Quantifying twelve fungal isolates associated with maize root and crown rot complex in South Africa

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Colossians 3:23-24

[23] Whatever you are doing, work at it with enthusiasm, as to the Lord and not for people, [24] because you know that you will receive your inheritance from the Lord as the reward. Serve the Lord Christ.

ABSTRACT

Maize is South Africa's most important crop contributing to dietary staple, livestock feed and to the gross domestic product of the country as an export crop. Root and crown rot on maize in South Africa threaten the optimal production of this staple food. A complex of fungal pathogens is responsible for causing these diseases and the best management strategies need to be applied to prevent yield loss. These pathogens have certain environmental preferences and conditions in which they thrive. Altering these conditions through applying cultivation practices in different climatic regions in the country together with other management strategies can limit root and crown rot. For these practices to be efficient the different pathogens need to be known and evaluated separately. How these pathogens co-exist in the different environments, as well as the mechanism by which the inoculum of each pathogen change over and between seasons, should be known. The overall aim is to understand the disease complex causing root and crown rot and its succession over time, to quantify the disease incidence and severity and to formulate management strategies accordingly. Limited research like this has been done for disease complexes and through this study many shortages will be identified and opportunities will arise for better and more research to optimize management of root and crown rot on maize. In this study, the influence of tillage and no-till, mono-cropping and crop rotation, dryland and irrigation, different localities (provinces) and tissue specificity on the presence and abundance of twelve commonly known fungal pathogens of root and crown rot in South Africa *(Curvularia eragostidis*, *Exserohilum pedicellatum*, *Fusarium chlamydosporum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. verticillioides*, *Macrophomina phaseolina, Pythium* species, *Phoma* species, *Rhizoctonia solani* and *Trichoderma* species) were studied. Visual evaluations and disease ratings, DNA extractions and qPCR (quantitative Polymerase Chain Reaction) technology, being effective, quick and precise, were used to separately analyse each pathogen with these above-mentioned variables.

Overall the complex showed significant root preference compared to crowns. In conventional cultivation practices the qPCR results showed that *Phoma* spp*., Pythium* spp., *F. oxysporum* and *F. chlamydosporum* were the most prominent and *Phoma* spp*., F. chlamydosporum, Pythium* spp. and *F. oxysporum* were prevalent in conservation agricultural practices. There was a significant tillage x province interaction for *F. oxysporum* (P=0.00), irrigation x province interaction for *E. pedicellatum* (P=0.02) and *R. solani* (P=0.04)*. F. verticillioides* showed significant differences between different rotated crops (P=0.01). *R. solani* was found significantly more in no-till fields compared to tilled fields, and between rotations with different crops (P<0.0001). From three cultivars (BG 3292, IMP 50-10 B and DKC 61-94 BR), BG 3292 had the lowest root and crown rot severity ratings and the highest root and crown plant-biomass. For *C. eragostidis*

(P=0.00) and *E. pedicellatum* (P=0.03) a significant locality x sampling date interaction was indicated, while *F. oxysporum* had significant cultivar x plant part x locality interactions (P=0.04). *Phoma* spp. were significantly affected by the sampling date and plant part interaction (pathogen presence increased in the roots with time and decreased in the crowns) (P=0.00) and *Pythium* spp. with the sampling date x plant part x locality interaction (pathogen presence increased in the roots of maize plants at Vaalharts with time and decreased in the crowns of maize plants at Vaalharts and Potchefstroom) (P=0.00). *Trichoderma* spp. showed the highest order interaction that contributed to the infection: sampling date x plant part x cultivar x locality (P=0.01). This study revealed the value of using molecular technology in studying the different variables contributing to the occurance and severity of these diseases (the fungi present and to which degree it contributes to the root and crown rot disease complex).

Key terms: Cultivation practices, disease, DNA extractions, fungal pathogen complex, interactions, management strategies, qPCR analyses, root and crown rot

OPSOMMING

Mielies is Suid-Afrika se belangrikste graangewas en dra by as stapelvoedsel, dierevoer en tot die land se ekonomie as uitvoer gewas. Wortel- en kroonvrot op mielies bedreig die optimale produksie van mielies in Suid-Afrika. ‘n Kompleks van swampatogene veroorsaak hierdie siektes en optimale bestuurstrategieë moet toegepas word om enige verlies aan opbrengs te beperk. Die swampatogene het verskillende omgewings voorkeure en kondisies waar hul die beste oorleef. Deur die voorkeure te onderdruk of verander deur verbouings praktyke in verskillende klimaatstreke saam met die toepassing van ander bestuurstrategieë, kan die siekte beperk word. Die praktyke en strategieë sal slegs effektief wees as die verskillende swampatogene bekend is, individueel en hul interaksie, geanaliseer kan word, asook die hoeveelheid van elk en hoe dit in en oor seisoene verander. Die algehele doel van die studie is om die siektekompleks wat wortel- en kroonvrot op mielies veroorsaak en die opvolging in en oor seisoene beter te verstaan, om die siekte voorkoms en graad te kwantifiseer en om bestuurstrategieë daarvolgens te formuleer. Huidiglik is die tipe navorsing wat reeds gedoen is op siekte komplekse beperk en deur die studie sal nog tekortkominge geïdentifiseer word en geleenthede geskep word vir beter en verdere navorsing om die bestuur van wortel- en kroonvrot te optimaliseer. In die studie is die effek van bewerking teenoor geen bewerking van lande, droë teenoor besproeiing, weefsel voorkeur, slegs mielies teenoor gewas rotasie, en verskillende lokaliteite (provinsies) ondersoek wat die voorkoms en hoeveelheid van die twaalf swampatogene beïnvloed wat in Suid-Afrika bekend is om wortel- en kroonvrot op mielies te veroorsaak (*Curvularia eragostidis*, *Exserohilum pedicellatum*, *Fusarium chlamydosporum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. verticillioides*, *Macrophomina phaseolina, Pythium* spesies, *Phoma* spesies, *Rhizoctonia solani* en *Trichoderma* spesies). Visuele evaluerings en siekte graderings, DNA ekstraksies en qPCR tegnologie is gebruik om die swamme individueel met bogenoemde veranderlikes te analiseer deur vinnige, effektiewe en akkurate molekulêre prosesse en protokolle te gebruik.

Tydens die studie is daar definitiewe voorkeur in die teenwoordigheid van die kompleks spesies in die wortels eerder as die krone waargeneem. Waar gewone verbouingspraktyke toegepas is, het die qPCR resultate gewys dat *Phoma* sp.*, Pythium* sp., *F. oxysporum* en *F. chlamydosporum* die prominentste voorgekom het, en *Phoma* sp.*, F. chlamydosporum, Pythium* sp. en *F. oxysporum* waar bewarings verbouingspraktyke toegepas is. Daar was beduidende verbouing x provinsie interaksie vir *F. oxysporum* (P=0.00), besproeing x provinsie interaksie vir *E. pedicellatum* (P=0.02) en *R. solani* (P=0.04) uitgewys*. F. verticillioides* het beduidende verskille tussen die verskillende geroteerde gewasse gehad (P=0.01). *R. solani* was merkwaardig meer in die onbewerkte lande teenoor die geploegde lande, en waar gewas rotasie voorgekom het

(P<0.0001). Drie mieliekultivars is gebruik (BG 3292, IMP 50-10 B and DKC 61-94 BR), waar BG 3292 die laagste wortel- en kroonvrot waardes en die hoogste wortel- en kroonbiomassa gehad het. *C. eragostidis* (P=0.00) en *E. pedicellatum* (P=0.03) het beduidende lokaliteit x monsterneming datum interaksies gehad en *F. oxysporum* beduidende kultivar x plant deel x lokaliteit interaksies (P=0.04). *Phoma* sp. is beduidend deur die monsterneming datum x plant deel interaksie beïnvloed (patogeen teenwoordigheid het toegeneem in die wortels met tyd en afgeneem in die krone met tyd) (P=0.00). *Pythium* sp. het beduidende monsterneming datum x plant deel x lokaliteit interaksie gehad (patogeen teenwoordigheid het toegeneem in mielie wortels by Vaalharts met tyd en afgeneem in die mielie krone by Vaalharts en Potchefstroom met tyd) (P=0.00). *Trichoderma* sp. het die hoogste orde interaksie gehad: monsterneming datum x plant deel x kultivar x lokaliteit (P=0.01). Hierdie studie het die waarde van die gebruik van molekulêre tegnologie in die analisering van die effek van verskillende veranderlikes op die voorkoms en graad van wortel- en kroonvrot aan die lig gebring, asook watter swampatogene tot watter graad bydra tot die siektekompleks.

DECLARATION

I declare that the dissertation submitted by me for the degree Magister Scientiae in Environmental studies 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: 

Date: 17/02/2019

Karla-Mart Beyers

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APS- The American Phytopathological Society

CA Conservation Agriculture

CDI- Crown Disease Index

CTAB- Cetyl Trimethyl Ammonium Bromide

DEP- DNA Extraction Buffer

DAP- Days After Plant

FAO- Food and Agricultural Organization for Africa

JADAFA- Joint Agribusiness Department of Agriculture Forestry and Fisheries Forum for Africa

LSD- Least Significant Difference

MNTSB- Maize No-till Soybean

MNTSG- Maize No-till Sorghum

MRORSB- Maize Rip-on-Row Soybean

MRORSG- Maize Rip-on-Row Sorghum

OECD- Organisation for Economic Cooperation and Development

OMAFRA- Ontario Ministry of Agriculture Food and Rural Affairs

PPRI- Plant Protection Research Institute

RDI- Root Disease Index

SADC- South African Development Community

SAGIS- South African Grain Information Services

SAS- Statistical Analyses Software

SAWS- South African Weather Service

LIST OF APPENDIX TABLES

CHAPTER 1

INTRODUCTION ON MAIZE AS GRAIN CROP AND THE INFLUENCE OF ROOT AND CROWN ROT

1.1 Maize production and importance

Maize is the third most planted and important crop in the world with regards to production area, high yields (with variations between growing seasons) and having an annual production in 2010 of 12.8 million tons in South Africa (SAGIS, 2011). Between the years 2000 and 2014 the total production of maize ranged between 6.5 -12.5 million tons, with consumption of maize increasing on an annual basis (Figure 1.1) (USDA-FAS, 2014).

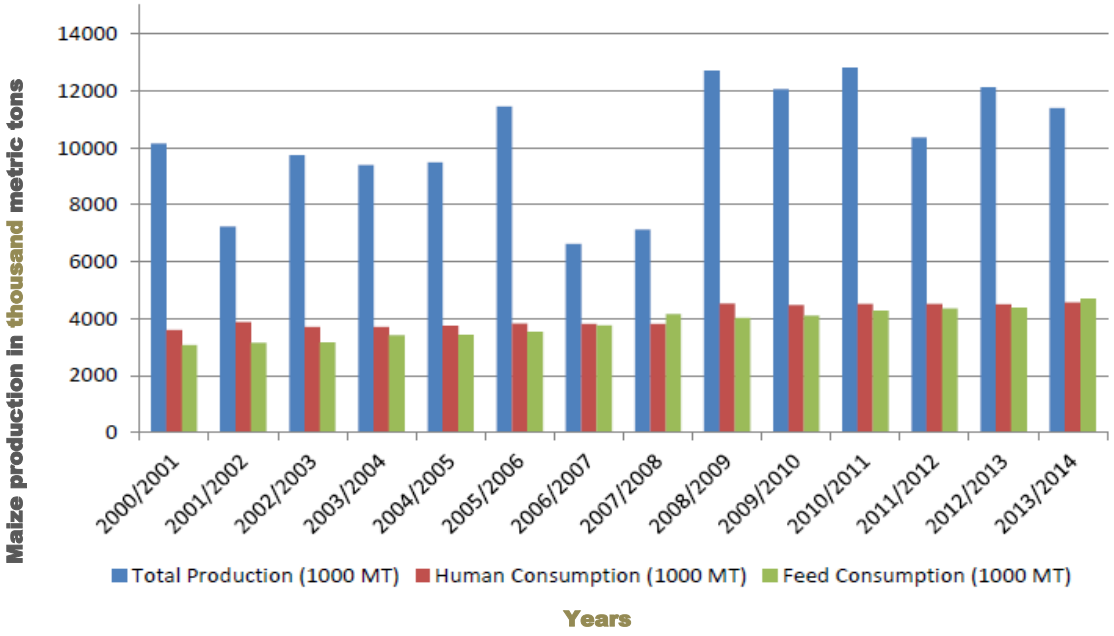

Figure 1.1: South Africa's maize production and consumption from 2001/2002 - 2013/2014 (USDA-FAS, 2014).

In 2002, South Africa contributed 1.5% to the total world maize production. Four provinces: Free State, Mpumalanga, North West and Gauteng supply more than 85% of the National output (FAO Aquastats, 2014). Maize is economically important for South Africa and the surplus is exported to other countries in Africa. In 2014, 330 000 hectares of maize were harvested and 14 982 000 tons of maize were produced in South Africa alone. Inevitably, maize is one of South Africa's major

staple food sources. Six distinct activities make up South Africa's maize marketing value chain: production, storage, trading, processing, retailing and consumption. Here, in South Africa, maize is used for household consumption, in the livestock industry and for export (maize products and grain) purposes (JADAFA, 2015). It is estimated that the world population will surpass the nine billion mark by 2050, with the growth rate being the highest in developing countries. Concerns are raised that the maize demands will surpass the supply as it is indicated that the maize demand will double in developing countries by 2025 (Cairns *et al*., 2013).

1.2 Soil borne diseases of maize

Disease occurrence on plants is usually dependant on three major factors being the host, pathogen and environment and is referred to as the disease triangle (Huber & Haneklaus, 2007). A disease triangle (Figure 1.2) can be formulated where the host plant, pathogen and environment forms the three corners as interacting components, resulting in a disease if all components are present and the conditions of each favourable. This disease triangle can be used to calculate or predict diseases that affect optimum food/grain production and yield (Huber & Haneklaus, 2007). A maize plant (host) infected with a fungus (pathogen) at environmental conditions allowing plant growth and pathogen survival completes the image of disease development in the form of a triangle. This triangle can also be used to prevent and manage disease; by altering one or more of the factors, the possibility of disease occurrence and development is less likely.

