as reproductive stages. Stages VE-V10 represents the leaf collar stages, in which the stages are defined according to presence of leaf collars, while VT-R6 represents the reproductive stages where stages are defined according to

kernel development (Wise *et al.*, 2016) (Figure 4.1). The lack of information of the succession of the FGSC that occur in maize stems throughout a maize growing season, as well as the possible resistance to different maize cultivars are also unknown. The interactive effect of *B. fusca* and FGSC in *Bt* and non-*Bt* hybrids in maize stems has also not been studied in South Africa. Therefore the aim of this study was to understand how the FGSC changes in stems over the season, and when this complex might become a problem during the season. Secondly, the possible correlation of FGSC infection with stem borers on *Bt*- and non-*Bt* maize hybrids in maize stems was also evaluated.

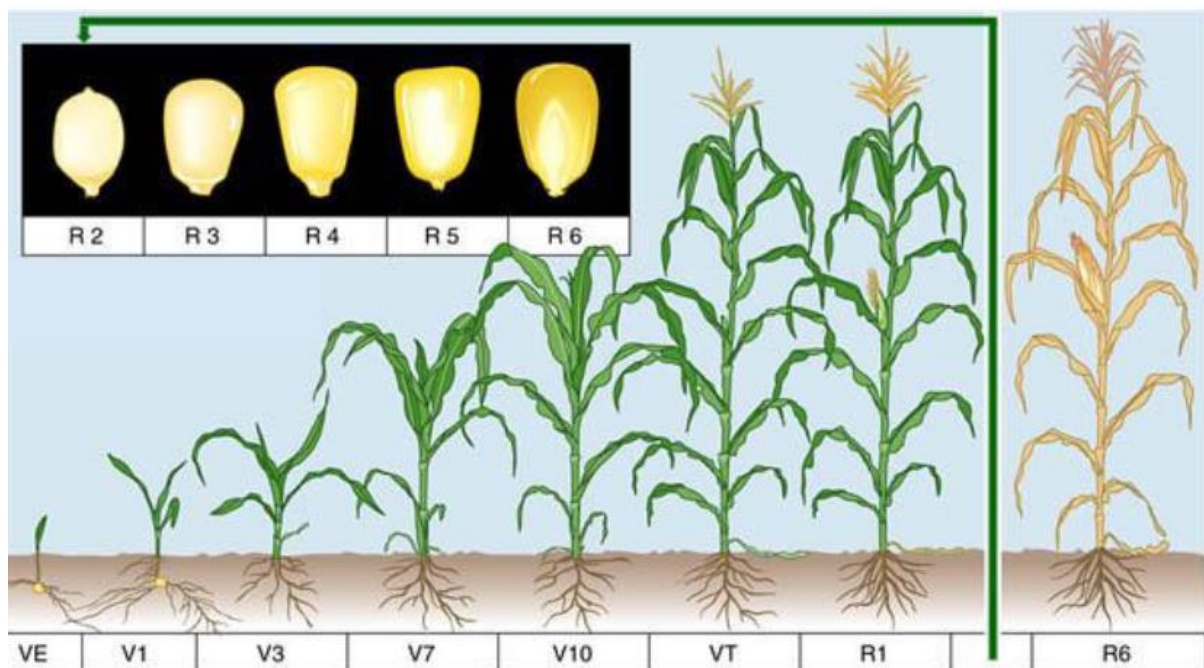


Figure 4.1: Vegetative (V) and reproductive (R) growth stages of maize. The vegetative stages begins with the emergence stage (VE) during which most of the growth remains below the soil surface, followed by the Vn stages which are based on the number of leaves (n) on the maize plant- this could go to V10 or up to V15 depending on maize hybrid, and lastly, the VT stage, at which the tassel is completely visible and the plant has reached full height. The reproductive stages are: silking stage (R1), blister stage (R2), milky stage (R3), dough stage (R4), dent stage (R5) and physiological maturity stage (R6) (<http://odells.typepad.com/blog/corn-growth-stages.html>).

4.2 Materials and methods

4.2.1 Field trials

Three yellow maize cultivars, BG 3292 (Non-Bt) (Pannar, South Africa), DKC 61-94BR (*Cry1A.105* and *Cry2Ab2* stacked *Bt*- genes) (Monsanto, South Africa) and IMP 50-10BR (*Cry1Ab Bt*-gene) (Agricol, South Africa) were planted in the 2016/17 maize planting season at two localities in Potchefstroom (irrigated) (North West) at the Agriculture Research Council-Grain Crops Institute (26° 74' 50" S, 27° 07' 45.1" E) and in the Vaalharts area (dryland conditions) (Northern Cape) at the Agriculture Research Council experimental farm (27° 56' 37.7" S, 24° 50' 28.7" E) that served as two trial replications in field conditions. The field trials were planted in a complete randomised block design (15 m x 15 m) with three different maize cultivars replicated three times. Intra-row spacing were 90 cm and inter-row spacing were 30 cm.

4.2.2 Sampling

Ten random plants with visual natural occurring stem borer damage, if present, were sampled out of each rep of each cultivar six times throughout the season on 21-, 55-, 70-, 80-, 90- and 100 days respectively after the maize were planted. The maize plants with visual damage of stem borers for each cultivar of each rep were also counted and recorded with every sampling stage that served as percentage stem borer damage. The 21 days after planting represented the seedling stage, at 55 days the plant is at 10 leaf stage, at 70 days it is approximately the last vegetative stage (VT), the entire tassel has emerged and the silks are now starting to emerge beyond the husk leaves. Eighty days after plant the kernels had a blister-like appearance, 90 days after plant represented the milk and soft dough-like stages and at 100 days was the dent stage going to physiological maturity. These specific days were selected to get an overview of the succession of FGSC as well as stem borers throughout a maize growing season.

The ten plants from each replicate of each cultivar were sampled, pooled, cut into small pieces and ground together to a powder using liquid nitrogen, before taking a sample from the powder for DNA isolation.

4.2.3 DNA extraction

DNA was extracted from field samples using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Möller *et al.*, 1992) as follows; 0.25 ml of fine ground stems were added to 2 ml centrifuge tubes (Eppendorf, Hamburg, Germany). One ml of DNA extraction buffer (DEB) (0.2 M Tris HCl, 0.75 M NaCl, 0.025 M EDTA (pH 8) 0.025 M SDS) was added to the fungi in the tube and crushed with a sterile pipet tip for about 30 sec. The samples were frozen in the -80°C freezer for 1 hour. After the samples have been frozen it is placed in boiling water for 5 minutes. 600 µl phenol: chloroform: iso-amylalcohol (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were centrifuged for 20 min at 14 000 rpm. The top aqueous layer was removed and added to a new tube, 200 µl of CTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris (pH 8), 20 mM EDTA, 0.2% β-mercaptoethanol (pH 8)) and 400 µl phenol: chloroform: isoamylalcohol (25:24:1) was added to the new tube and mixed by inversion. The samples were once again centrifuged for 15 min at 14 000 rpm, after which the aqueous layer was removed for a second time and added to a new tube. Sixty µl 3 M Sodium acetate and 800 µl 100% ice cold ethanol was added to the supernatant. After samples have been mixed by inversion it was centrifuged for 10 min at 14 000 rpm. The remaining supernatant was discarded and 500 µl of 70% ethanol is added to wash the DNA pellet. The samples were centrifuged for one last time for 5 min at 14 000 rpm, after which the supernatant were discarded and the tube were then left open to allow the pellet to air dry the pellet in a laminar flowhood for approximately 1 hour. Fifty µl of Low TE buffer (0.1 mM EDTA; 10 mM Tris) (Applichem, Germany) and 2 µl RNase were added to the tube with the pellet and vortexed until the pellet has dissolved. The samples were quantified and DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, South Africa) and the quality was determined with A260/A280 ration of between 1.7-2.0.

4.2.4 Quantification of FGSC members in field trial samples using quantitative polymerase chain reaction (qPCR)

After the DNA was extracted, the samples were diluted to 10 ng/µl. qPCR was performed with Bio-Rad CFX 96 (United States, Hercules, USA) using SYBR[®] Green protocol and the fungal mass was quantified as pg/µl for *F. graminearum*. The SYBR[®] Green protocol was done under the following conditions and reaction mix; 10 µl Bio-Rad iTaq Universal SYBR[®] Green Supermix (Hercules, USA), 11.75 µl H₂O, 2 µl DNA (10 ng) and 0.625 µl of the forward primer

(10 μ M) (Fgram379-fwd (5'-CCATTCCCTGGGCGCT-3') and reverse primer (10 μ M (FgramB411-rev (5'-CCTATTGACAGGTGGTTAGTGA CTGG-3')) (Nicolaisen *et al.*, 2009), designed to amplify the translation elongation factor (EF-1 α) gene of *F. graminearum* sensu lato. The PCR cycles consisted of an initial step at 94°C for 10 min followed by 40 cycles of 94°C for 30 sec; 60°C for 30 sec and 72°C for 30 sec before two final extension steps at 95°C for 30 sec and 40°C for 30 seconds.

4.2.5 Statistical analysis

A reference isolate of *F. graminearum* (13.08) was provided by the University of Stellenbosch and was used to produce DNA standards for the quantification of the inoculated FGSC isolates in maize grain and stems. The standard curve of the reference isolate was created by the dilution of the isolate's DNA in water 4x, 16x, 64x, 465x and 1024x to 20 000 pg/ μ l, 5000 pg/ μ l, 1250 pg/ μ l, 312.5 pg/ μ l and 78.125 pg/ μ l respectively. Each sample were replicated three times. The C_t value of the standard curve was used as DNA concentration for the samples. To ensure that the samples could be used, the efficiency was between the optimal range of 90-110%, the R^2 value <0.95 and the slope at -3.32 (Figure 4.2 and Figure 4.3).

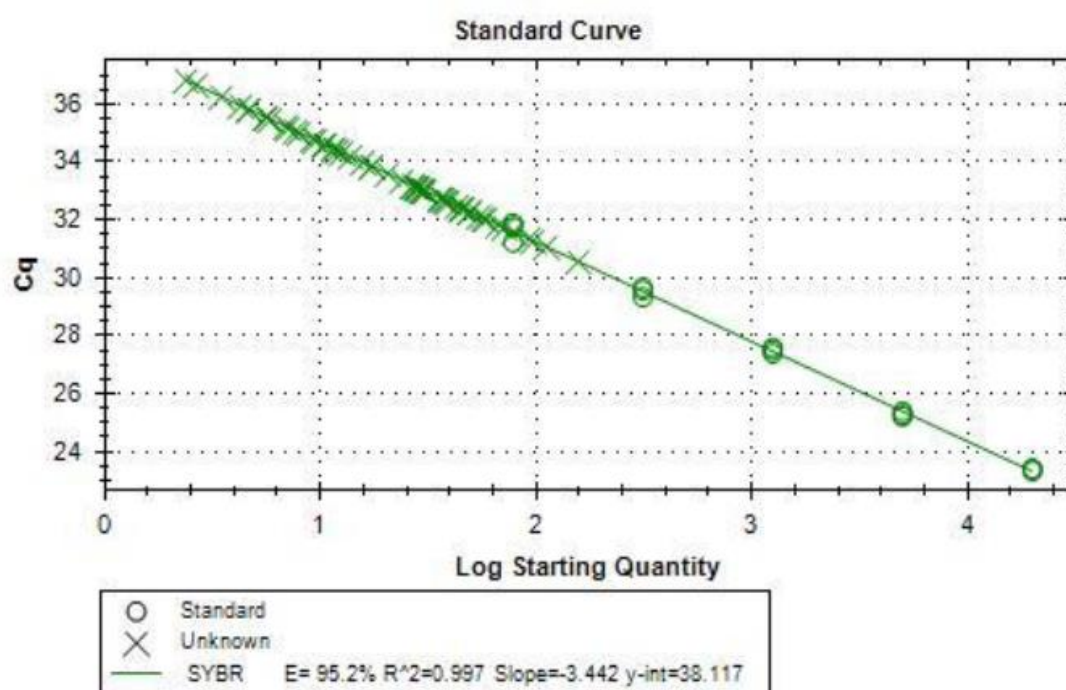


Figure 4.2: Standard curve analysis demonstrating the analytical specificity of the quantitative real-time PCR assay performed with FgramB470-fwd/ FgramB411-rev (Nicolaisen *et al.*, 2009) primers representing the FGSC members in maize stems.

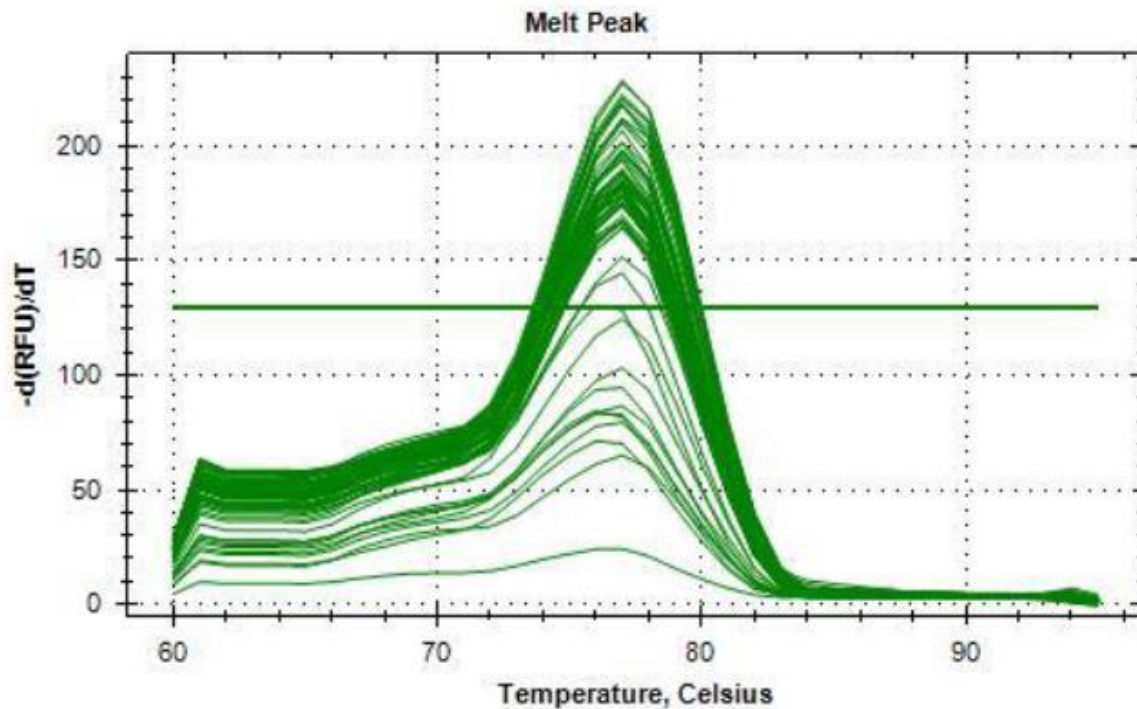


Figure 4.3: Melt curve analysis demonstrating the analytical specificity of the quantitative real-time PCR assay performed with FgramB470-fwd/ FgramB411-rev (Nicolaisen *et al.*, 2009) primers representing the FGSC members in maize stems.