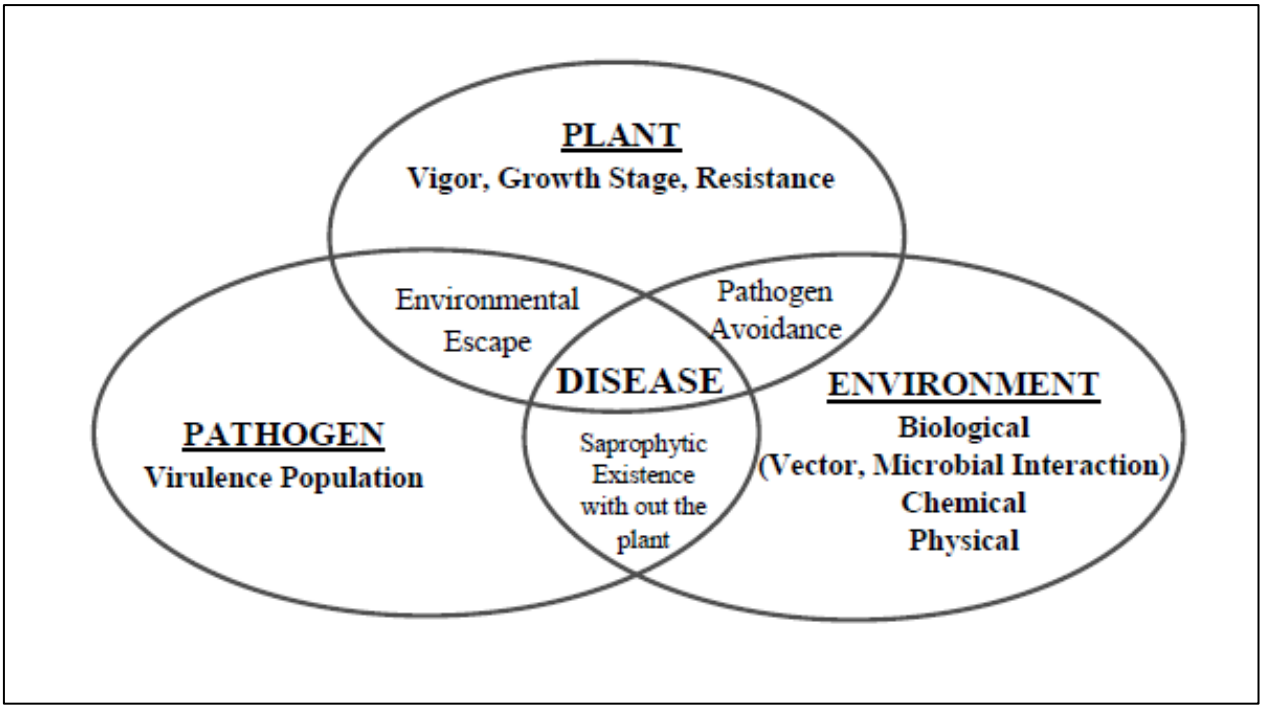

Figure 1.2: A disease triangle representation with its interacting components (Huber & Haneklaus, 2007).

Maize is affected by soil borne diseases like root, crown and stem rots that are known but poorly understood diseases due to the fact that these rots aren't caused by a single organism, but are the result of more than one fungal pathogen (disease complex). Stalk and root rot of maize is frequently associated with each other and Whitney & Mortimore (1957) noticed that maize roots may be totally diseased without having stalk rot, but that stalk rot always occurs with root rot. Also, the succession of a disease complex constantly changes because of pathogen precursors in the complex, differing climate conditions, soil conditions, cultivation practices, maize plant resistance and the growth stages of the maize. General symptoms of root- and crown rot are rotted roots, roots reduced in size and roots showing lesions, while the above ground parts show slow emergence, stunted and wilted plants, lodging and death (Wise *et al.,* 2016). General penetration by pathogens of the roots and crowns occurs directly through cell walls, wounds and natural openings of the plant and occurs more commonly under stress conditions. Not all penetration leads to infection, only susceptible cells and tissue will become diseased. Pathogens can be classified as biotrophic, necrotrophic or hemibiotrophic based on their lifestyles as types of parasitism (Divon & Fluhr, 2006). These pathogens all procure nutrients used for growth, multiplication, invasion and colonisation to a certain extent (Agrios, 2005). The biotrophic pathogens, who are completely dependent on the host in order to complete their life cycle, procure nutrients from living host cells by developing haustoria (specialized infection structures). Necrotrophs derive their nutrients from sacrificed cells (no specialized infection structures present) and hemibiotrophs occupy the living host cells briefly before switching to the necrotrophic lifestyle (Divon & Fluhr, 2006).

1.2.1 Pathogen component

Many pathogenic fungi can be present in a disease complex that can cause the disease on its own or in synergy with the other pathogenic fungi (White, 1999). Fungi are found everywhere in nature, recycling nutrients from organic matter. Most fungi are strict saprophytes with few causing diseases in plants and humans. Further categorization occurs with regard to the type of infection: saprophytic fungi as opportunistic pathogens (enter wounds or as result of weakened host condition) and true pathogens (depend on plant or human tissue for its nutrients but can survive outside the host too) (De Lucca, 2007). Fungal plant pathogens that cause diseases have been shown to reduce grain yields and quality, influencing nutrient availability (the uptake and distribution of nutrients are reduced in the plants) which result in food security and safety issues (mycotoxin production) (Bankole & Adebanjo, 2003). There are three groups of pathogens which include: true polyphagous fungi which have a large range of host species such as *Pythium* spp, *Verticillium* spp. and *Sclerotium* spp., species that are polyphagous and host specific like

Rhizoctonia solani (Kühn) and monophagous pathogens that only have one host or host group (Coetzee, 2015).

Certain fungi cause rot which is the softening, disintegration and discoloration of the succulent parts of plant tissue (Agrios, 2005). Symptoms generally caused by fungi are tissue necrosis, uneven growth and stunting of specific organs or the entire plant. Root, crown and stem rot in the field are often misinterpreted as the aboveground symptoms correspond with many other diseases and deficiencies (White*,* 1999). Belowground symptoms are rots, discolouration, and reduced root systems. The aboveground symptoms include poor germination, poor seedling vigour, and reduction in plant stand, plant lodging and ultimately reduced yield. According to Summer & Bell (1982) lodging caused by root rot occurs at the soil surface and stalk rot generally between the fourth and fifth internode of the maize plant. For every twenty-five percent of disease severity of root, crown and stem rot of maize, caused by soilborne pathogens, yields can show losses of up to two tons per hectare. Ten to thirty percent loss often occurs due to stalk rot complexes (Agrios, 2005).

Twelve fungal pathogens have been identified, through research done at the Agricultural Research Council of South Africa, as the most common associated with root and crown rot on maize in South Africa (Craven & Nel, 2016, Smit, 1998). The twelve fungal pathogens identified are: *Curvularia eragostidis* (Hennings) J.A. Meyer, *Exserohilum pedicellatum* (Henry) K.J. Leonard & E.G. Suggs, *Fusarium chlamydosporum* (Wollenw. & Reiking), *Fusarium equiseti* (Corda) Sacc., *Fusarium graminearum* (Schwabe), *Fusarium oxysporum* (Schlectend), *Fusarium verticillioides* (Sacc.), *Pythium* species (Drechsler), *Phoma* species (Sacc.) Boerema, Dorenbosh & Kesteren, *Rhizoctonia solani, Trichoderma* species, *Macrophomina phaseolina* (Tassi) Goid (Table 1.1). These twelve pathogens (although not the only pathogens present) are regarded as the most prominent and fundamental fungal maize root- and crown rot pathogens, forming a complex (Smit *et al.,* 1997; Smit, 1998; Smit & McLaren, 1997, Lamprecht *et al.,* 2008).

Table 1.1: Twelve fungal isolates with its known maize disease associations.

Fungal species	Diseases associated	References
Curvularia eragostidis (Hennings) J.A. Meyer	*Curvularia* leaf spot Stalk and root rot (secondary stalk invader)	(Shurtleff *et al*., 1993) (White, 1999)
Exserohilum pedicellatum (Henry) K.J. Leonard & E.G. Suggs	*Helminthosporium* root rot Seed rot seedling blight Cob-rot	(Shurtleff *et al*., 1993) (Gilbert, R.L., 2003)

*F*usarium *chlamydosporum* (Wollenw. & Reiking)	Root, crown and stalk rot Ear rot	 (Morales-Rodriguez *et al.,* 2007)
*F*usarium *equiseti* (Corda) Sacc.	Minor root rots, crown and stalk rot Fusarium head blight	(Shurtleff *et al*., 1993) (Nicolaisen *et al.,* 2009)
Fusarium graminearum (Schwabe)	Gibberella ear and stalk rot Seed rot-seedling blight Fusarium head blight	(Shurtleff *et al*., 1993) (White, 1999) (Logrieco *et al.*, 2003)
Fusarium oxysporum (Schlectend)	Minor root rots and wilts Minor stalk rots Fusarium head blight Seedling blight and root rot	(Shurtleff *et al*., 1993) (Jiménez-Fernández *et al*., 2010) (Nicolaisen *et al*., 2009) (White, 1999)
*F*usarium *verticillioides* (Sacc.)	Fusarium ear, cob and stalk rot Fusarium kernel, root and stalk rot, seed rot and seedling blight Fusarium head blight Leaf scorch	(Shurtleff *et al*., 1993) (Christensen *et al*., 2014) (Logrieco *et al.,* 2003) (De Luca, 2007) (White, 1999) (Nicolaisen *et al*., 2009)
Pythium species (Drechsler)	Pythium root rot (*P. arrhenomanes* Drechs. *P. graminicola* Subramanian) Pythium stalk rot (*Pythium aphanidermatum* (Edson) Fitzp. *P. butleri* L. Subramanian) Seed rot- seedling blight (death), damping off	(Shurtleff *et al*., 1993) (White, 1999)
Phoma species (Sacc.) Boerema, Dorenbosh & Kesteren	Minor leaf spots Root and crown rot, red root rot Pyrenochaeta stalk and root rot (*Phoma terrestris* E.M. Hans.) Red root rot	(Shurtleff *et al*., 1993) (White, 1999)
Rhizoctonia solani (Kühn)	Banded leaf and sheath spot , banded sheath blight Rhizoctonia root, ear and stalk rot	(Shurtleff *et al*., 1993) (White, 1999)

	Seed rot- seedling blight and damping off, failure to germinate	
Trichoderma species	Trichoderma ear and root rots Stalk rots (secondary stalk invader), seedling blight, seed rot	(Shurtleff *et al*., 1993) (White, 1999)
Macrophomina phaseolina (Tassi) Goid	Charcoal rot Seed rot-seedling blight Root and stem rot	(Shurtleff *et al*., 1993) (White, 1999) (Francl, 1998)

The most common fungi, worldwide, on maize roots are the *Fusarium* species (Smit, 1998). *Fusarium* species occur more in soil that has not been ploughed and tillage practices affect the extent to which the healthy roots are invaded by *Fusarium* species (Smit, 1998). The tillage practices affect the physical soil properties and soil microflora present in the soil, which directly contributes to the favourability of invasion conditions for the pathogens. Many of the *Fusarium* species also produce mycotoxins that can cause acute and chronic diseases in humans and animals (Jurado *et al*., 2006). Mycotoxins are toxic secondary metabolites produced by fungi and apart from health threats, it holds economic implications as grain quality and utility are influenced negatively (Bankole & Adebanjo, 2003).

F. verticillioides (previously known as *F. moniliforme*) is widespread in maize producing areas in South Africa and can produce fumonisin as a secondary metabolite. *F. verticillioides* grows systemically from the roots upwards towards the stalk and into the ears of maize causing rots. Infection levels can be extremely high, up to 100%, and can survive on crop residue in or on the soil and can overwinter in seeds (pedicel, endosperm and/or embryo). *F. verticillioides* can be dispersed through asexual spores (macroconidia and microconidia) through the air (wind), insects (stalk borers such as: *Chilo partellus* (Swinhoe, 1885) and *Busseola fusca* (Fuller, 1901)), or on plant debris and seeds (Ortiz *et al*., 2015). *F. verticillioides* causes the most damage as stalk and ear rot of maize and is also associated with rot in sugarcane, rice and asparagus to name a few (Summerell *et al.,* 2011). *F. oxysporum* has subgroups (formae speciales) as pathogens to plants that cause crown and root rot, damping off and vascular wilts (Summerell *et al*., 2011). Summerell *et al.* (2011) stated that *F. oxysporum* does not affect cereals and grain crops to a large degree and is regarded to be of little relevance in terms of mycotoxin production. *F. oxysporum* also has saprophytic members that act as secondary invaders and colonise necrotic roots. *F. equiseti* is a ubiquitous soil saprophyte frequently occurring in sub-tropical and tropical areas and is less

frequent in temperate regions (Kosiak *et al.,* 2005). *F. equiseti* is a cosmopolitan soil inhabitant which commonly colonises damaged plant tissue (Summerell *et al*., 2011). The secondary metabolites (trichothecene, zearalenone and butenolide) that they produce often differ in quantity and toxicity (Kosiak *et al*., 2005). *F. equiseti* produces type A trichothecene mycotoxins, but is not regarded as a major plant pathogen of maize (Summerell *et al*., 2011). *F. graminearum* causes diseases like head blight of wheat, barley, oats, stalk and ear rot of maize as an airborne pathogen (Summerell *et al*., 2011). *F. graminearum* cause stem and ear rot on maize, which reduces grain quality and yield, and produce zearalenone, deoxynivalenol and nivalenol, type B-trichothecenes (mycotoxins) that can be produced in the field or in stored grain (Koncz *et al.*, 2008). The last of the *Fusarium* species; *F. chlamydosporum* is common in the soil of warmer, dry areas (semi-arid, arid, grasslands). *F. chlamydosporum* is seen mostly as a secondary invader and has been isolated from various plant parts, but does not produce mycotoxins (Summerell *et al*., 2011).

C. eragostidis is a pathogen of maize, tea, yam, passion fruit, striga and various grass species (Zhu & Qiang, 2011), and produces conidia that begin to germinate on grass leaves and penetrate at conjunction grooves of epidermal cells or directly through the stomata. *C. eragostidis* produces phytotoxins with bio-control characteristics as a mycoherbicide. The mycoherbicide was first reported for *Digitaria sanguinalis* (crabgrass), showing great potential to control this noxious weed. The fungus kills weeds rapidly due to its high virulence (Jiang *et al.,* 2008). *C. eragostidis* produces secondary metabolites in chemical classes based on use, toxicity and structure with different biological activities (phytotoxic, antifungal and cytotoxic). X,β-dehydrocurvularin is one of the compounds produced by *C. eragostidis* and used for its bio-activity (natural bio-herbicide) (Jiang *et al.,* 2008). This compound lowers the chlorophyll content and photosynthetic capacity of crabgrass, inhibits chlorophyll *a* fluorescence and seed germination and decreases the photophosphorylation and Mg^{2+} ATPase activity, in crabgrass and maize (Jiang *et al.,* 2008).

Phoma is a large genus of fungi with widespread geographical distribution and occurs in various ecological niches. Some of the species are harmless, but most are fungal plant pathogens on crops of economic importance like oilseed crops and various *Brassicaceae* (Aveskamp *et al.,* 2008). There are 110 of the described 3 000 taxa that are classified as pathogenic species (Zimowska, 2011). *Phoma* spp. are usually ascociated with maize leaf spot, but the presence in maize roots have been observed throughout research.

According to Sivanesan (1987) *E. pedicellatum* attacks species of *Echinochloa, Oryza, Paspalum, Setaria, Sorghum, Triticum* (causes dark brown root lesions) and *Zea mays* L., where root rot is induced. Distribution of these fungi has been recorded in Egypt, India, Pakistan, South Africa, USA and Australia. *Rhizoctonia* is a sterile fungal genus (incapable of producing spores) and a

soil inhabitant (basidiomycete) that exists primarily as mycelium or small sclerotia. *Rhizoctonia* is common in warm, moderately moist soil. *R. solani* as soilborne pathogen has a wide host range (ornamental plants and trees) causing diseases worldwide, but the yield of sugar beet and maize are the most influenced by this pathogen (Abbas *et al*., 2014). *R. solani* is seen as a collective species (consists of a number of more or less unrelated strains) of which the strains can be distinguished due to the fusion of touching hyphae (Agrios, 2005). This pathogenic fungus causes root and stem rots, damping off but seldom leaf blight. It occurs in the soil as hyphae and sclerotia, infects the plant resulting in decay and results in brown to red-brown lesions in the roots at or just below the soil. The stems start to rot just above the soil and also have brown-red lesions. In favourable conditions the lesions will enlarge forming cankers that can encircle the entire stem (Agrios, 2005). Infected plants appear wilted during the day because of inadequate water and nutrient absorption.