The qPCR values of the Starting Quantity (SQ) of stems that were sampled and the stem borer damage percentage in two field trials were used in a general Analysis of Variance (ANOVA), using the mean of three reps to compare locality, cultivar and days after plant. Means of significant differences were compared using Fisher's protected t-test and least significant differences (LSD) at a 5% significance level ($P < 0.05$) (Snedecor and Cochran, 1967). Statistical procedures were performed using GenStats 15th Edition SP 1 (64 bit). Regression graphs were analysed using NCCS statistics with significant differences ($R^2 < 0.811$). Models used were Holliday ($Y = \frac{1}{A+B \times X + C \times X \times X}$) and Cubic ($Y = A + B \times X + C \times X^2 + D \times X^3$).

4.3 Results

4.3.1 Stem borer damage on maize plants

Significant differences between locations, cultivar as well as days after plant of percentage stem borer damage were shown ($P < 0.05$) (Figure 4.4). Vaalharts had significantly higher stem borer damage compared to Potchefstroom at 55, 70, 80, 90 and 100 days after plant on cultivars BG 3292 and IMP 50-10BR (Figure 4.4). In Potchefstroom the stem borer damage significantly increased from sampling dates for BG 3292 and thus with each sampling date significantly more stem borer damage was monitored on BG 3292 at 21, 55, 70, 80, 90 and 100 days after plant respectively. The same tendency was observed on IMP 50-10BR (Table 4.1; Figure 4.5). A similar trend was observed in Vaalharts with an increase in stem borer damage over sampling dates on BG 3292 and IMP 50-10BR at 21, 55, 70, 80, 90 and 100 days respectively (Table 4.2; Figure 4.6).

There was a trend for the stem borer damage that increased on the plants in Potchefstroom (Figure 4.5) and Vaalharts (Figure 4.6) for the two cultivars BG 3292 and IMP 50-10BR. Cultivar, DKC 61-94BR, had no stem borer damage and no stem borer survival was recorded on this cultivar in Potchefstroom as well as in Vaalharts (Figure 4.5 and Figure 4.6). In Potchefstroom, stem borer damage in BG 3292 differed significantly from IMP 50-10BR, although there is resistance against this last mentioned cultivar- there is still a degree of susceptibility in this location (Table 4.1). In Vaalharts, more stem borer resistance could be seen on the IMP 50-10BR cultivar which are not significantly different from BG 3292 over the season (Table 4.2). On cultivars BG 3292 and IMP 50-10BR there was an increase in stem borer damage (Figure 4.6 and Figure 4.7). The DKC 61-94BR cultivar differed significantly from the other two cultivars with no stem borer survival or damage recorded on this cultivar at both localities (Figure 4.5 and Figure 4.6).

Table 4.1: Significant differences ($P<0.05$) of stem borer damage between cultivars and days after plant in Potchefstroom ($F_{(10;34)}= 62.51$; $P<0.001$).

Days after plant		21	55	70	80	90	100
FGSC colonisation concentration (pg/ μ l)	BG 3292	0 a	24.03 cd	39.57 f	50.67 g	53.77 gh	55.33 h
	IMP 50-10BR	0 a	10.23 b	22 c	26.7 d	31.33 e	33.77 e
	DKC 61-94BR	0 a	0 a	0 a	0 a	0 a	0 a

Table 4.2: Significant differences ($P<0.05$) of stem borer damage between cultivars and days after plant in Vaalharts ($F_{(10;34)}= 61.39$; $P<0.001$).

Days after plant		21	55	70	80	90	100
FGSC colonisation concentration (pg/ μ l)	BG 3292	0 a	50.43 b	75.77 d	83.53 e	92.90 fg	96.47 g
	IMP 50-10BR	0 a	44.67 b	65.10 c	73.53 d	79.57 de	85.77 ef
	DKC 61-94BR	0 a	0 a	0 a	0 a	0 a	0 a

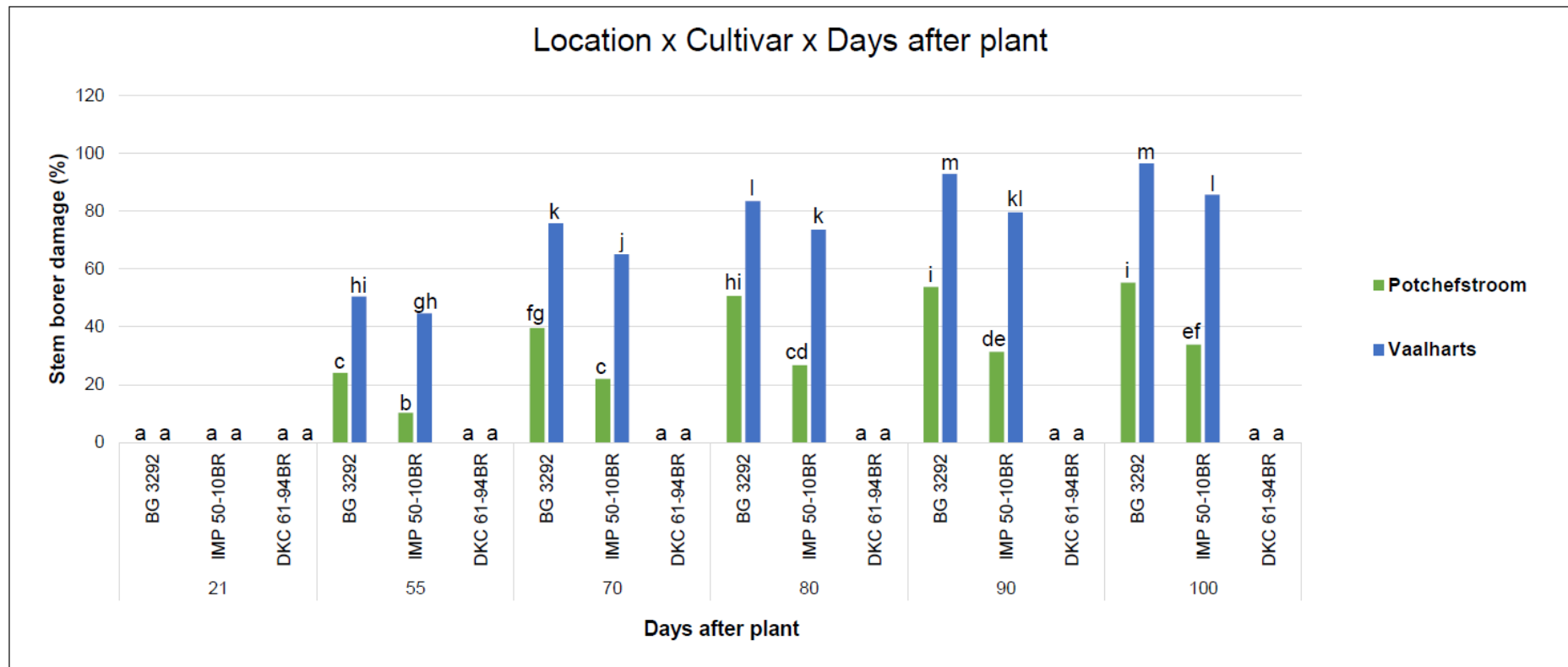


Figure 4.4: Significant differences ($P<0.05$) of stem borer damage between locations, cultivars and days after plant ($F_{(5;72)} = 11.81$; $P<0.001$).