Some *Trichoderma* spp. are economically important as they produce enzymes, antibiotics and are often used as bio-control agents. These species are important in humic acid synthesis, stimulation of plant growth, fungal community structure regulation and produce degrading xenobiotics. Several *Trichoderma* spp. can either be mycoparasite colonizers of other fungi or occur directly on the host plant (Kulling *et al.,* 2000). They occur in soil worldwide, in different ecosystems and over wide ranges of climatic conditions in the soil and on aerial parts of plants (Cordier *et al.,* 2007). Plant pathogenic fungi may be inhibited by *Trichoderma* spp. through inducing resistance and causing plant defence reactions, or by antibiosis and direct confrontation as myco-parasites (Anees *et al.,* 2010). *Trichoderma* spp. are known as secondary invaders in maize, although it has been reported as pathogens on the seedlings and stalks (Lipps & Deep, 1991). There is also a definite interaction between some *Trichoderma* and *Fusarium* species on maize plants (Lipps & Deep, 1991).

M. phaseolina has been reported to infect 500 crop plants worldwide as an opportunistic soilborne fungal pathogen (Babu *et al.,* 2011). *M. phaseolina* causes seedling blight, charcoal rot, and root and stem rot (Francl, 1998). It is said to be a seed, soil and stubble borne fungus, with evidence that it is primarily root inhibiting and produces black sclerotia of 1-8 mm in diameter (Khan, 2007). *M. phaseolina* can survive for extended periods under dry soil conditions (more than 10 months). According to Khan (2007), the disease severity is related directly to the population of the viable sclerotia in the soil, while the mycelium is not seen as a major inoculum source. The fibro vascular system of roots and basal internodes are affected by the pathogen which impedes nutrient and water transport to upper plant parts. Before the plant is invaded, the pathogen's fitness depends on its ability to survive in the soil, competing with other micro-organisms, utilising organic material and colonising the root rhizosphere of the host plant. In South Africa there has been an increase

in reports of maize infected by *M. phaseolina* in drought stricken parts and it thus an important pathogen in dry season (Craven, 2016).

Pythium spp. occur worldwide in surface water and in the soil where they live saprophytically on dead plant and animal materials or parasitically on plant roots. Almost all plants are susceptible to *Pythium* root rot. Free water is required for the zoospores of the pathogen to spread (swim) and infect plant seeds and young seedlings. The roots (important for nutrient and water uptake) are infected and killed first, leading to stunted, yellow, wilted plants. The root tips turn brown and die as the microscopic thick walled spores colonise the root cells (Agrios, 2005).

1.2.2 Environmental component

The environment plays an important role where biotic and abiotic factors greatly affect the outbreak and occurrence of diseases. About 40 percent of arable land in the world has unpredictable and low rainfall, 60 percent of this land is situated in developing countries (Govaerts *et al.,* 2007) and South Africa can also be classified with such conditions. The soil in North West and Free State Province are mostly shallow sandy soils, making production practices very important to maintain top soil and soil moisture which is usually lost by erosion and runoff water (Smit, 1998). Most of the South African soils have the characteristic of being very vulnerable to degradation and have a low recovery potential, meaning any small land management error has the chance to be devastating with little chance of complete recovery (Goldblatt, 2010). In South Africa 91% of arable land is under dryland production and dryland fields are susceptible to soil degradation. The most severe degradation in commercial farming areas occurs in the Western and Northern Cape provinces, due to wind and water erosion (Department of Environmental Affairs, 2007). Cook and Papendick (1972) found in their study that most soil borne pathogens survive these conditions and infect plants in the upper 24 centimetre of soil (tillage layer), where moisture stress also commonly occurs. *Fusarium* crown and root rots can cause severe plant death and yield losses, but are often more acute in dry soils when the crops are already stressed because of the low soil moisture (Cook & Papendick, 1972).

Environmental conditions (geographic location, temperature, rainfall) create suitable or non-suitable conditions for the crops as well as the pathogens. Diseases are commonly known to appear and develop in warm, wet conditions and heavy nitrogen fertilization causes plants to be more readily attacked by some pathogens such as *Rhizoctonia* spring blight on winter wheat (Huber & Haneklaus, 2007). Thus, the abiotic environmental factors that have the most severe influence on disease occurrence and development are temperature and moisture on the plant surface, with soil nutrients, light and soil pH being important for plant health (Agrios, 2005).

Dominant fungi that attack maize differ across soil types and across different bio-climatic conditions (Lamprecht *et al*., 2008). Du Toit (1968) and Chambers (1987) noted that maize root rot is more severe when the plant experiences times of stress, especially drought conditions and where excessive soil water occurs for longer periods of time.

Different pathogens have different temperature preferences. The temperature determines the formation of spores in a unit plant area and the time it takes for the spores to be released (reproduction) (Agrios, 2005). The change in climate can potentially alter the host plant's physiology and resistance and influence the developmental stages of the pathogens. Diseases can be presented by a cycle that consists out of the following: inoculum survival, infection, latency period, reproduction and dispersal, where each is greatly influenced by environmental conditions. Pathogens produce new inoculum after infection which can be dispersed to other sites causing new infections. When one infection cycle occurs per crop season it is referred to as monocyclic pathogens and more than one infection cycle per crop season is caused by polycyclic pathogens (APS, 2017). It is also important to consider some pathogens inoculum build up over seasons, which is referred to as polyetic diseases. Most soil borne pathogens causing root rots, vascular wilts and other diseases are monocyclic, but generally have survival structures (sclerotia, chlamydospores and oospores) in the soil or mycelium found in crop residues also making it polyetic. The inoculum of these monocyclic pathogens are dispersed by cultivation and the newly produced inoculum will only be dispersed when the soil is cultivated again, for the next crop season, showing only one infection cycle per season (APS, 2017).

Moisture in the form of rain, irrigation water, relative humidity and dew are necessary for fungal germination, spore formation, longevity, activation, spread and penetration of the host plant. Moisture also makes the plant more succulent and affects the suitability of it to the pathogens which will influence the disease severity (Agrios, 2005). Areas in the North West and Free State province that produce 75% of the country's maize, consisting of mostly sandy soils are included in the estimated 25% of soils that are highly susceptible to wind erosion (Goldblatt, 2010). The pH of the soil can influence the pathogen's life cycle and the disease cycle and make the host plant more susceptible by altering the nutritional status of the plant and by restricting nutrient absorption. Contradictory information exist on water stress, where Cook (1973) found that many plant pathogens are stimulated rather than inhibited when low osmotic water potentials occur, but Summer (1968), found that the root weight is significantly more in low moisture treatments (root weight as parameter of root-rot), meaning less root rot when dry conditions occur. In dryer conditions enough air and unsaturated pores occur for better root development compared to oversaturated and wet soils (Agrios, 2005). In extreme dry conditions the roots' development will again be restricted as no available moisture would be acquired for nutrient and mineral uptake

and normal root growth. In saturated soils the root systems were severely decayed and discoloured indicating favourable conditons for fungal development leading to some rots. According to Summer & Bell (1986) dry land maize showed less root diseases than irrigated maize fields.

Nutrients in the soil are important for optimal plant growth (harvest) and defence methods against pathogens. Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) are macro-elements while iron (Fe), boron (B), copper (Cu), manganese (Mn), zinc (Zn), molybdenum (Mo) and silicon (Si) are micro-nutrients essential for plant health. The nutrients function together as a delicate balanced interdependent system with the environment and plant genetics and this balance is needed to occur for optimum plant response and resistance mechanisms to occur (Huber & Haneklaus, 2007). The plant incorporates these elements for defence methods and from various studies *Fusarium* spp. and the diseases inflicted by this pathogen increases when too much ammonium fertilizer is used (Huber & Haneklaus, 2007). The amount and the form of N as well as the other elements are essential for sufficient uptake by the plants. Stalk and root rot of maize may increase with excess N as the plant's resistance decreases because the pathogen activity can be enhanced (Graham & Webb, 1991) or physiological sufficiency of other nutrients may be imbalanced (Warren *et al.,* 1980). Calcium can reduce diseases caused by *Rhizoctonia, Pythium myriotylum, F. solani* and *F. oxysporum* as it effects cell wall composition and reduces penetration by these pathogens (Bateman & Basham, 1976, Kelman *et al.,* 1989). As long as Ca levels remain sufficient, Mg will also be important for structural integrity of cell components (both required for effective structural integrity and good plant health) and can reduce plant susceptibility to macerating enzymes produced by these above mentioned pathogens (Csinos & Bell, 1989). Mg contributes to healthy maize plant development and the maturation of the plant, while Ca is essential for healthy foliage and cob quality. Healthy plants are less susceptible to root and crown rot pathogens (Csinos & Bell, 1989).

Some of the fungal species studied have previously recorded environmental preferences (abiotic conditions): *F. oxysporum* occurs more commonly in temperate areas and in improved pasture soils (Summerell *et al.,* 2011). *F. verticillioides* grows optimally in dry, hot climatic conditions (Summerell *et al.,* 2011) that correspond to the climate experienced in the North Western parts of South Africa, while *F. graminearum* growth in South Africa is also favoured by warm weather (25˚C to 28˚C) and high humidity, usually occurring in irrigated fields (Greyling & Flett, 2013). *M. phaseolina* occurs readily under stress conditions like drought and high temperatures (Smit, 1998). Where soil moisture is high (water-logged) the host plant's ability to defend itself against the pathogen is reduced due to the reduction in available oxygen and lowered soil temperature (Agrios, 2005). *Pythium* spp, mostly affects maize seed and seedlings and its presence is

enhanced through long periods of soil saturation, unfavourable low temperatures for the host plant, when mono-cropping occurs and when too much nitrogen is present in the soil (decreased host plant resistance due to nutrient imbalances and enhanced pathogen activity), (Graham & Webb, 1991). *R. solani* causes the most damage and symptoms occur on their host plant when the soil is wet but not flooded (65% even soil saturation) and at temperatures ranging from 12°C to 32°C (Agrios, 2005). *E. pedicellatum* reduces or occurs less under stress conditions like drought and is more prevalent when optimum plant growth conditions occur (Smit, 1998). It is evident that different species will have different climatic condition preferences and the cultivar of the host plant will determine the relative humidity, soil moisture and temperature requirements for optimal growth of the plant and determine when it becomes more susceptible to pathogen species (Khan, 2007).

1.2.3 Host plant component

Cultivar selection has many considerations in terms of resistance, planting time and the region where maize is planted which directly affects the fungus and diseases (root and crown rot). Every farmer's primary purpose is to get the highest possible yield with selected maize cultivars. The cultivars are grouped into regions due to temperature differences and rainfall across the country and choices should be made accordingly for the best possible yield. Alternating crops and cultivars can secure biodiversity and delay possible disease resistance. Cultivars with resistance to insects and other stress conditions directly affect the growth and regulation of the maize, and determines to which extent the plant is vulnerable to diseases, thus a well-planned and researched cultivar selection is required each year/season by farmers as it greatly contributes to reducing and minimizing the risk of diseases. No maize cultivars with resistance to root and crown rot caused by this complex of fungal species are available on the market, because it has not been screened yet. Seed treatments are preferred because of the root and crown rot complexity.

1.2.4 Other factors

1.2.4.1 Conventional tillage

Conventional tillage is defined as a tillage system that uses cultivation (soil tillage, ploughing, harrowing and removing plant residue) as a major means to prepare the seedbed and for weed control to achieve optimum crop growth (OECD, 2001). It is known that this practice leaves less than 15% crop residue on the soil surface before the next crop is planted (EA, 2003). According to Bennie & Botha (1986) and Memon *et al.* (2013), deep tillage practices are used to improve the soil's physical properties, soil aeration and water infiltration as it breaks the hard pans. It also enhances the root growth and improves nutrient availability by the acceleration of mineralization

processes ultimately leading to better plant growth and higher crop yields. Tillage as a crop production factor influences or contributes up to 20% towards production. In double cropping systems, tillage should be reduced, because the time for seedbed preparation is short and production costs can be minimized (Ehsanullah *et al.,* 2015). Deep tillage and crop rotation with deep-rooted crops can be used to reverse sub-soil compaction (Motavalli *et al.,* 2003). Although all the trends are moving to less tillage some may still use it as it results in the highest yields in the short run. With regard to maize diseases and yield reduction, root and crown rot often occurs when the root growth of the maize is restricted by soil compaction. *Fusarium* spp. in maize sub-crown mesocotyls and roots thus tend to be more abundant in no-till systems than ploughed fields (Lipps & Deep, 1991).

1.2.4.2 Conservation agriculture

Conservation agriculture (CA) is defined as management principles that result in more sustainable agricultural production, less production costs and increased profitability (FAO, 2010). CA aims to minimize soil and water loss by having at least 30% crop residues present on the field through the year. CA has three central themes: 1) systematic crop rotation, 2) permanent soil cover by crop residues, 3) minimum tillage (zero tillage) that forms the basis of its advantages (Rusinamhodzi, 2015). No-till and crop residue conservation can improve water infiltration, conserve soil moisture, reduce soil erosion, enhance soil structure, result in higher organic matter and carbon content in the soil and more stable soil temperatures will occur (Ceja-Navarro *et al.,* 2010). It also results in lower production costs (less fertilizer, fuel and water requirements) compared to the removal of crop residue and conventional tillage (Ceja-Navarro *et al.,* 2010). CA optimizes the use of the seasonal cropping window by making earlier field entry possible (Hobbs *et al.,* 2008). Limitation of the tillage practices will influence the soil borne pathogens in various ways, taking into account their survival strategies (Craven *et al.,* 2016). The pathogens with survival structures and which remain viable in the soil and on plant residue are most affected by the different tillage practices (Govaerts *et al.,* 2007). Reduced tillage practices will lead to more fungi and micro-organisms in the soil because more plant residues are present in the upper soil layers (Brussaard *et al*., 2007). Summer *et al.* (1981) recorded decreasing incidence of soil borne diseases where residues were ploughed in. *Fusarium* spp. causes more severe damage if the soil is compacted and if sub-surface tillage pans cause restricted root growth (Agrios, 2005). Herman (1984) also reported that *Fusarium* spp. were more common in unploughed soil. *R. solani* were also more commonly found in cereals grown where conservation tillage was implemented (Weller *et al.,* 1986).

1.2.4.3 Mono-cropping systems

Mono-cropping is the continual planting of the same crop over several seasons and has more disadvantages than advantages (Sithole *et al.,* 2016). Planting monoculture results in increased inoculum build-up of soil borne pathogens causing many diseases (Boosalis & Doupnik, 1979) and can potentially be the reason for major yield decreases, especially on grain crops (Smit *et al.,* 1997). Monoculture maize depletes nutrients more readily as the roots inhabit the same zone in the soil continuously (decrease root development). One example is *Pythium* spp. that causes root rot and are considered important as it lowers maize yields in soils where continuous mono-cropping occurs (White, 1999).

1.2.4.4 Crop rotation

Crop rotation as cultivation practice has many advantages, but also many considerations and implications. The major benefits are known to be the reduction in plant diseases with specific reference to those caused by soil borne pathogens and the opportunity to increase soil fertility through the crops chosen for rotation (e.g. legume crops) (Hobbs *et al.,* 2008). The aim, with regard to plant diseases, of crop rotation is to improve natural mortality through the reduction of suitable host tissue for the pathogens (Coetzee, 2015). The choice of crops used is the most important part as the crop will determine the host range of the pathogen complex. Crop rotation is effective for some pathogen species in reducing the increase of their population levels, but is less effective when high pathogen population densities have already been established. The survival of soil borne pathogens range from mycelium that can be dormant to specialized structures (e.g. sclerotia of *Sclerotinia sclerotiorum*) with varying time periods (Coetzee, 2015). The periods or seasons of rotation are thus complex and depends on the pathogens present. Crop rotation also leads to a greater variation in the number of fungal species due to the variety of available organic material for decomposition. Thus, host plant preference and crop rotation may not be as useful to reduce inoculum potential for fungi that occur over wide host and environmental ranges as adaption and establishment occur readily (Summer & Bell, 1986). Smit *et al.* (1997) stated that the influence of rotation systems on fungal pathogens that are isolated from maize roots is complex as no cropping system showed preference towards all fungi or single fungi. The different fungi are affected differently by the various cropping systems. Crop rotation will only have an effect on the fungus population over an extended period (long-term) of application. Economically, crop rotations need to be evaluated in terms of the value of the rotation crop compared to the primary crop and the period for which the rotation will occur so as to benefit the farmer in the present and future (Coetzee, 2015). Lastly crop rotation can include beneficial allelopathy and biocontrol agents through the production of siderophores (Fe^{3+} binding) and

antibiotic agents, nutrient competition, niche exclusion and through systemic acquired resistance (Coetzee, 2015).

M. phaseolina has a wide host range that suggests that it is a non-host specific fungus (Khan, 2007). *M. phaseolina* showed the highest number of isolations from a maize-sunflower-maize rotation compared to rotations with soybean and groundnuts (Smit *et al.,* 1997). *F. equiseti*, *F. oxysporum* and *Pythium* diseases were more abundant in monoculture maize (Smit *et al.,* 1997).

1.3 Management strategies to minimize maize rots

To manage diseases one or more of the three components of the disease triangle (Agrios, 2005) (virulent pathogen, susceptible host, and favourable environment) should be manipulated and used in combination with other strategies (Foround *et al.,* 2014). An example of such a combination would be host resistance, fungicides and crop rotation to reduce disease development and the inoculum levels in the field (Agrios, 2005). General management strategies can be to reduce the stress on the maize plants also taking into consideration the life cycle, host preference and performance under certain environmental conditions. Constant changes of maize hybrids' genes, cultural practices, climate, pathogen population evolvement and introduction of new pathogens makes disease specific and combination management essential, while constantly adapting to these changes. When environmental conditions are ideal for disease development, the variety of resistance, rotation and inoculum levels tend to be more critical than the tillage practice applied (Lipps & Deep, 1991).

1.3.1 Chemical control

Effectively controlling and managing maize rots is difficult due to the wide spectra of fungi associated and its variability (between locations, soil types, climates etc.). Chemical control seems to be the easiest way out, but it is usually not economically justifiable and precise enough for certain fungi. Chemical control (sprays, dust, seed-coatings) is most effective for controlling many fungal diseases. Nematodes that often aggravate root diseases by physical damage to plant roots by using thestylet to pierce the maize plant and to acquire nutrients and via this tissue damage secondary fungal infection can take place (Niblack, 2003). The use of nematicides for nematode control is mostly limited by environmental or cost concerns (White, 1999). Integrated management is required to effectively control fungal pathogens, insect pests and weeds that affect the host crops. (Agrios, 2005). Seed treatments can improve plant emergence, prevent the transmission of seedborne pathogens, it can protect above ground plant parts (systemic action against infection by airborne pathogens, feeding insect pests and vectors of diseases), improves plant vigor and uniform crop growth and it fulfils the phytosanitary requirements for prevention of

pathogen spread (Munkvold *et al.,* 2014). Maize seed treatments are almost universally standard including combinations of an insecticide, a nematicide and four fungicides that give good protection across wide pathogen spectrums and prevents damage to seed and seedlings by feeding pests (Munkvold *et al.,* 2014). The use of herbicides and pesticides give the plant opportunity to grow optimally and defend itself against possible diseases like rots. Herbicides and pesticides have various negative effects on the environment and even compromise human health with known chronic effects like cancer, respiratory diseases, neurological effects, diabetes, genetic disorders and fetal diseases (Andersson *et al.,* 2014), thus trends to decrease the use of these chemicals are common. Current implementation of no-till practices on the contrary promotes herbicide application again as more weeds occur with less cultivation practices applied, having important impacts on the presence and abundance of root and crown rot pathogens (Smit, 1998).GM (Genetically Modified) maize can have glyphosate and glufosinate-ammonium herbicide tolerance which reduces chemical control and Bt-maize can be planted that limit chemical pest control (Phipps & Park, 2002). GM maize also showed that Bt-maize (containing the insecticidal protein for the control of the maize stalk borer) had significantly less *Fusarium* symptoms than other non-Bt-maize hybrids (Flett & Ncube, 2015).

Registered fungicides in South Africa on maize include Artea and Score (Syngenta), Acanto Plus 280 SC (Du Pont), Nativo (Bayer), Abacus (SASF), Aroxy 250 SC, Azur Top 325 SC, Captan FS, Colloso, Defender 250 EC and Solo (Arysta), most of the fungicides being systemic. These fungicides are not specific for the fungus focused on and are more readily used for wilts and blight diseases. The seeds can be coated with systemic fungicides to protect maize seedlings, but it works out quickly (three to maximum four weeks) as the plants grow (White, 1999).

1.3.2 Biological control

Biological control per definition is the control of a pest or disease by introducing or maintaining natural enemies or predators. In terms of diseases and especially root and crown rot biological control can be applied through fungi (applied additionally or sometimes already present) that enhance plant growth and development, increases crop productivity, gives rise to resistance to abiotic stresses and helps in nutrient use and uptake through the roots, thus making the plant less susceptible to any other pathogen that might cause disease (Eilenberg *et al.*, 2001). No bio-control agents, except *Trichoderma* spp.*,* are currently available for farmers, but potential micro-organisms, nematodes and fungi already exist and are found commonly in the soil that can be used to reduce disease severity and soil borne pathogen presence (Agrios, 2005). *Trichoderma* spp. compete for space and nutrients with other soil micro-organisms and fungi (Harman *et al.,* 2004). *Trichoderma* spp. also showed promise to reduce plant parasitic nematodes, limiting

secondary infections when less damage is inflicted with the lower nematode numbers (Windham *et al.,* 1989). Some *Trichoderma* spp. can control *Fusarium* spp.*, Pythium* spp. and *R. solani* induced diseases (Cordier *et al.,* 2007). *Phoma* spp*, T. koningii, A. alternata, Beauveria bassiana* and *Acremonium strictum* endophyte isolates from maize roots shows great benefits to host plants as promoter of health, improving potential growth and acting as bio-control agent of which *Trichoderma koningii* and *Alternaria alternata* have the most potential (Sinha *et al*., 2012). Another example is the endophytic bacterium, *Bacillus subtilis* that occupy the same ecological niche within the maize plant as *F. verticillioides* and can reduce mycotoxin accumulation and rots during the vertical transmission growth phase, acting as a competitive exclusion principle (Bacon *et al.,* 2001). *C. eragostidis* can also be used as a mycoherbicide, being able to control crabgrass for example (Jiang *et al.,* 2008).

1.3.3 Cultural control

Cultural control measures in general include planting crop (maize) hybrids with good resistance or tolerance (no known resistant maize cultivars available for root rots), planting pathogen-free seed, destroying plant parts/residue that harbour pathogens, destroying alternative host plants in the surrounding area, the use of clean equipment and tools and making sure there is good drainage in the fields and aeration for the plants (Oerke & Dehne, 2004). To manage insect pests and implement good weed control, planting at appropriate plant densities and having balanced nitrogen and potassium fertilizer application programs in place are also part of good cultural control strategies (Oerke & Dehne, 2004).

Cultivation practices like crop rotation and tillage (categorized as mechanical control, together with howing) can be used accordingly (OMAFRA, 2009). Tillage has little effect on *Rhizoctonia* rots, but the disease tends to be more in irrigated, well managed maize fields (White, 1999). *F. graminearum* can be managed through crop rotation once established whether organic matter and other crop residues are utilised for overwintering (survival) by the pathogen. Removing stubble will lower the disease incidence in the following planting season. Crop residue can be chopped into smaller pieces to expose bigger surfaces for microbial activity leading to higher decomposition rates of crop residue (Sims & Frederick, 1970). CA practices which retain crop residue enhances soil fauna and flora, also leading to enhanced residue decomposition (Chan & Heenan, 2006).

The nutrients in the soil (environmental part) influences microbial growth, plant growth and pathogen presence, adequate nutrition will lead to high disease tolerance. It is thus an important disease control factor as nutrient balances need to be established for optimum growth responses (Huber & Haneklaus, 2007). Calcium and magnesium function in balance with each other and

increase structural integrity, cell wall components, membranes and resistance of the middle lamella of plant cells which suppresses macerating diseases caused by various pathogens (*P. myriotylum* and *R. solani*) (Bateman & Basham, 1976). Silicone together with other components gives rise to cells with thicker cell walls and is important for potassium availability and magnesium mobility. Nitrogen and sulphur can change the biotic and abiotic environment in the soil, which leads to specific nutrient uptake and enhance genetic resistance (Huber & Haneklaus, 2007). An excess of nitrogen may lower natural disease resistance specifically to stalk rots in maize, because other nutrient imbalances will occur, and pathogen activity can be enhanced (Huber & Haneklaus, 2007). Creating the best environment for optimum plant growth by means of fertilization for example (minimize stress conditions) will also aid as it makes the host less susceptible.

In terms of GM crops, research showed that the resistance to different diseases may show association with each other (Mesterhazy & Kovacs, 1998) and future research development for GM crops should include the possible interactions between the pathogens causing the same and different diseases. The use of insecticides or Bt-maize to timely control insects can help to reduce *F. verticillioides* infection and root rot. *Pythium* spp. has no commercial resistant plant varieties yet, but can be controlled using good drainage measures, intercropping and crop rotations (Agrios, 2005). *Pythium* spp. can also be managed with effective seed treatments (from the same or different chemical group). *Rhizoctonia* diseases are difficult to control, avoiding poorly drained, wet areas and allowing enough space between plants for good aeration can slightly aid in management. Crop rotation of three years with less suitable hosts may reduce the build-up of the pathogen. Pathogens, especially *Phytophthora* and *Pythium* species, easily spread through irrigation water and where maize is planted under irrigation, it is essential to make sure clean water is used (Perry, 2006).

1.4 Techniques for evaluation of soil borne diseases

Plating on different agar variations were regularly used for the evaluation of soil borne pathogens. This technique is very time consuming, limits execution to seasonal availability, makes it difficult to include variables and leaves space for human error in the identification and quantification of soil borne pathogens, ultimately leading to slow progress in knowledge gained for soil borne diseases. Molecular techniques like conventional Polymerase Chain Reaction (PCR) use agarose gels or other post-PCR detection methods which also lacks some precision (Nolan *et al.,* 2013). Real-Time PCR / Quantitative (qPCR) is now an easier more precise method that can be used for DNA and RNA quantification. With traditional PCR the end-point (plateau) from the PCR phases is used for data collection, while qPCR use the exponential growth phase. More

advantages of qPCR include an increase in reseptor fluorescent signal which is directly proportional to the number of generated amplicons, the cleaved probe provides a permanent record amplification of the amplicon (with probe-based qPCR), the range of detection is increased and no post-PCR processing is required (Nolan *et al.,* 2013).

1.5 Conclusion

In a world and country with growing population levels, food demands, degrading land and challenging environmental conditions, understanding soil borne fungal pathogens on maize is important. The control of these root, crown and stem diseases in maize are difficult due to these diseases being caused by more than one fungal pathogen. Each pathogen can differently interact with other fungal pathogens with the environment and the host plant. The fungal pathogen complex changes over time (during and over seasons) and the severity and presence of diseases and pathogens are influenced by various variables (such as climatic conditions, farming practices, soil types, cultivar susceptibility). Evaluating the complex changes under different environmental conditions and knowing the distribution of these fungal pathogens in South Africa together with tissue specificity will aid in achieving clarity on how to better manage soil borne diseases in maize.

1.6 Aim and objectives of this study

1.6.1 General aim

The aim of this study is to use molecular techniques with improved identification and quantification ability to research different factors contributing to root and crown rot severity of maize in South Africa.

1.6.2 Specific objectives

The specific objectives were to:

- determine the effect of different cultivation practices on twelve commonly known root and crown rot fungal pathogens of maize in South Africa
- determine tissue specificity between roots and crowns for the twelve fungal pathogens
- determine the dominance and cultivation preferences of the twelve fungal pathogens in Gauteng, North West, Free State, KwaZulu-Natal, Mpumalanga and the Northern Cape
- evaluate the succession of the fungal pathogen complex for the first time during a maize growing season
- to understand the influence of different localities and cultivars on the fungal complex and disease symptom development

The results of the study are presented in three chapters with the following titles:

- Chapter 2: The effect of tillage, no-till and crop rotation on the composition of maize root- and crown rot fungi
- Chapter 3: Interaction of farming practices and the composition of the root and crown rot complex in different provinces in South Africa using qPCR
- Chapter 4: Evaluation of the succession of maize soil borne fungal complex causing root and crown rot using different cultivars

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CHAPTER 2

THE EFFECT OF TILLAGE, NO-TILL AND CROP ROTATION ON THE COMPOSITION OF MAIZE ROOT- AND CROWN ROT FUNGI

2.1 Abstract

Maize serves as a staple food source and helps generate financial income in South Africa. Root and crown rot diseases threaten optimum maize production and food security as yield losses of up to 1.8 tons/ha for every 25% disease severity have been observed (Nel & Lamprecht, 2011). The diseases in the fungal pathogen complex are poorly understood due to these diseases being influenced by crop precursors, climate, soil conditions, cultivation practices, maize plant resistance and the plant growth stage. In a country with risk factors such as limited arable land, soil degradation and drought many farmers are implementing conservation agriculture (CA) that includes crop rotation and limited tillage. In the past the influences of these practices on root and crown rot diseases were difficult to study but with qPCR identification and quantification of fungi might be more feasible. In this study the root and crown rot fungal pathogen complex consisting of the 12 most commonly occurring pathogens, were analysed under conventional and various CA practices. Trials were conducted in the Free State province (Kroonstad) during the 2015 and 2016 seasons, plant biomass and the root and crown disease severity were determined. qPCR was performed using hydrolysis probe or SYBR Green protocols (technology) to quantify the target DNA for 12 fungal pathogens. Statistical analysis was performed using ANOVA, Levene's and the Shapiro-Wilk test. The plant biomass and disease severity results showed significant differences in the conventional CA practices in the different seasons. The qPCR analyses for the conventional practices showed that *Phoma* spp., *Pythium* spp., *F. oxysporum* and *F. chlamydosporum* were the most prominent of the fungi tested. In the CA treatments *Phoma* spp., *F. chlamydosporum, Pythium* spp. and *F. oxysporum* were the most prominent of the fungi tested. In this study multiple factors influencing the root and crown rot complex were studied. Season, tillage and plant part played a significant part in the infection and occurrence of the different fungal species in the soil borne diseases. The importance of gathering multiple seasons' data was highlighted and this will hopefully give better insight into the effect of cultivation practice and crop rotation on soilborne pathogens and their resultant impact on root and crown rot development.