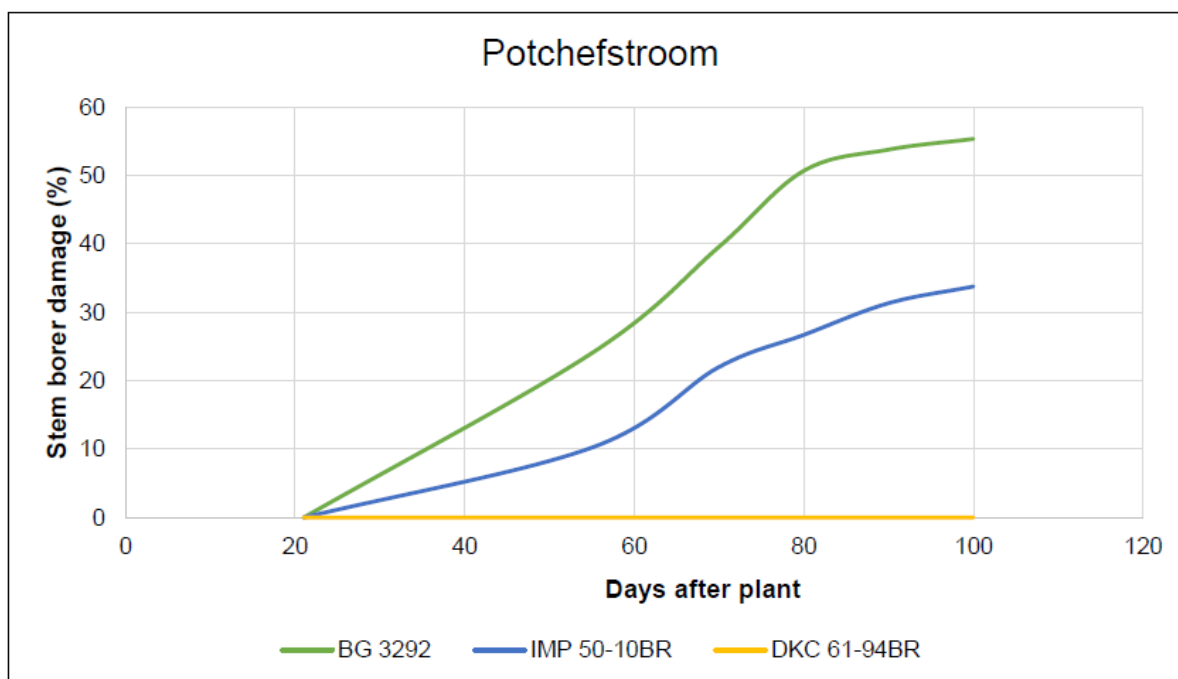


Figure 4.5: The tendency of stem borer damage for three cultivars over time in Potchefstroom ($F_{(10;34)} = 62.51$; $P < 0.001$).

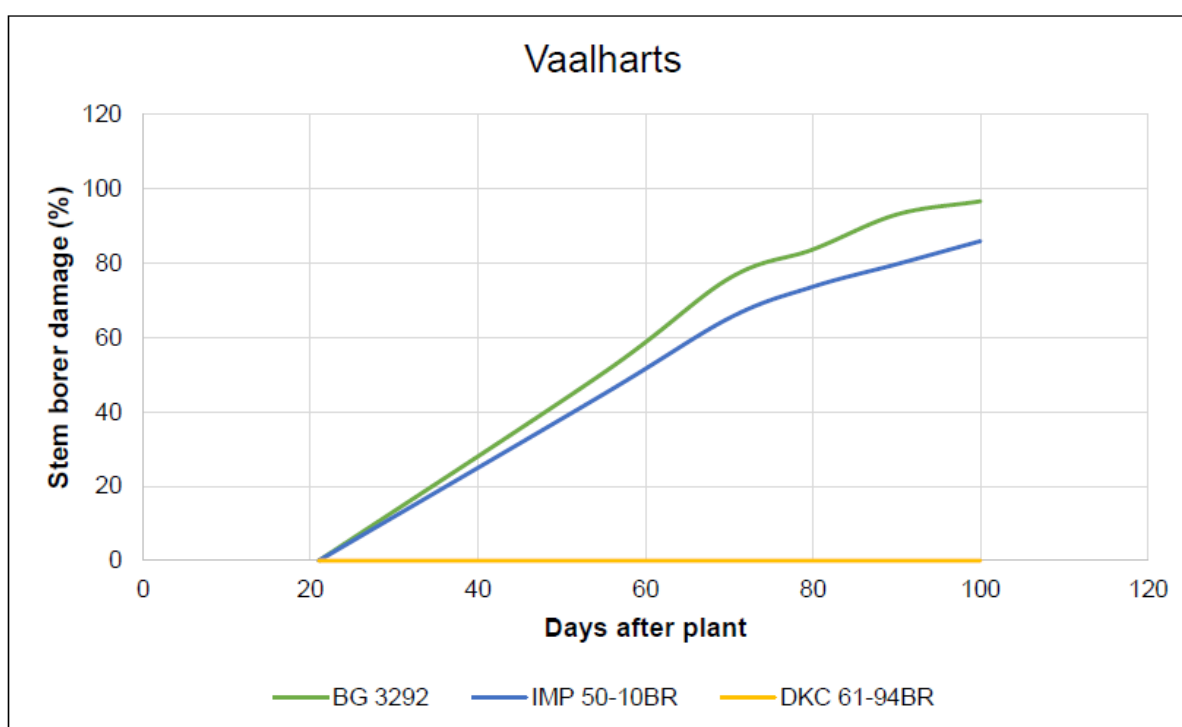


Figure 4.6: The tendency of stem borer damage for three cultivars over time in Vaalharts ($F_{(10;34)} = 61.39$; $P < 0.001$).

4.3.2 Fungal colonisation - quantitative polymerase chain reaction concentrations (qPCR)

At the different days after planting there were no significant difference in the FGSC concentration measured in the three cultivars used at different localities respectively. However, significant differences in the FGSC concentrations were measured for all three cultivars together across the season between different localities (Figure 4.7). The FGSC concentration was significantly higher at 55 days in Potchefstroom than at 90 days after plant. FGSC concentration at Vaalharts from 21-80 sampling days after plant was significantly higher than at 90 and 100 days after plant. Also, at 21, 70 and 80 days after plant in Vaalharts the FGSC concentration was higher than the concentration at 55 days. The FGSC concentration was significantly higher in Vaalharts than in Potchefstroom at 21, 55, 70 and 80 days after plant (Figure 4.7).

In Potchefstroom there was no significant difference between the cultivar and days after plant, but only significant differences between different sampling days after plant. On day 70 the pathogen accumulation was the highest and differed significantly from day 21, 80, 90 and 100 (Table 4.3). Vaalharts also showed no significant differences between cultivars and days after plant, but also just between the sampling days after plant. As at Potchefstroom, the highest pathogen accumulation was at day 70 and differed significantly from day 55, 90 and 100 (Table 4.4).

Table 4.3: Significant differences ($P < 0.05$) of FGSC concentration between days after plant in Potchefstroom ($F_{(5;34)} = 19.50$; $P < 0.001$).

Days after plant	21	55	70	80	90	100
FGSC colonisation concentration (pg/ μ l)	30.29 a	60.87 b	61.53 b	20.61 a	26.60 a	22.59 a

Table 4.4: Significant differences ($P < 0.05$) of FGSC concentration between days after plant in Vaalharts ($F_{(5;34)} = 13.548$; $P < 0.001$).

Days after plant	21	55	70	80	90	100
FGSC colonisation concentration (pg/μl)	178.63 c	104.63 b	195.79 c	157.01 bc	16.94 a	34.04 a

Succession analysis of the FGSC fungal colonisation concentration for Potchefstroom showed an increase from 21 to 70 days after plant, then the FGSC concentrations decreases and reached a plateau at 100 days after plant in all three cultivars (Figure 4.8). The succession analysis for the FGSC fungal concentration measured in Vaalharts, showed a high concentration at 21 days and a similar trend in Potchefstroom was observed for the three cultivars (Figure 4.9).

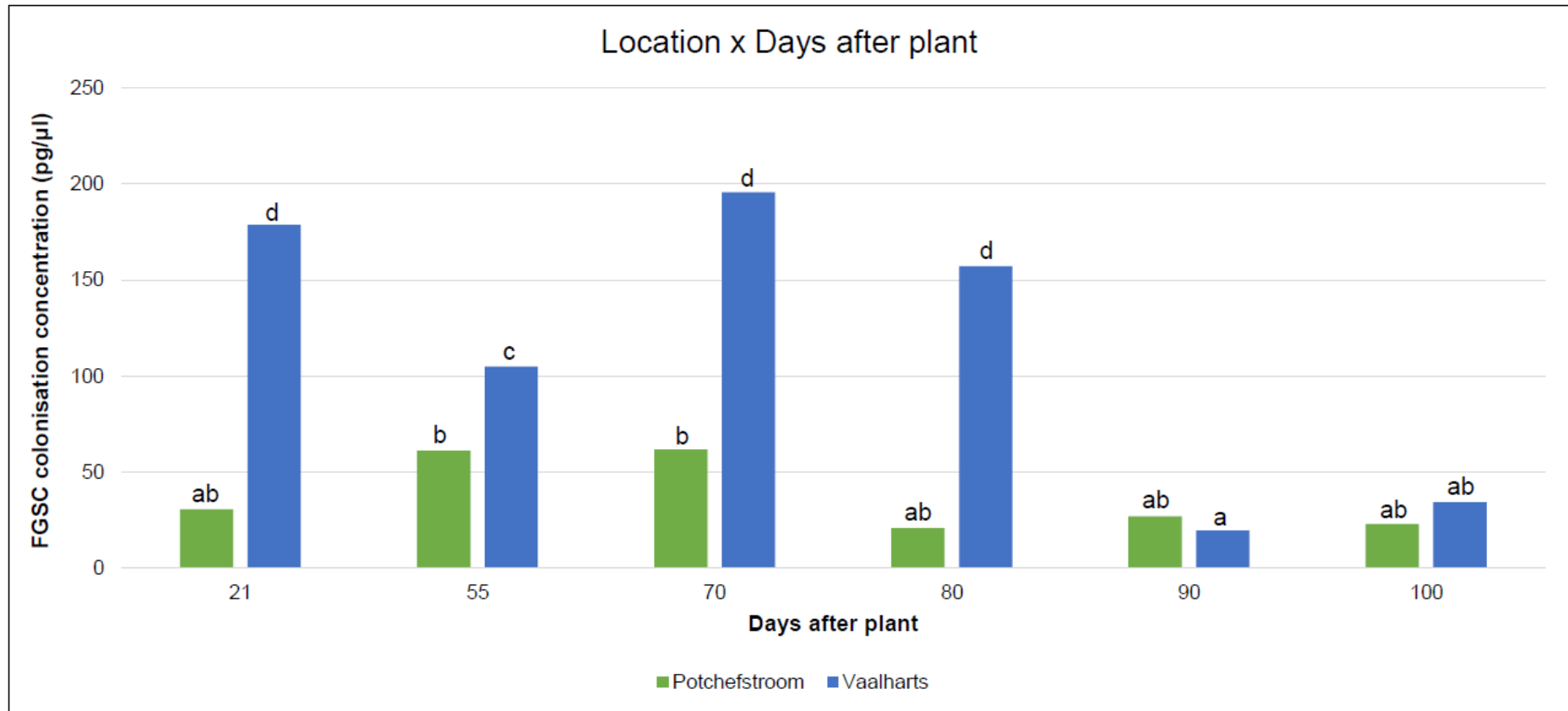


Figure 4.7: Significant differences ($P < 0.05$) of FGSC concentrations between locations and days after plant ($F_{(5;72)} = 11.81$; $P < 0.001$).

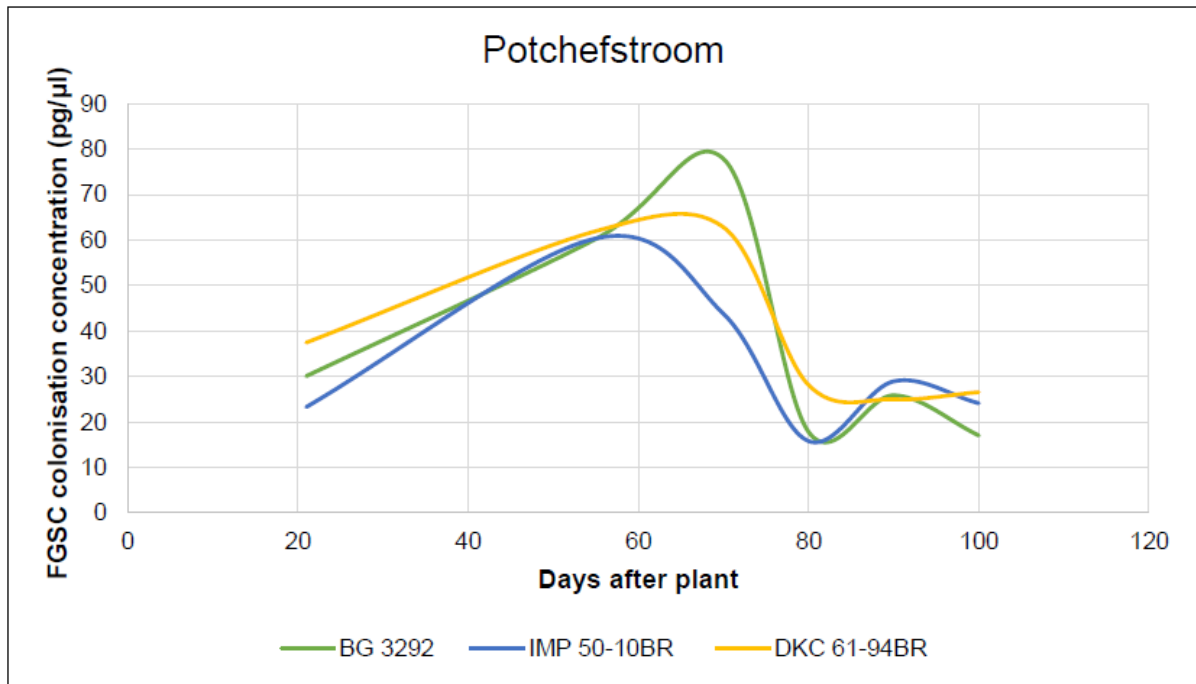


Figure 4.8: The tendency of FGSC accumulation concentration for three cultivars over time in Potchefstroom.