Key words: Conservation agriculture (CA), crop rotation, cultivation practices, disease, fungal pathogens, hydrolysis probes, root and crown rot, SYBR Green

2.2 Introduction

Maize is the third most important grain crop worldwide and is consumed as a staple food source with high energy properties in South Africa (Dwivedi *et al*., 2015). Maize yields decline because of various climatic conditions and stress factors which include drought, pests, diseases and low soil fertility (Cairns *et al.,* 2013). Diseases such as root and crown rot and their impact on maize yield are not well understood. It is important to note that root rots occur on every maize plant every season (White, 1999). Every fungus differs in virulence in root rots. The soil borne fungal pathogens causing root and crown rot vary in the ability to reduce the growth and survival of the plants (White, 1999). The fungal pathogens that cause evident reduction in plant growth and health because of the rots will lead to yield loss and have economical implications. Root rots are a disease complex that involves more than one fungus occurring on the roots that will be different at certain host growth stages, under different environmental conditions, genotypes and because of previous crops planted (Wise *et al.,* 2016). In SA the most commonly occurring fungal pathogens on maize are: *Curvularia eragostidis*, *Exserohilum pedicellatum*, *Fusarium chlamydosporum*, *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Macrophomina phaseolina, Pythium* species, *Phoma* species, *Rhizoctonia solani* and *Trichoderma* species. Research to prevent yield loss due to these diseases is becoming more essential as food demands rise for the ever-growing human population.

Different cultivation practices are being applied during maize production. Conventional tillage practices include all varieties of ploughing which influences the soil environment. Tillage in general is ploughing or harrowing land to cultivate crops, where no-till is when crops are planted directly in the soil without ploughing, usually used in association with herbicides as weed control (Sithole *et al.,* 2016). Conservation Agriculture (CA) is more readily used today as it counteracts yield loss through soil management, soil health maintenance and effective use of economic and natural resources. CA practices like no-till or reduced tillage, crop rotation and retaining crop residue are proposed as better methods to produce crops as it improves the soil moisture and nutrients in the soil (Ceja-Navarro *et al*., 2010). Crop rotation is defined as a system that varies successive crops in a certain order on the same land, mostly to prevent depletion of soil nutrients and for weed, pest and disease control (Shaalan *et al.,* 2014).

Roughly 600 million hectares (40%) of the world's cropland experience unpredictable and low rainfall, developing countries making up 60% of the 600 million hectares (Govaerts *et al.,* 2007). In South Africa where approximately 90% of maize is being cultivated in the Highveld region regular drought conditions occur because of varying and unpredictable seasonal rainfall (du Toit *et al.*, 2000). In addition, South Africa also has high temperatures and shallow soils, stressing the

importance of preserving soil moisture and topsoil for cultivation, hence the use of CA practices (Smit, 1998). Each cultivation practice can increase or decrease root and crown rot diseases in maize. Govaerts *et al*. (2006) observed that with monoculture planting higher levels of maize root rot occur in no-till (NT) systems compared to conventional tillage. The effect of crop rotation, soil borne fungi and diseases are complex, the results vary and sometimes contradict each other.

Traditionally root and crown rot diseases have been analysed by plating out on agar media (Pasche *et al*., 2013). The use of qPCR offers an alternative method for more effective, time efficient and accurate data generation to better understand these diseases under different cultivation practices (Pasche *et al*., 2013). The aim of this study was to investigate the effects of till, no-till and crop rotation on the twelve commonly occurring fungal isolates that cause root and crown rot of maize in South Africa.

2.3 Materials and methods

2.3.1 Locality and plots sampled

Two field trials were planted in the Free State province (Kroonstad, Springboklaagte, 27° 42' 47'' S 26° 59' 38'' E) for two seasons (2015 and 2016). Trial 1 consisted of monoculture maize with till (rip on row) vs. no-till. Trial 2 was crop rotation (maize/ soybean, maize/ sorghum) with till vs. no-till. Trial 1 was planted with four replicates on eight different plots (two plots per replicate) while trial 2 contained two replicates on eight different plots (four plots per replicate). The maize cultivar DKC 7845 Bt was used at both field trails. No-till has been implemented since the 2012-2013 season on the selected trial areas. Each plot had five 100 m rows, with 1.2 m spacing between the rows and included a border row on each side. The row next to the border row was allocated for destructive sampling. Soil analysis were done and the fertilizer applied to trial 1 included: Urea (46%) before plant (application followed by rod weeder with spikes), 15:10:6 (31) +0.5% Zink (Zn) +4% Sulphur (S) +0.15% Boron (B) with planting and Urea (46%) four weeks after emergence (application combined with mechanical weeding). The fertilizer application for trial 2 was: Urea (46%) before plant (application followed by rod weeder with spikes), 15:10:6 (31) +0.5%Zn+4%S+0.15%B with planting and Urea (46%) four weeks after emergence (application combined with mechanical weeding). The soybean and sorghum only had 15:10:6 (31) +0.5%Zn+4%S+0.15%B applied with planting. The trial plots were dependent on seasonal rain.

2.3.2 Sampling and biomass of plant material

Sampling of root and plant material was done at approximately 100 days after planting (DAP). Fifteen random plants were taken from allocated rows within each plot, roughly implying that every twentieth plant was sampled in the first season (2015). Thirty random plants were taken from

allocated rows within each plot, roughly implying that every tenth plant was sampled in the second season (2016). The mass of the belowground material (roots and crowns still attached) was determined for the plants sampled within each plot and the plant biomass subsequently expressed as kg.plant^{-1}. The roots were washed under running water and the disease symptoms for the roots and crowns were visually rated.

2.3.3 Disease ratings

Disease incidence was established using the percentage of plants sampled for each treatment and replicates that demonstrated visual discolouration (some degree of rot) for the roots and crowns. The root and crown rot severity ratings were done seperately in percentage from the plants sampled, by using the root and crown disease index (RDI, CDI) that is based on the adjusted scale ranging from 0-5 (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = <25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, and 5 = dead or totally wilted) The disease severity was calculated as the product of disease incidence x RDI or CDI (Soonthornpoct *et al*., 2001).

2.3.4 Fungal pathogen complex evaluation

2.3.4.1 DNA extraction

After visual screenings were done of the washed roots and crowns, representative samples of diseased roots and crown material were put together from each set of 15 plants sampled for the first season and 30 plants sampled for the second season. The samples were cut into smaller pieces and stored at -80˚C until DNA exctractions could be done. The samples were crushed using liquid nitrogen in a mortar and pestle. DNA was extracted using the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Möller *et al*, 1992). Approximately 0.25 ml ground material was added to 2-ml centrifuge tubes (Eppendorf, Hamburg, Germany) together with 1 ml DNA extraction buffer (DEB: 0.2 M Tris HCl (Tris(hydroxymethyl)aminomethane hydrochloride), 0.15 M NaCl (sodium chloride), 0.025 M EDTA (ethylenediamine tetra-acetic acid), 0.5% SDS (sodium dodecyl sulfate). The DEB-plant material mixture was frozen in the -80° C freezer for 1 hour, followed by heating in boiling water for 5 minutes. In the following step, 600 µl phenol: chloroform: isoamylalcohol (IAA) (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes. The top aqueous layer was removed and added into a new tube. In the following step 200 µl 2xCTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris pH 8, 20 mM EDTA, 0.2% β-mercaptoethanol pH 8.0) and 400 µl chloroform:IAA (24:1) were added to the tube and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes, the top aqueous layer removed and added to a new tube. Immediately after, 60 µl 3M Sodium acetate and 800 µl 100% ice cold ethanol were added

to the supernatant, inverted and centrifuged for 10 minutes at 14 000 rpm. The supernatant was then discarded and 500 µl of 70% ethanol added to wash the DNA pellet. The samples were then centrifuged for 5 minutes at 14 000 rpm. The pellet was left to dry in the laminar flow (1 hour) and resuspended in 50 µl Low TE buffer [10 mM Tris pH 8 (T8443), 1 mM EDTA] (AppliChem, Germany). The samples were then stored at -80°C until the DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and the quality determined with the A_{260}/A_{280} ratio.

2.3.4.2 Quantification of fungal species

The DNA samples were diluted to 10 ng/µl for further use with water. qPCR was performed with a Bio-Rad CFX 96 thermal cycler (United States, Hercules, USA) using hydrolysis probes (Taqman® probe) and/or SYBR Green protocols and the fungal content was quantified as pg/µl for the following species: *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. verticillioides*, *M. phaseolina, Pythium* spp., *Phoma* spp., *R. solani* and *Trichoderma* spp. with the primer sets and protocols used (Table 2.1). The primers and probes used were designed to target the translocation elongation factor 1α (*TEF*1) gene of *F. equiseti*, *F. graminearum*, *F. verticillioides*, *F. oxypsorum* and *T. longibrachiatum*. The internal transcribed spacer region (ITS) of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *M. phaseolina*, *P. sorghina*, *P. periilum* and *R. solani* was used for primer and probe design. (Schoeman, 2016). The amplification product of SYBR Green and Hydrolysis probe protocols were sequenced (Schoeman, 2016) in order to confirm the specificity of the amplified product (amplicon) and to ensure that the correct fungal species are identified.

2.3.4.2.1 SYBR Green protocol

Quantification was carried out using SYBR Green in 25 µl reaction volumes containing 10 µl Bio-Rad iTaq Universal SYBR Green supermix (Hercules, USA), 0.4 µM of the forward and reverse primers, 0.8 ng/µl DNA and RNase/ DNase free water (Biolab Diagnostics, Wadeville, S.A.). The PCR cycles consisted of denaturation 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, 95°C for 30 sec and 40°C for 30 seconds. A melt curve was done at 60°C to 95°C, with an increment of 1.0°C for 10 sec followed by the plate read. The SYBR Green protocol was used for the quantification of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *F. graminearum*, *F. oxysporum*, *Pythium* spp., *Phoma* spp., *R. solani, Trichoderma* spp. and *M. phaseolina* as the SYBR Green bind to double stranded DNA, generate detectable fluorescence, and as result the amount of signal is proportional to the amount of double stranded DNA present. The primer sequences are summarized in Table 2.1.

2.3.4.2.2 Hydrolysis probe protocol

Quantification with hydrolysis probes was carried out in 20 µl reaction volumes containing 10 µl Bio-Rad iQ Supermix, 0.3 µM of the forward and reverse primers, 0.2 µM of the probe, 0.8 ng/µl of DNA and RNase/ DNase free water (Biolab Diagnostics, Wadeviile, S.A.) The denaturation took place at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and annealing at 60°C for 1 min (with plate read). The hydrolysis probe protocol was used for *F. verticillioides* and *F. equiseti* for optimum quantification results (with Taqman probes). The probe sequences can also be found in Table 2.1.

Table 2.1: Twelve fungal pathogens, separate primer and probe sets and melt temperatures.

Fungal pathogen	Forward primer	Reverse primer	qPCR technique (probe if applicable)	Melt temperature	Culture	References
C. eragostidis	CurASCF 5'-GCC CAA AGA CTC GCC TTA AA-3'	CurASCR 5'- GAT GGA TTG CTG GCC TCT TTA G-3'	SYBR	82 °C	PPRI 5447	Schoeman (2016)
E. pedicellatum	ExPBASF 5'- AGC CGG CCT ACT GGT TTC -3'	ExPBASR 5'- CCT ACC TGA TCC GAG GTC AA-3'	SYBR	82 °C	PPRI 10037	Schoeman (2016)
F. chlamydosporum	F.chl ASCF 5'- CAC ATA TTC AAC GCC AAG ACA C-3'	F.chl ASCR 5'- TGT ATC TTC TTC TCT TCA CCC TTC -3'	SYBR	78 °C	PPRI 4580	Schoeman (2016)
F. equiseti	Feqi ASDF 5'- TTA CAC TCA TAA CCT TCT CAT GC-3'	Feqi ASDR 5'- CAA TGA TGA GAA TAG CGC AAT CG-3'	Taqman probe Fequi probe 5'- /5Cy5/CA TGT ATT CCA GAC GCT CCC GGT C/3IAbRQSp/ Cy5	-	PPRI 7735	Schoeman (2016)
F. graminearum	Gram2F 5'- CCC TCT TCC CAC AAA CCA TT-3'	Gram2R 5'- GCT TCC TAT TGA CAG GTG GTT A -3'	SYBR	77 °C	M13.08	Schoeman (2016)
F. oxysporum	FoxyASCF 5'- CTC TCC TCG ACA ATG AGC AT -3'	FoxyASCR 5'- GGT CTG TGA AAC GAT GTC AGT A -3	SYBR	78 °C	PPRI 7729	Schoeman (2016)
F. verticillioides	Vert1F 5'- CGC GTT TCT GCC CTC TC -3'	Vert1R 5'- TCG GAT GGT TAG TGA CTG CT -3'	Taqman probe Verti probe 5'- /5TEX615/CC ACA ACC TCA CTG AGC TCA TCG T/3IAbRQSp/-3' Taxas Red	-	MRC 826	Schoeman (2016)
M. phaseolina	MacPASCF 5'- GCA ATC CTG TCG GAC TGT T-3'	MacPASCR 5'- GCG ATG CCG ATA CCA AGA T-3'	SYBR	79 °C	PPRI 1051	Schoeman (2016)
Phoma spp.	PhoASCF 5'- GCT CTG GTG TCT ACA ATG G -3'	PhoASCR 5'- GTC AGT TCT AGT ACC TCG TTG AAG -3'	SYBR	78 °C	PPRI 10098	Schoeman (2016)
Pythium spp.	PytASDF 5'- GGT TAC GCC TGG AAG TAT GT -3'	PytASDR 5'- CAA TCA AAC AAC CGA CGA CTA C -3'	SYBR	78 °C	PPRI 20772	Schoeman (2016)

R. solani	RhizASCF 5'- TGT TAT GCT TGG TTC CAC TCG-3'	RhizASCR 5'- GGA CTA TTG GAA GCG GTT CAT C -3'	SYBR	77 °C	PPRI 10376	Schoeman (2016)
Trichoderma spp.	TriASDF 5'-GGG TGC GTA TTC CAT CAA TCA -3'	TriASDR 5'- CAC GGT GGT CGA CTT TCC-3'	SYBR	80 °C	PPRI 9138	Schoeman (2016)

2.3.4.2.3 Data analysis

Pure cultures of fungal pathogens were obtained from the National databank PPRI (Plant Protection Research Institute). Standard curves were generated for each fungal pathogen by diluting each species' DNA, (approximately 10ng/µl) 4x, 16x, 64x, 256x and 1024x, and the extracted maize DNA (target DNA) were compared to these standard curves. Three independent qPCR runs were done for each sample. Using the standard curve, the C_t value was transformed into DNA concentration for each fungal pathogen in each trial analysed. To ensure that the unknown samples fall in the acceptable range compared to the standard curve of the known fungal pathogen, the efficiency was between 90-110% (optimal), the R^2 >0.95 (optimal) and the slope -3.32 (optimal). Melt curve values of the unknown samples that differed more than ± 1 °C, were not used in the analysis. (Appendix A).

2.3.5 Statistical analysis

Statistical analysis included using Levene's test for the homogeneity of variances for the data of the two years. Where the variability in observations of the two years was of comparable magnitude the analysis of variance of the two years' observations could validly be carried out (John, 1997). The separate measurements of the roots and crowns were added as a sub-plot factor in the variance analysis. The Shapiro-Wilk test was used to test the normality (Shapiro, 1965). Student's t-Least Significant Difference was calculated at the 5% level to compare treatment means of significant effects. SAS v9.2 statistical software was used to do all the analysis (SAS, 1999). Analysis of variance (ANOVA) was conducted with significance at *P=0.05*. Pearson's correlation analysis was conducted between root and crown disease index values and the fungal frequencies obtained for each season and plant part respectively.