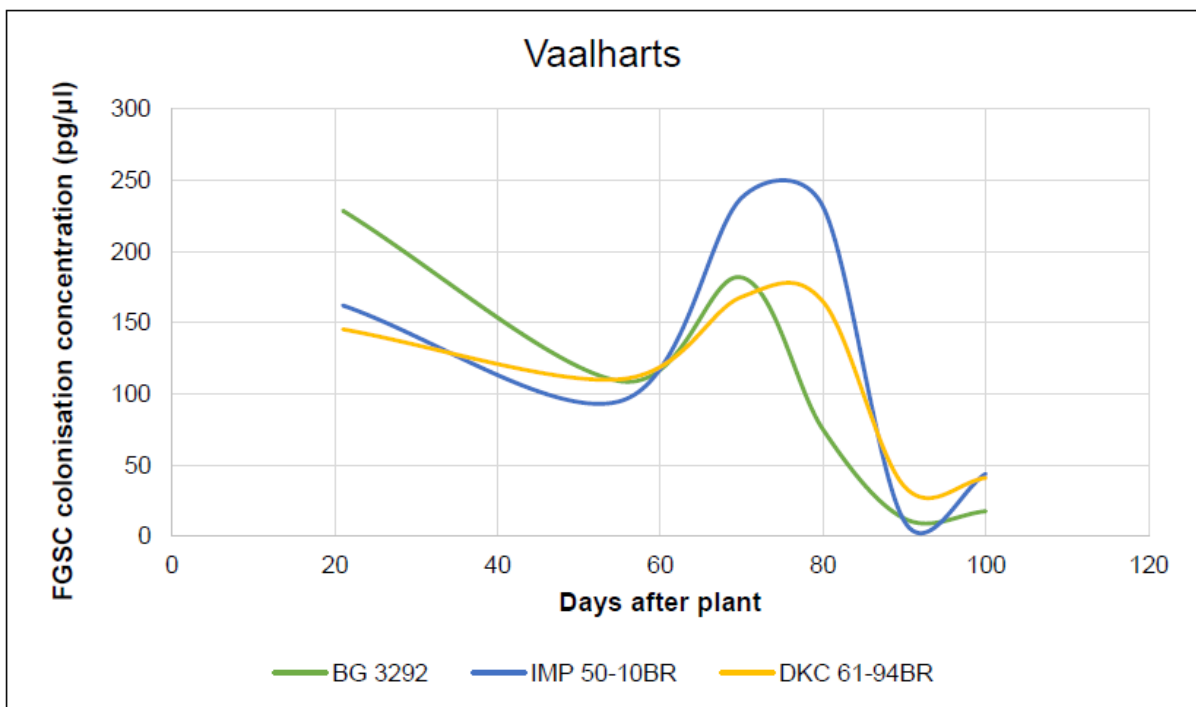


Figure 4.9: The tendency of FGSC accumulation concentration for three cultivars over time in Vaalharts.

4.3.3 Regression between stem borers and FGSC colonisation

Significant differences ($R^2 > 0.811$) were only shown for cultivars IMP 50-10BR in Potchefstroom and BG 3292 in Potchefstroom and Vaalharts. The highest FGSC presence was observed with approximately 10% stem borer damage at any given time in the season (Figure 4.10) while the highest FGSC colonisation for BG3293 in Vaalharts was at 0% stem borer damage (Figure 4.11). The highest fungal colonisation in Potchefstroom of cultivar BG3292 was present with an approximate 35% stem borer damage (Figure 4.12).

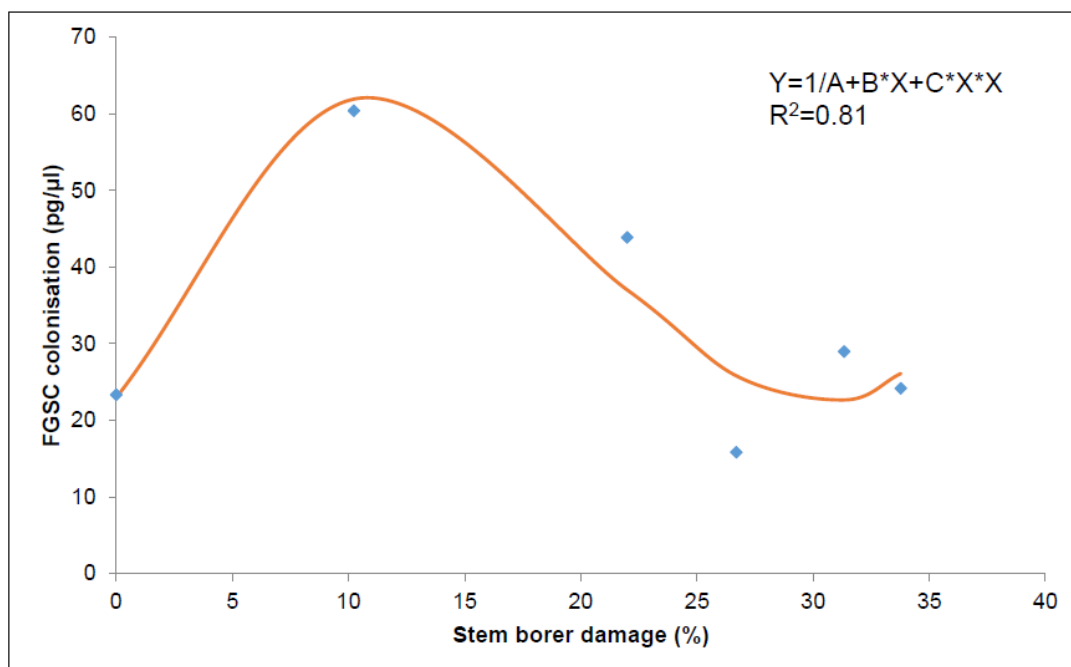


Figure 4.10: Regression of FGSC colonisation and stem borer damage of cultivar IMP 50-10BR in Potchefstroom using Cubic's model.

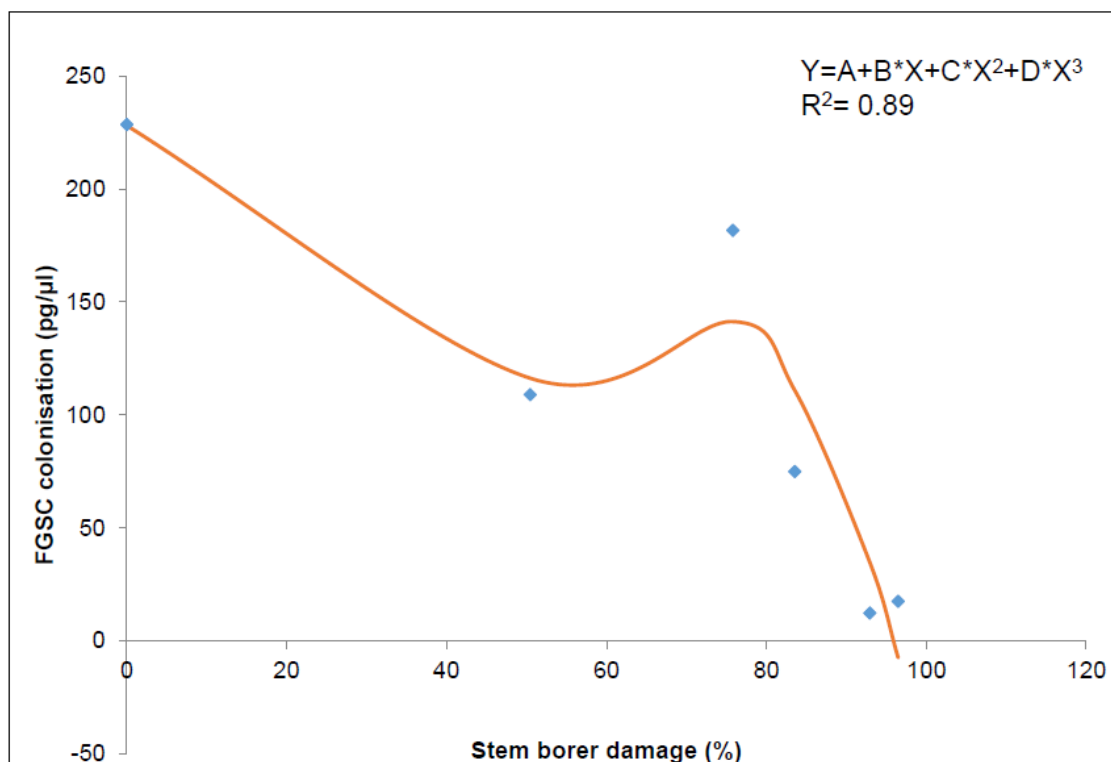


Figure 4.11: Regression of FGSC colonisation and stem borer damage of cultivar BG3292 in Vaalharts using Cubic's model.

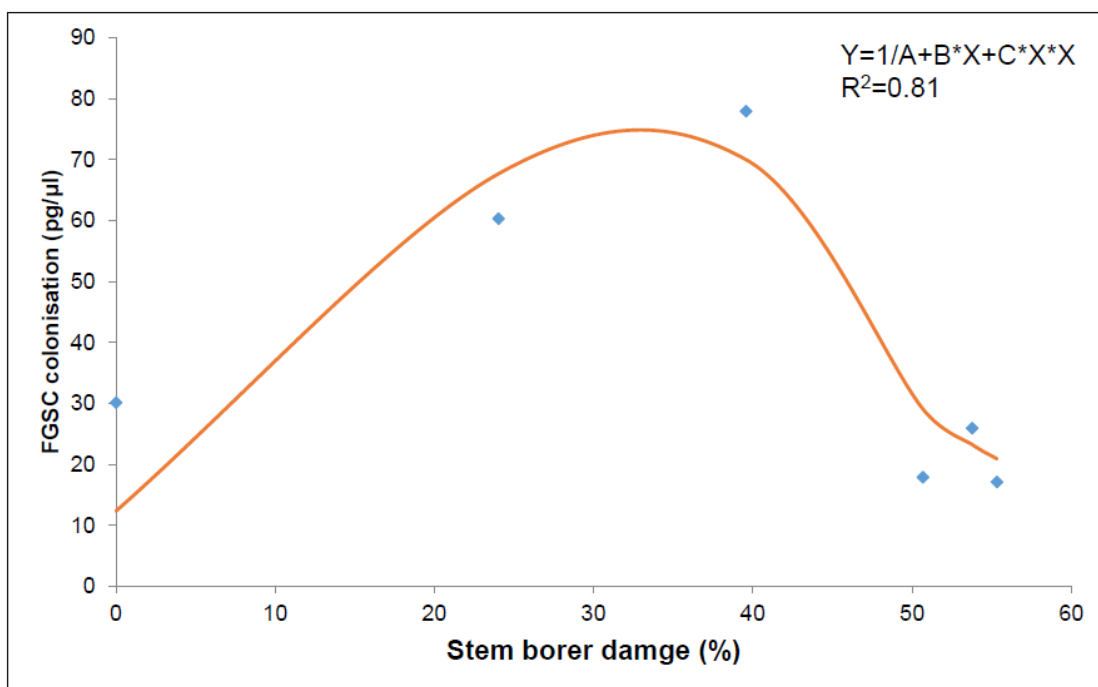


Figure 4.12: Regression of FGSC colonisation and stem borer damage of cultivar BG3292 in Potchefstroom using Holiday's model.

4.4 Discussion

In this study it was found that both FGSC and the stem borer damage increased significantly in stem tissue during the growing season. Thus, the fungal-and insect pathogens were confirmed to be serious biotic threats to maize production. The disease and insect pressure were much higher in Vaalharts than in Potchefstroom and indicated that serious disease and insect epidemics may break out in this area threatening food safety and security.

Vaalharts irrigation scheme is known for its abundance in maize stem borers (Van Wyk *et al.*, 2007), therefore also the first resistance was noted on these stem borer populations against the *Bt*-hybrid expressing *Cry1Ab* in this area (Van Rensburg, 2007). The results retrieved from this study not only confirmed the resistance against this *Bt*-hybrid, but also showed the abundance of stem borers in Vaalharts compared to the stem borers noted in Potchefstroom. The maximum of maize stem borers and damage found in Vaalharts and Potchefstroom was 96.7% and 55.77% respectively.

The FGSC concentrations were also significantly higher in Vaalharts than in Potchefstroom. Vaalharts maize production area is thus more vulnerable to diseases caused by the FGSC complex. The increase of FGSC in the western part of South Africa will force producers to reinvent and adapt different control and cultivation practices in order to prevent major disease epidemics from occurring. A study by Broders *et al.* (2007), confirmed the findings of several studies (Leslie *et al.*, 1990; Lipps and Deep, 1991 and Windels *et al.*, 1988) that *F. graminearum* found in maize debris left on the soil after harvesting provided sufficient inoculum for seedlings. Similarly, *F. graminearum* was also found inside the soil on diseased and dead seedlings. The causal agent of *Gibberella zea* stalk rot can survive and overwinter on crop residue in the soil in the absence of the crop host, where it infects the plant through the roots causing root rot as well as stalk rot (Jackson-Ziems *et al.*, 2014). This also explains the high amount of fungi present at seedling stage especially at Vaalharts.

Certain diseases of maize can be more or less problematic at different developmental stages of maize throughout the season. A significant increase of the accumulation and succession of FGSC members throughout the maize planting season in maize stems was shown and peaked at 70 days after planting. This pattern was observed both in Potchefstroom and in Vaalharts.

At 70 days after plant the maize plant is at its most vulnerable point for disease development as this is where grain fill is taking place. Stalk infections, which usually develop at the basis of the leaf sheaths or around the supporting roots, occur shortly after pollination and when the plant is stressed. It is also possible that the fungus may enter through the roots and grow up into the lower stem (White, 1999a). If maize plants get stressed during this period the maize plant will absorb nutrients from stalk tissue and remobilize carbohydrates from the stalk to complete grain fill (Dodd, 1980; White, 1999; Wise *et al.*, 2016).

Many studies have shown that with increased stem borer damage there was increased fungal infections (Munkvold and Desjardins, 1997; Munkvold *et al.*, 1999; Flett and Van Rensburg, 1992). Insects, such as stem borers, create wounds in maize stalks which enable pathogens easier access to the maize plants, thus creating secondary diseases (Smeltzer, 1958; Bosque-Pérez and Mareck, 1991; Dowd, 1998; Munkvold, *et al.*, 1999; Rutherford *et al.*, 2002; Jackson-Ziems *et al.*, 2014; Wise *et al.*, 2016).

In this study there was an increase in stem borer occurrence, as well as FGSC fungal concentration during the growing season. No correlations could be found between these two biotic factors. Correlation between FGSC and stem borer occurrence was more prevalent on specific cultivars in Potchefstroom, namely IMP 50-10BR and BG 3292, with the last-mentioned cultivar in Vaalharts shown on regression graphs (Figure 4.10-4.12). However, no stem borer damage was found in DKC 61-94BR over the season at both localities, and yet the fungal concentration of this cultivar had no significant difference to the other two cultivars. The relationship between FGSC and stem borer occurrence was inverted as the FGSC fungal concentration reduced significantly up to physiological maturity, while stem borer occurrence increased significantly up to this growth stage.

During the maize growing period, the plant also requires nutrients at different stages of plant growth, and the highest nutrient uptake for maize stalks from the soil takes place at the end of the vegetative growth stage and at the start of grain fill (Figure 4.13) (Ritchie *et al.*, 1986; Ross, *et al.*, 2013). This is also the stage at which the most FGSC was found in maize stalks in this study. Thus, the accumulation of FGSC in the stalks found in this study is in coherence with how the plant requires nutrients throughout the growing cycle from the soil for optimal growth during a season.