2.4 Results

2.4.1 Monoculture maize

2.4.1.1 Plant biomass

The analysis of variance indicated that the plant biomass (kg/plant) was not significantly affected by the tillage practices applied (i.e. ROR versus NT) and/or the seasons (Table 2.2).

Table 2.2: Analysis of variance on the impact that tillage systems (ROR vs. NT) had on plant biomass over two seasons (2015 and 2016).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season	1	0.001	0.001	0.03	0.88
Rep(Season)	6	0.317	0.053	1.28	0.39
Tillage system	1	0.114	0.114	2.75	0.15
Season*Tillage system	1	0.045	0.045	1.09	0.34

Insignificant observations occur where the plant biomass of the NT maize was slightly lower compared to the ROR maize plant biomass in the 2015 season, with a less noticeable difference in the 2016 season (Figure 2.1).

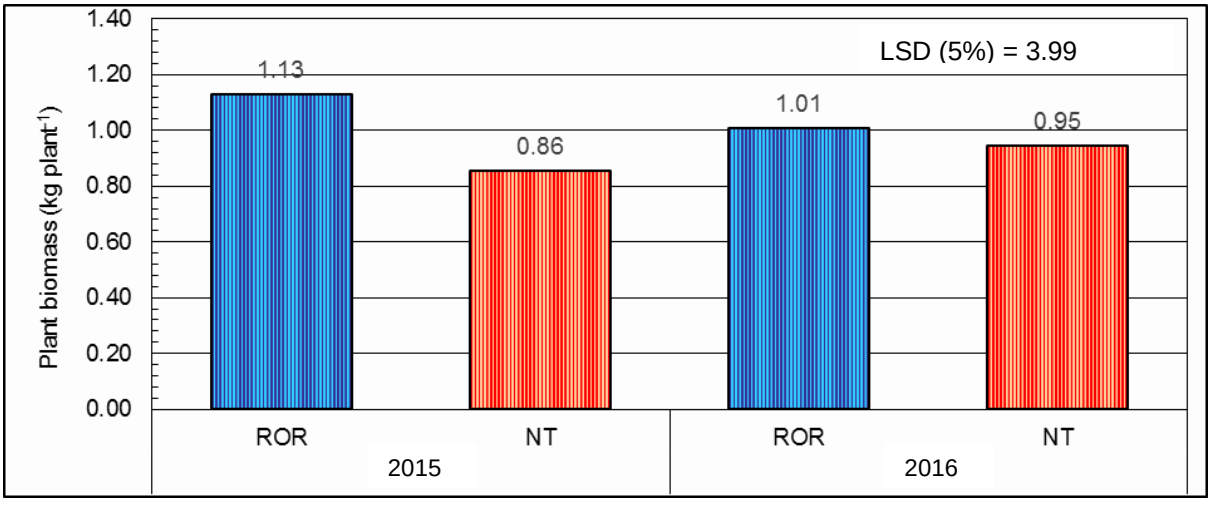


Figure 2.1: Plant biomass as measured for ROR and NT treatments during the 2015 and 2016 seasons respectively.

2.4.1.2 Root and crown disease severity ratings

Over the two seasons of the trial the NT treatments (which started in the 2012-2013 season) resulted in high, but not significant, root rot severity with scores/ratings of 134 rising to 159, while the crown rot severity rating remained low and also did not differ significantly between the treatments or over the seasons (NT: 29 and 24, ROR: 31 and 28) (Figure 2.2).The analysis of variance indicated a significant impact of the tillage systems on root and crown rot for the plant parts (P=0.03) (Table 2.3). A highly significant difference in the disease ratings was observed between the different plant parts (P<0.0001). Further analysis indicated a significant difference between the roots and crowns for both tillage practices, with a significant difference in disease severity of the roots between NT and ROR treatments (Table 2.4). No-till practices had significantly higher root disease severity averages compared to ROR (NT: 146.5 compared to ROR: 115.4), while the crowns resulted in no significant differences between the two treatments (Table 2.4).

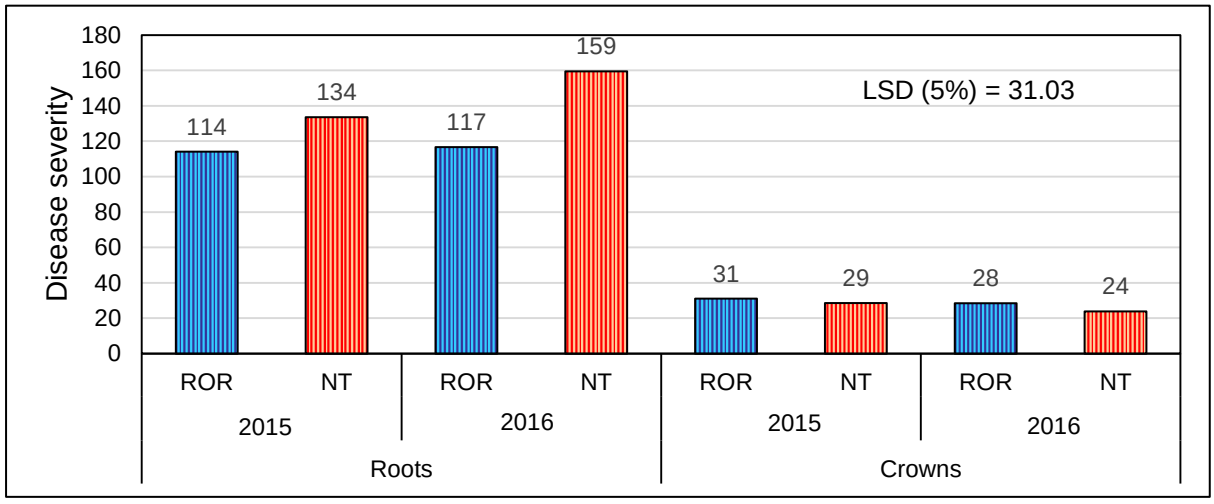


Figure 2.2: Disease severities observed in the roots and crowns for the ROR and NT treatments during the 2015 and 2016 seasons respectively.

Table 2.3: Analysis of variance on the impact that tillage systems (ROR vs. NT) had on root and crown rot development over the 2015 and 2016 seasons combined.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season	1	223.2	223.2	0.51	0.50
Rep (Season)	6	3383.3	563.9	1.28	0.39
Tillage	1	1521.0	1521.0	3.46	0.11
Season*Tillage	1	224.9	224.9	0.51	0.50
Error (a)	6	2641.3	440.2		
Plant Part[a]	1	84802.0	84802.0	209.00	**<0.00**
Season*Plant part	1	640.3	640.3	1.58	0.23
Tillage*Plant part	1	2406.8	2406.8	5.93	**0.03**
Season*Tillage*plant part	1	323.7	323.7	0.80	0.39
Corrected total	31	101036.3			

[a] - Plant part referring to root as opposed to crown

Table 2.4: T-test for the root and crown disease index severity averages of the whole species complex for the 2015 and 2016 seasons under No-till and Rip-On-Row practices (Significance P≤0.05).

Disease severity	NT	ROR
Roots	**146.5 a**	115.4 b
Crowns	26.2 c	**29.7 c**

2.4.1.3 Fungal pathogen complex

The qPCR analysis showed that *Phoma* spp., *Pythium* spp., *F. chlamydosporum* and *F. oxysporum* were the most prominent of the twelve fungi after quantification of the root and crown plant samples from the trial (Figure 2.3). Over the two seasons the ratio of the fungi based on their concentration was similar, but was generally higher in the 2016 season (Figure 2.3).

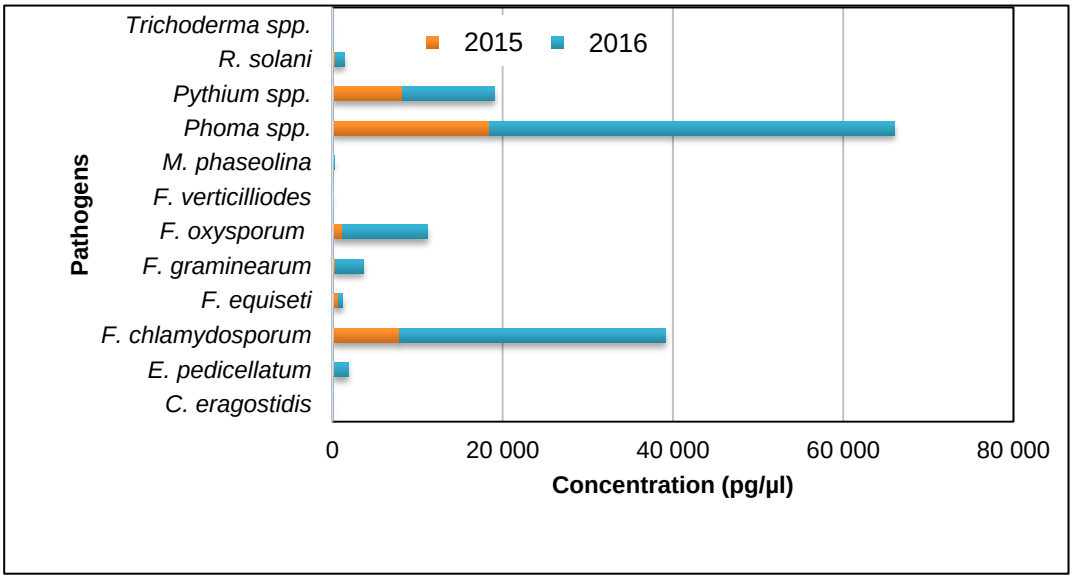


Figure 2.3: Average concentrations of twelve known soil borne pathogens as observed in the roots and crowns of all treatments and plant parts combined over two seasons (2015 and 2016).

Phoma spp. (P= 0.37) and *F. chlamydosporum* (P=0.84) had the highest concentrations (pg/µl) of the detected pathogens. The concentrations were also higher in the 2016 season compared to the 2015 season. No definite trend could be observed between the two cultivation practices for both the two plant parts and cultivation practices (Figure 2.4). The Pearsons correlation analysis could not link any of the fungi with the root and crown rot severities that were observed. Low severity observations or other fungal pathogens (which were not included in the study) could be responsible for the observed disease.

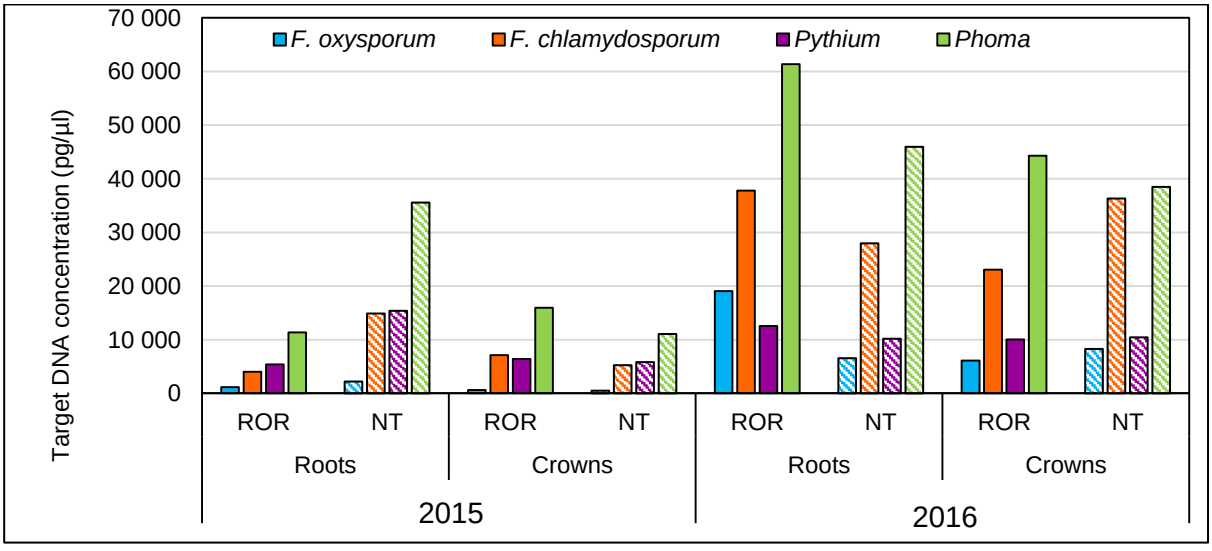


Figure 2.4: The target DNA concentration of the four prominent fungi (*F. oxysporum, F. chlamydosporum, Pythium* spp. and *Phoma* spp.) measured from the roots and crowns of the plants sampled from the ROR and NT treatments respectively for two seasons (2015 and 2016).

The season x tillage interaction was significant for *F. oxysporum* (P=0.01), *C. eragostidis* (P=0.01) and *E. pedicellatum* (P=0.04) (Table 2.5). *F. oxysporum* target DNA was significantly higher in the 2016 season and under NT practices (12 593 pg/µl) compared to ROR in the second season (7 414 pg/µl) and to ROR (1 351 pg/µl) and NT (894 pg/µl) in the 2015 season. *C. eragostidis* target DNA was significantly different for NT and ROR between the seasons, but not between the cultivation practices within the season. For the 2015 season, *C. eragostidis* target DNA was significantly lower (ROR: 1.6 pg/µl, NT: 1.4 pg/µl) compared to the 2016 (ROR: 2.9 pg/µl, NT: 3.1 pg/µl) season. *E. pedicellatum* target DNA was significantly lower for NT and ROR in 2015 comapared to 2016, but not between the cultivation practices within each season. For the 2015 season *E. pedicellatum* was significantly lower (ROR: 206 pg/µl, NT: 169.6 pg/µl) compared to the 2016 (ROR: 1 268.7 pg/µl, NT: 2 123.5 pg/µl) season (Table 2.6).

Table 2.5: Analysis of variance of *C. eragostidis, E. pedicellatum* and *F. oxysporum* that were significantly influenced by ROR and NT treatments respectively over the two seasons.

Species	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
C. eragostidis	**Season x Tillage**	3	0.52843055	0.52843055	2.52	**0.02**
E. pedicellatum	**Season x Tillage**	3	1588472.49	1588472.49	3.05	**0.04**
F. oxysporum	**Season x Tillage**	3	6356908	63565908	10.73	**0.02**

Table 2.6: Season (2015 and 2016) x tillage (NT and ROR) t-test interaction table showing the effects on target DNA of *F. oxysporum, C. eragostidis* and *E. pedicellatum* Significance P≤0.05).

	2015		2016	
Pathogen	ROR	NT	ROR	NT
F. oxysporum	1351.0 c	894.0 c	7414.0 b	12593.0 a
C. eragostidis	1.6 b	1.4 b	2.9 a	3.1 a
E. pedicellatum	206.0 b	169.6 b	1268.7 a	2123.5 a

Season x plant part interaction had a significant effect on *F. graminearum* (P=0.05), *M. phaseolina* (P=0.02) and *Trichoderma* spp. (P=0.04) (Table 2.7) infection. *F. graminearum* target DNA was significantly higher in the crowns (6 396 pg/µl) in the 2016 season compared to the crowns (703 pg/µl) in the 2015 season, while the roots showed no significant difference between the seasons. *M. phaseolina* target DNA was significantly different for the roots and crowns between the

seasons, but not between the different plant parts within the season. For the 2015 season, *M. phaseolina* target DNA was significantly higher (roots: 126.7 pg/µl, crowns: 112.1 pg/µl) compared to the 2016 (roots: 94.7 pg/µl, crowns: 93.4 pg/µl) season. *Trichoderma* spp. were significantly higher for the 2016 season in the roots (34.4 pg/µl) compared to the 2015 season (roots: 10.4 pg/µl, crowns: 13.4 pg/µl) (Table 2.8).