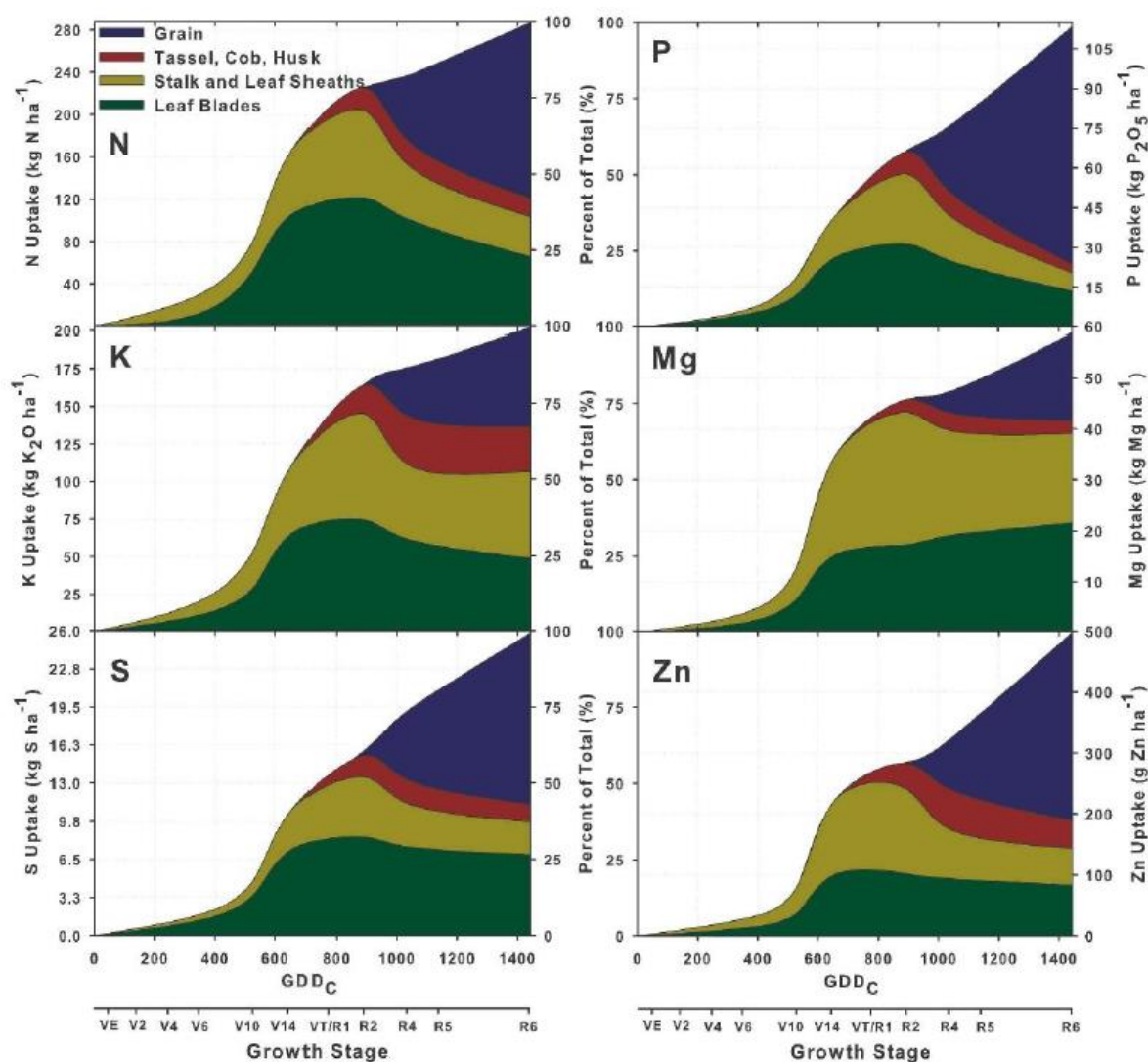


Figure 4.13: Nutrient uptake from soil from different maize plant parts over a growing season (http://cropphysiology.cropsi.illinois.edu/research/nutrient_uptake.html).

The overall FGSC colonisation concentration and stem borer occurrence of Vaalharts were also significantly higher than in Potchefstroom. Thus, the results have shown that maize in Vaalharts may have required more nutrients and water from the soil as this locality was very dry with limited rain. This could also clarify the high percentage of stem borers, as this maize in Vaalharts could have been more vulnerable to insects due to the nutrient- and water deficiency. The maize plants in Vaalharts was also more stressed and more vulnerable to pathogens because of limited water and high stem borer occurrence.

In this study FGSC and stem borers were confirmed as major biotic stressors of maize production. Various factors contributed to the occurrence and levels of stem borers and FGSC,

respectively, such as locality, cultivar and maize growth stage. For prevention of major epidemics these factors should be considered and strategies must be developed to ensure successful maize production in South Africa.

4.5 Conclusion

Diseases in maize can be more problematic at different developmental stages throughout the season. A definite pattern of the accumulation of FGSC fungal concentration in maize stems was recorded in Vaalharts and Potchefstroom. The most vulnerable stage of when the highest pathogen concentrations were measured in maize stems was at 70 days after planting when the plant is busy with grain fill. The fungal accumulation pattern is also in coherence with the plants requirements for nutrients in the soil which suggests that the plant is infected through the fungal infested soil and not by direct stem borer damage.

High stem borer occurrence was also noted and increased significantly throughout the season. With this study correlation between stem borer damage and FGSC fungal concentrations seemed to be dependent on cultivars used. The results showed that cultural practices such as controlling debris might reduce and help control FGSC occurrence. Important information was gathered in this study as to when stem disease might become more prevalent during periods of stress such as drought and insect damage.

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CHAPTER 5 - CONCLUSION

The increasing levels of *Fusarium graminearum* species complex (FGSC) in maize growing areas in South Africa is a threat to food security as well as a health hazard for humans and animals. The aim of this study was to elucidate the FGSC on maize. The importance of disease identification was highlighted in chapter 2. Seeing that two new maize infecting pathogens (*F. lunulosporum* and *F. cerealis*) were identified in this study, can be a warning for proper disease identification and management of future disease epidemic threats in the field. The use of PCR techniques to identify FGSC members was evaluated, but multilocus genotyping (MLGT) served as a more reliable source to correctly identify FGSC members on maize. The four FGSC members found in this study occurring on maize stems, roots and crowns and identified by the MLGT technique were *F. graminearum* (stems and crowns), *F. boothii* (stems and crowns), *F. graminearum* x *F. boothii* (stems, crowns and roots) and *F. meridionale* (stems).

Known FGSC members occurring on maize in South Africa is limited to either the maize ears or roots or to specific regions in South Africa. This study provided clarification of the preference of certain plant parts and locations of members of the FGSC through artificial inoculation in maize stems and ears. The ability of FGSC members to successfully infect not only ears, but also the stems of maize was revealed. The most virulent isolate was *F. graminearum* which was inoculated onto maize ears collected from diseased maize isolates from KwaZulu-Natal. In some cases the stem inoculations showed more colonisation than inoculated ears. *F. graminearum* x *F. boothii* was also associated with maize stems and grain for the first time and proved that it can grow and cause disease on maize ears and stems. Thus, these three FGSC members may cause serious epidemics in maize and farmers should be equipped to effectively control these pathogens. Some of the inoculated isolates were able to produce mycotoxins such as nivalenol and zearalenone which can seriously affect human and animal health. The mycotoxin producing potential of the hybrid species, *F. graminearum* x *F. boothii* producing nivalenol was proven for the first time. The production of these different mycotoxins is a serious health risk not only for humans, but infected grains and stems are also a big health risk for animals and management of this species complex is highlighted.

Stem borers on maize and fungal infections on maize have been studied on separate occasions before, but this study provided information for the first time about the possible interaction of these two biological threats on maize. Stem borer data in corporation with the pathogens

showed no direct interaction in joined competition. However, the succession of FGSC pathogen in maize stems provided important and valuable data in which a definite pattern of FGSC accumulation concentration in maize stems was recorded at different times throughout the growing season. In Vaalharts and Potchefstroom maize were most vulnerable to pathogens in the stages that the maize plant was busy with grain fill (± 70 days). High stem borer occurrence was also noted at increased levels throughout the season. This study also indicated that stem borer and FGSC interactions could be dependant on cultivars used.

This study provided valuable data about identification methods for identifying FGSC, plant part preference as well as mycotoxin production and when fungal infections in stems might become a problem during the growing season with possible stem borer interactions. The aims of this study were successfully investigated and achieved.