Table 2.7: Analysis of variance of *F. graminearum, M. phaseolina* and *Trichoderma* spp. that were significantly influenced in the roots and crowns respectively over the two seasons.

Species	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
F. graminearum	**Season x Plant part**	3	63278466.6	63278466.63	2.58	**0.04**
M. phaseolina	**Season x Plant part**	3	355.63114	355.63114	1.46	**0.02**
Trichoderma spp.	**Season x Plant part**	3	20402145.4	20402145.4	0.48	**0.04**

Table 2.8: Season (2015 and 2016) x plant part (roots and crowns) t-test interaction table showing the effects on the target DNA of *F. graminearum, M. phaseolina* and *Trichoderma* spp. (Significance P≤0.05).

	2015		2016	
Pathogen	Root	Crown	Root	Crown
F. graminearum	26.0 b	703.0 b	93.0 b	6396.0 a
M. phaseolina	126.7 a	112.1 a	94.7 b	93.4 b
Trichoderma spp.	10.4 b	13.4 b	34.4 a	14.4 b

Lastly a significant season x tillage x plant part interaction occurred for *F. verticillioides* (P=0.01), *F. equiseti* (P=0.01)*, F. chlamydosporum* (P=0.01), *Phoma* spp. (P=0.02) and *R. solani* (P=0.02) (Table 2.9). *F. verticillioides* had no significant differences in the 2015 season, but was significantly higher in the 2016 season under NT practices for the crowns (154.2 pg/µl) compared to the roots (49.5 pg/µl) under the same cultivation practice in the same season and under ROR practices for the same season (roots: 71.7 pg/µl, crowns: 57.3 pg/µl). *F. equiseti* did not differ significantly in the 2015 season between ROR (roots: 672.9 pg/µl, crowns: 597.7 pg/µl) and NT (roots: 1 132.4 pg/µl, crowns: 306.5 pg/µl) practices. The roots and the crowns under NT differed significantly from each other, but under ROR no significant difference between the roots and crowns occurred. For the 2016 season there were no significant differences between the roots and cultivation practices (NT: 482 pg/µl, ROR: 828 pg/µl), and between the crowns and cultivation

practices (NT: 711.6 pg/µl, ROR: 194 pg/µl). *F. chlamydosporum* was significantly higher in the crowns under NT for the 2016 season (36319.0 pg/µl) than in the 2015 season (14 883 pg/µl). It was also significantly higher in the roots under ROR in the 2016 season compared to the 2015 season. No significant differences could be seen between the cultivation practices within seasons. *Phoma* spp. had no significant difference during the 2015 season for the roots under NT (35 564 pg/µl) compared to the crowns under NT (11 062 pg/µl) and the roots (11 378 pg/µl) and crowns (15 933 pg/µl) under ROR of the same season. Significant differences between the roots under ROR (61 344 pg/µl) in 2016 and the roots (11 378.0 pg/µl) and crowns (15 933.0 pg/µl) under ROR in the 2015 season and the crowns (11 062.0 pg/µl) under NT in the same season occurred. *R. solani* was significantly lower in the 2015 season for the roots under ROR (120.9 pg/µl) compared to the crowns (1 565.0 pg/µl) under NT of the 2016 season. No other significant differences were observed (Table 2.10).

Table 2.9: Analysis of variance of *F. verticillioides, F. equiseti, F. chlamydosporum, Phoma* spp. and *R. solani* that were significantly influenced by ROR and NT treatments respectively over the two seasons for the roots and crowns.

Specie	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
F. verticillioides	**Season x Tillage x Plant part**	4	8707.97527	8707.97527	3.86	**0.01**
F. equiseti	**Season x Tillage x Plant part**	4	1302264.436	1302264.436	7.45	**0.01**
F. chlamydosporum	**Season x Tillage x Plant part**	4	63743205	63743205	3.8	**0.01**
Phoma spp.	**Season x Tillage x Plant part**	4	1129375.156	1129375.156	1.39	**0.02**
R. solani	**Season x Tillage x Plant part**	4	492779.82	492779.82	0.69	**0.02**

Table 2.10: Season (2015 and 2016) x tillage (NT and ROR) x plant part (roots and crowns) t-test interaction table showing the effects on target DNA of *F. verticillioides, F. equiseti, F. chlamydosporum, Pythium* spp., *Phoma* spp. and *R. solani* (Significance P≤0.05).

	2015				2016			
	NT		ROR		NT		ROR	
Pathogen	Root	Crown	Root	Crown	Root	Crown	Root	Crown
F. verticillioides	14.6 b	4.4 b	12.4 b	15.2 b	49.5 b	154.2 a	71.7 b	57.3 b
F. equiseti	1132.4 a	306.5 b	672.9 ab	597.7 ab	482 b	711.6 ab	828.0 ab	194.5 b
F. chlamydosporum	14883.0 bc	5247.0 c	4016.0 c	7117.0 c	27966.0 ab	36319.0 a	37767.0 a	23088.0 abc
Phoma spp.	35564.0 ab	11062.0 b	11378.0 b	15933.0 b	45955.0 ab	38485.0 ab	61344.0 a	44306.0 ab
R. solani	700.9 ab	433.0 ab	120.9 b	385.0 ab	477.2 ab	1565.0 a	856.4 ab	973.3 ab

2.4.2 Crop rotation trial

2.4.2.1 Plant biomass

The crop rotation trial showed no significant difference in plant biomass for the various crop rotations and the tillage practices for both seasons (Table 2.11).

Table 2.11: Analysis of variance of the impact that two crop rotation systems under ROR and NT treatments respectively had on the plant biomass over the two seasons.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season	1	0.0098	0.0098	1.52	0.26
Rep(Season)	2	0.0003	0.0002	0.02	0.98
Rotation system	3	0.0389	0.0129	2.01	0.21
Season*Rotation system	3	0.0735	0.0245	3.80	0.08

2.4.2.2 Root and crown disease severity ratings

The disease severity averages for both seasons were significantly higher in the roots compared to the crowns for MRORSB (Maize/Soybean rotation under ROR), MNTSB (Maize/Soybean rotation under NT) and MRORSG (Maize/Sorghum rotation under ROR) cultivation practices. No significant difference could be observed between the roots and crowns for the MNTSG rotation (Table 2.10). The maize/soybean rotation under ROR of the roots had the highest disease severity average (152.5 pg/µl) and the maize/sorghum rotation under ROR of the crowns had the lowest disease severity average (10.1 pg/µl) (Table 2.12). No significant differences could be observed between the disease severity levels under the four cultivation practices applied over the two seasons.

Table 2.12: T-test mean root and crown disease severity averages for the 2015 and 2016 seasons under the four cultivation practices applied (MRORSB, MNTSB, MRORSG and MNTSG) (Significance P≤0.05).

Disease severity	MRORSB	MNTSB	MRORSG	MNTSG
Roots	152.5 c	129.2 c	137.0 c	110.2 bc
Crowns	12.4 a	22.4 a	10.1 a	28.0 ab

MRORSB – Maize/Soybean rotation under ROR; MNTSB – Maize/Soybean rotation under NT; MRORFS – Maize/Sorghum rotation under ROR; MNTFS – Maize/Sorghum rotation under NT

The analysis of variance indicated a highly significant impact of the different plant parts (P<0.0001) on disease severity over the 2015 and 2016 growing seasons under the conditions of two different crop rotation systems and different cultivation practices (Table 2.13). However, no

significant interactions were observed between disease severity and the crop rotation systems or seasons.

Table 2.13: Analysis of variance on the impact of two crop rotation systems under ROR and NT treatments respectively on root and crown rot disease severity (all species combined) over the 2015 and 2016 seasons. (Significance P≤0.05).

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season	1	486.4	486.4	0.41	0.54
Rep (Season)	2	3 536.2	1 768.1	1.50	0.23
Rotation system	3	745.2	248.4	0.21	0.89
Season*Rotation	3	2 047.6	682.5	0.58	0.65
Error (a)	6	7 051.9	1 175.3		
Plant Part[a]	1	103 991.0	103 991.9	102.50	**<0.00**
Season*Plant part	1	478.8	478.9	0.47	0.51
Rotation*Plant part	3	3 812.4	1 270.8	1.25	0.35
Season*Rotation*plant part	3	632.8	210.9	0.21	0.89
Corrected total	31	130 899.9			

[a] - Plant part referring to root as opposed to crown

Individually the species that differed significantly between the roots and crowns under ROR and NT treatments respectively were *F. oxysporum, F. equiseti, M. phaseolina* and *E. pedicellatum* (Table 2.14).

Table 2.14: Analysis of variance on the impact of two crop rotation systems under ROR and NT treatments respectively on root and crown rot disease severity over the 2015 and 2016 seasons. (Significance P≤0.05).

Specie	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
F. oxysporum	**Plant part**	1	80916357.6	80916357.6	8.19	**0.02**
F. equiseti	**Plant part**	1	2701431.934	2701431.934	5.10	**0.05**
M. phaseolina	**Plant part**	1	528.3637	528.3637	7.74	**0.02**
E. pedicellatum	**Plant part**	1	22401096.69	1129375.156	6.43	**0.03**

2.4.2.3 Fungal pathogen complex

The qPCR results were similar to the mono-culture maize trial where *Phoma* spp., *Pythium* spp., *F. chlamydosporum* and *F. oxysporum* were the most prominent of the twelve fungi. Their concentration rankings over the two seasons were similar, but were higher in the 2016 season (Figure 2.5). *Phoma* spp., *F. chlamydosporum* and *F. oxysporum* concentrations were noticeably, but not significantly, higher in ROR treatments in the same season (Figure 2.5).

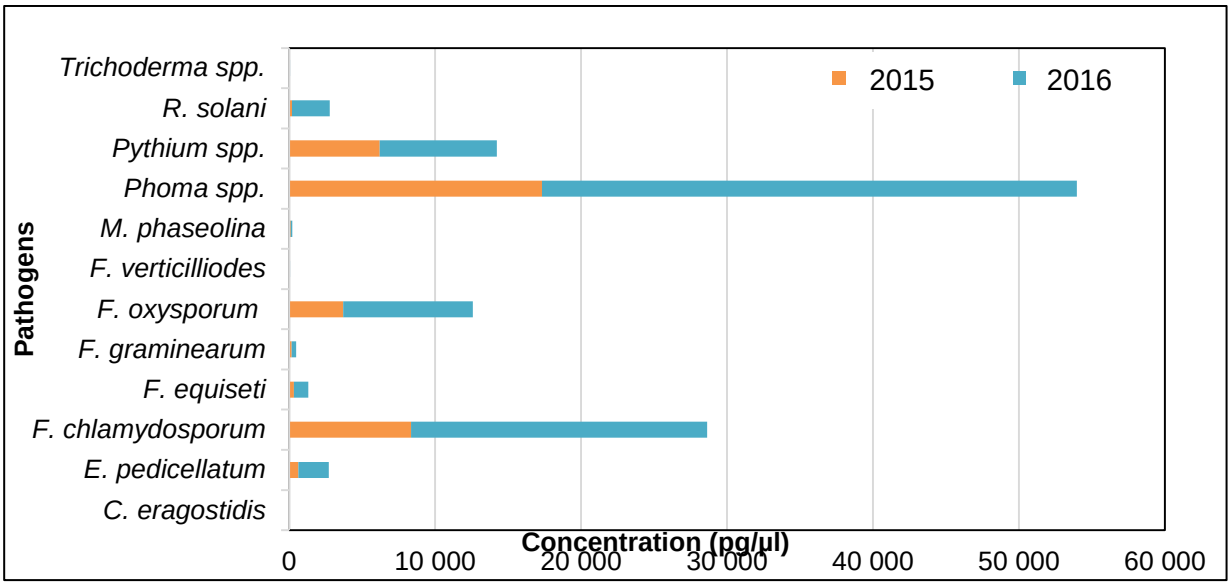
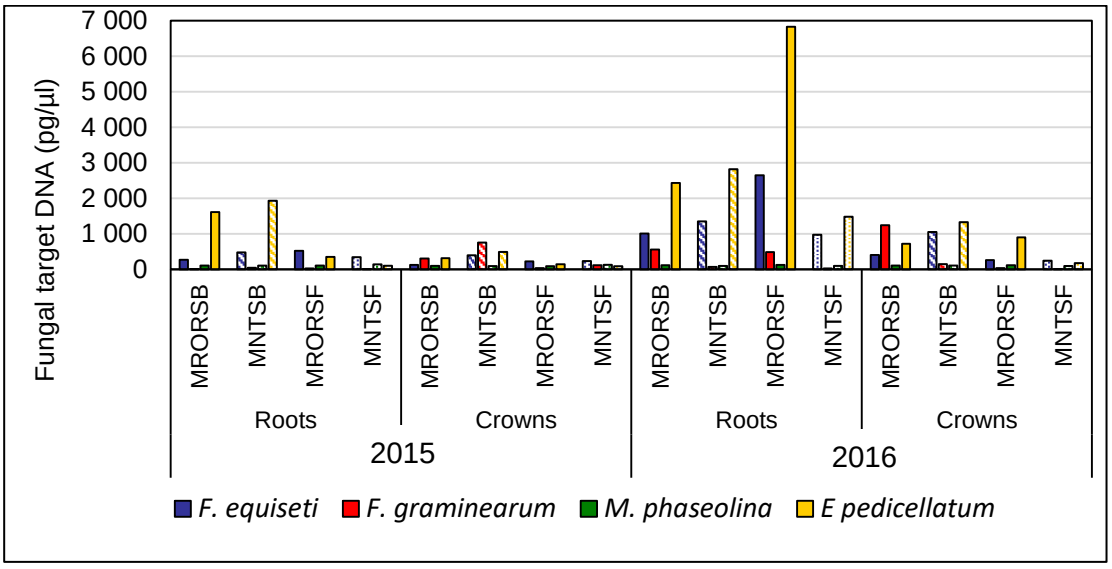


Figure 2.5: Average concentrations of twelve known fungi (soil borne pathogens) as observed in the roots and crowns of all treatments combined over two seasons of the crop rotation trial.

Additionally, *E. pedicellatum* occurred in higher concentrations in the roots in 2016 when ROR treatments with sorghum rotations were applied (Figure 2.6). *F. equiseti*, *F. graminearum* and *M. phaseolina* had a target DNA lower than 10 000 pg/µl in maize roots and crowns of four rotation-tillage treatments during 2015 and 2016 (Figure 2.6).



MRORSB – Maize/Soybean rotation under ROR; MNTSB – Maize/Soybean rotation under NT; MRORFS – Maize/Sorghum rotation under ROR; MNTFS – Maize/Sorghum rotation under NT

Figure 2.6: Fungal target DNA less than 10 000 (pg/µl) in maize roots and crowns of four rotation-tillage treatments during 2015 and 2016.

The season x plant part interaction had a significant effect on *F. oxysporum* (P=0.05), *F. equiseti* (P=0.01)*, F. chlamydosporum* (P=0.01), *M. phaseolina* (P=0.02), *Phoma* spp. (P=0.03), *R. solani* (P=0.02) and *E. pedicellatum* (P=0.03) (Table 2.15) infection. *F. oxysporum* was significantly higher in the roots (11 794 pg/µl) of the 2016 season compared to the crowns (6 012 pg/µl) of the same season and both plant parts of the 2015 season (roots: 3 978 pg/µl, crowns: 3 400 pg/µl). Similarly, *F. equiseti,* was significantly higher in the roots (1 491.6 pg/µl) of the 2016 season compared to the crowns (486.2 pg/µl) of the same season and both plant parts of the 2015 season (roots: 399.3 pg/µl, crowns: 242.4 pg/µl). *F. chlamydosporum* was significantly higher in the roots of the 2016 season compared to to both plant parts in the 2015 season. *M. phaseolina* was significantly higher in the roots of the 2015 season (111.2 pg/µl) compared to the crowns of the same season (99.7 pg/µl) with no significant difference compared to both plant parts in the 2016 season (roots: 107.7 pg/µl, crowns: 103 pg/µl). *Phoma* spp. was significantly higher in the roots (40 900 pg/µl) and crowns (32 380 pg/µl) of the 2016 season compared to the 2015 season (roots: 16 813 pg/µl, crowns: 17 847 pg/µl). *R. solani* was significantly higher in the crowns (4 060 pg/µl) of the 2016 season compared to both plant parts of the 2015 season (roots: 148 pg/µl, crowns: 220 pg/µl). *E. pedicellatum* was significantly higher in the roots (3 386.6 pg/µl) of the 2016 season compared to the crowns (778 pg/µl) of the same season and both plant parts of the 2015 season (roots: 994.3 pg/µl, crowns: 256.2 pg/µl) (Table 2.16).

Table 2.15: Analysis of variance of the significant interaction between the roots and crowns over the two seasons for *F. oxysporum, F. equiseti, F. chlamydosporum, M. phaseolina, Phoma* spp. *E. pedicellatum* and *R. solani*.

Specie	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
F. oxysporum	**Season x Plant part**	3	54160598.6	54160598.6	5.48	**0.05**
F. equiseti	**Season x Plant part**	3	1440060.242	1440060.242	2.72	**0.01**
F. chlamydosporum	**Season x Plant part**	3	65203210	65203210	1.3	**0.01**
M. phaseolina	**Season x Plant part**	3	89.0925	89.0925	1.31	**0.02**
Phoma spp.	**Season x Plant part**	3	182574440	182574440	1.21	**0.03**
E. pedicellatum	**Season x Plant part**	3	6997220	6997220	2.01	**0.03**
R. solani	**Season x Plant part**	3	16575772.1	16575772.1	1.55	**0.02**

Table 2.16: T-test target DNA of fungal pathogen qPCR values as measured on roots and crowns for season 2015 and 2016 for *F. oxysporum, F. equiseti, F. chlamydosporum, M. phaseolina, Phoma* spp., *R. solani* and *E. pedicellatum* (Significance P≤0.05).

Pathogen	Season 1		Season2	
	Root	Crown	Root	Crown
F. oxysporum	3978.0 b	3400.0 b	11794.0 a	6012.0 b
F. equiseti	399.3 b	242.4 b	1491.6 a	486.2 b
F. chlamydosporum	7781.0 c	8939.0 bc	23722.0 a	16855.0 ab
M. phaseolina	111.2 a	99.7 b	107.7 ab	103.0 ab
Phoma spp.	16813.0 b	17847.0 b	40900.0 a	32380.0 a
R. solani	148.0 b	220.0 b	1110.0 ab	4060.0 a
E. pedicellatum	994.3 b	256.2 b	3386.6 a	778.0 b

The plant part x management strategy interaction was significant for fungal infection of *C. eragostidis* (P=0.03) (Table 2.17). *C. eragostidis* was significantly higher in the roots where MRORSB (5.5 pg/µl) was implemented (Table 2.18). It differed significantly, from MNTSB (1.9 pg/µl) and MNTSG (1.9 pg/µl) and for all four treatments of the crowns (MRORSB: 1.9 pg/µl, MNTSB: 1.8 pg/µl, MRORSG: 1.7 pg/µl, MNTSG: 1.5 pg/µl) (Table 2.18).

Table 2.17: *A*nalysis of variance for *C. eragostidis* of the interactions between the roots and crowns over the two seasons and for the different cultivation practices.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season x Cultivation practices	3	1.02210	0.34070101	0.06	0.98
Season x Plant part	3	0.19572	0.19572991	0.04	0.84
Plant part x Cultivation practices	3	16.77200	5.59080000	1.21	**0.03**
Season x Cultivation practices x Plant part	6	0.31526	0.1058811	0.02	0.99

Table 2.18: *C. eragostidis* t-test target DNA for roots and crowns and for all four cultivation practices applied (MRORSB, MNTSB, MRORSB, and MRORSG) (Significance P≤0.05).

Pathogen	Root				Crown			
	MRORSB	MNTSB	MRORSG	MNTSG	MRORSB	MNTSB	MRORSG	MNTSG
C. eragostidis	5.5 a	1.9 b	2.1 ab	1.9 b	1.9 b	1.8 b	1.7 b	1.5 b

MRORSB – Maize/Soybean rotation under ROR; MNTSB – Maize/Soybean rotation under NT; MRORFS – Maize/Sorghum rotation under ROR; MNTFS – Maize/Sorghum rotation under NT

Trichoderma spp. (P=0.04) had significant 2-way interactions for season x cultivation practice (Table 2.19). *Trichoderma* spp. were significantly higher in the MRORSB cultivation practice of

2015 and the MRORSG cultivation practice of 2016, compared to the other treatments within and between the two seasons, but did not differ significantly compared to each other (Table 2.20).

Table 2.19: *A*nalysis of variance for *Trichoderma* spp. of the interactions between the cultivation practices over the two seasons.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season x Cultivation practices	3	1.02210	0.34070101	0.06	**0.04**
Season x Plant part	3	0.19572	0.19572991	0.04	0.84
Plant part x Cultivation practices	3	16.77200	5.59080000	1.21	0.98
Season x Cultivation practices x Plant part	6	0.31526	0.1058811	0.02	0.99

Table 2.20: *Trichoderma* spp. t-test target DNA for the 2015 and 2016 seasons and all four cultivation practices applied (MRORSB, MNTSB, MRORSB, and MRORSG) (Significance P≤0.05).

	Season 1				Season 2			
Pathogen	**MRORSB**	**MNTSB**	**MRORSG**	**MNTSG**	**MRORSB**	**MNTSB**	**MRORSG**	**MNTSG**
Trichoderma spp.	42.2 a	7.9 b	10.3 b	26.1 ab	24.0 ab	24.5 ab	44.5 a	19.2 b

MRORSB – Maize/Soybean rotation under ROR; MNTSB – Maize/Soybean rotation under NT; MRORFS – Maize/Sorghum rotation under ROR; MNTFS – Maize/Sorghum rotation under NT

F. verticilliodes had a significant 3-way interaction between season x plant part x cultivation practice (Table 2.21). *F. verticilliodes* was significantly higher during the 2016 season for the MRORSG cultivation practice in the roots and MNTSG cultivation practice in the crowns compared to the other treatments in the same season and for all the treatments and different plant parts of the 2015 season, but not compared to each other (Table 2.22).

Table 2.21: *A*nalysis of variance for *F. verticillioides* of the interactions between the roots and crowns over the two seasons and for the different cultivation practices.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Season x Cultivation practices	3	3786.77823	1262.25941	3.93	0.07
Season x Plant part	3	7.22990	7.22990	0.02	0.88
Plant part x Cultivation practices	3	5935.99183	1978.66394	6.55	0.06
Season x Cultivation practices x Plant part	6	6748.67836	2249.55945	7.45	**0.01**

Table 2.22: *F. verticillioides* t-test target DNA for the 2015 and 2016 seasons and all four cultivation practices applied and both plant parts (MRORSB, MNTSB, MRORSB, and MRORSG) (Significance P≤0.05).

	Season1								Season 2							
	Root				Crown				Root				Crown			
Pathogen	MRORSB	MNTSB	MRORSG	MNTSG	MRORSB	MNTSB	MRORSG	MNTSG	MRORSB	MNTSB	MRORSG	MNTSG	MRORSB	MNTSB	MRORSG	MNTSG
F.verticilliodes	5.2 bc	13.3 bc	3.0 bc	2.0 bc	0.0 c	9.6 bc	6.1 bc	0.0 bc	39.4 bc	23.7 bc	111.34 a	18.1 bc	40.7 b	20.2 bc	32.9 bc	98.6 a

MRORSB – Maize/Soybean rotation under ROR; MNTSB – Maize/Soybean rotation under NT; MRORFS – Maize/Sorghum rotation under ROR; MNTFS – Maize/Sorghum rotation under NT

2.5 Discussion and conclusion

The use of qPCR identified and quantified all twelve most commonly occurring root and crown rot soil borne fungal pathogens of maize. From this study the most prominent fungi were *Phoma* spp., *F. chlamydosporum, Pythium* spp, *F. oxysporum* and *F. graminearum* and are similar to the study of Chambers (1987). However, *Pythium* spp. was not included in the study of Chambers (1987) and *Exserohilum* and *Curvularia* spp. were not found in high abundance in this present study.

No significant differences were observed in the plant biomass for the different tillage practices and for the different management strategies which included various crop rotations. The expected outcome that certain practices (till, no-till, crop rotation) increase or decrease the occurrence of specific fungal pathogens showed very limited significance or definite results. This is because inconsistencies are common in the results over seasons for various parameters (Craven & Nel, 2016). Smit *et al*. (1997) state that research shows no single crop rotation system to favour or reduce all fungi. Some of the factors that could lead to this variation include different host growth stages and environmental factors (White, 1999). Lipps and Deep (1991) attributed these inconsistent fungal frequencies to variation in rainfall over the seasons, while Doupnik and

Boosalis (1980) added the impact that stubble had on water retention and the regulation of soil temperature in different climatic conditions.

Diseases and especially root and crown rot, for which the twelve most common pathogen species present in South Africa is known, are greatly influenced by seasonal changes (Craven & Nel, 2016). In this study, significant differences were also obtained between the cultivation practices. This could be due to the benefits of CA practices such as yield increase over a few years, soil nutrient conservation, retaining of soil moisture and soil temperature regulating to name a few (Sithole *et al*., 2016). The root and crown disease severity were significantly influenced by tillage systems and for different plant parts, especially for the MRORSB, MNTSB and MRORSG within one season, but not over the two seasons. In conservation practices monoculture of maize are common, as well as tilling of soil leading to variation in soil nutrients, moisture and temperature (Sithole *et al*., 2016). These differences affect the fungi occurring in the soil (Craven & Nel, 2016). As with the study of Craven & Nel (2016) fungal frequencies were inconsistent over seasons as well as for the plant part from which the fungi were obtained.

The fungal frequencies differ significantly between plant parts and could be indicative of tissue specificity of fungal pathogen. This could also explain the lack of correlation between visible lesions on the roots and crowns and fungal pathogen target DNA levels obtained with qPCR (Pearson's correlation analysis). Smit & McLaren (1997) recorded similar results where they also found that root discolorations did not give a good indication of the presence of root rot pathogens using the plating out on agar technique. In this study, it was clear that the fungal pathogens were more inclined to occur in the roots than the crowns.

In the tillage trial *F. oxysporum, C eragostidis* and *E. pedicellatum* had significant season x tillage interactions. *F oxysporum* were significantly higher in 2016 under NT, *C. eragostidis* and *E. pedicellatum* differed significantly between NT and ROR between the two seasons. *F. graminearum, M. phaseolina* and *Trichoderma* spp. had significant season x plant part interactions. *F. graminearum* differed significantly for the crowns between the seasons, *M. phaseolina* for the roots and crowns between the seasons and *Trichoderma* spp. for the roots between the seasons. *F. verticillioides*, *F. equiseti, F. chlamydosporum, Phoma* spp. and *R. solani* had significant season x tillage x plant part interactions. *F. verticillioides* were higher in 2016 under NT for the crowns compared to the roots, *F. equiseti* differed significantly for the roots and crowns under NT within the season, *F. chlamydosporum* were significantly higher in the crowns under NT for the 2016 season and for the roots under ROR in the 2016 season, *Phoma* spp. differed under ROR for the roots in 2016 and for the roots and crowns under NT in 2015, *R.*

solani were lower in the 2015 season for the roots under ROR. The statement of Memon *et al.,* (2013) that the maize plant is more susceptible to *Phoma* spp. infection in the dry season can link with the finding of *Phoma* being significantly higher where ROR practices were applied (lowering the soil moisture content available for the roots).

The crop rotation trial had a significant season x plant part interaction for *F. oxysporum, F. equiseti, F. chlamydosporum, M. phaseolina, Phoma* spp., *R. solani* and *E. pedicellatum*. Mean target DNA was higher in maize roots in the 2016 season, with the exception of *R.solani* where target DNA was higher in the maize crowns. *Phoma* spp. target DNA was equally high in the maize roots and crowns in the 2016 season. *M. phaseolina* were only significantly higher in the roots of 2015 compared to the crowns of 2015. *C. eragostidis* had a significant plant part x cultivation practice interaction, being higher in the roots where MRORSB were applied compared to all the other strategies for the roots and the crowns. *Trichoderma* spp. had a significant season x cultivation practice interaction and *F. verticillioides* had a significant season x plant part x cultivation practice interaction. *Trichoderma* spp. were higher in the MRORSB (2015) and MRORSG (2016) management strategies, while *F. verticillioides* were higher in 2016 for MRORSG in the roots and MNTSG in the crowns compared to the other variables. The maize/soybean rotation under ROR practices showed the highest significant differences with the highest root rot severity and maize/sorghum under ROR for the crown rot severity. The concentrations of the fungi declined in the crop rotation trials compared to the monoculture trials and where NT instead of ROR practices were applied. It thus shows possible long-term beneficial effects of crop rotation and conservation agriculture (lower pathogen concentrations in the roots and crowns). With the qPCR results, no direct correlation could be linked for one or more specific fungi responsible for the root and crown rot, which could be because of the overall low level of rot severity or because only twelve fungi were tested for. Each fungal species is also favoured by different interactions (varying between season, plant part, cultivation practice, tillage practice and combinations).

The results accordingly confirmed that multiple season data are required to study the impact of cultivation practices on soilborne pathogens and the impact they have on root and crown rot. Trends need to be drawn up for the fungi, or fungal complexes established in different regions to see the potential disease severity and incorporated with known CA practices for improved disease management of root and crown rot of maize in South Africa and worldwide.

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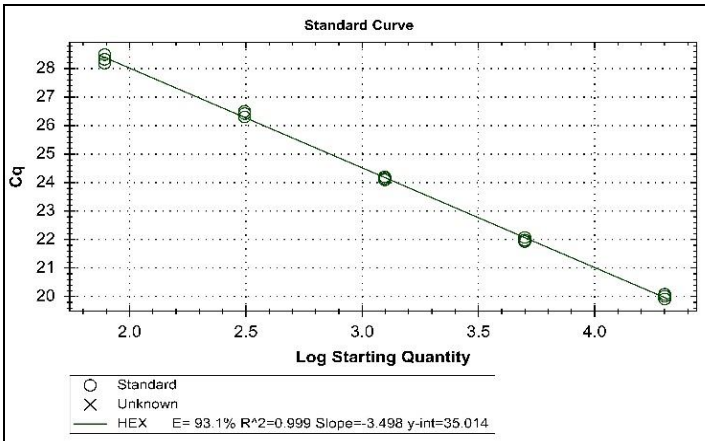
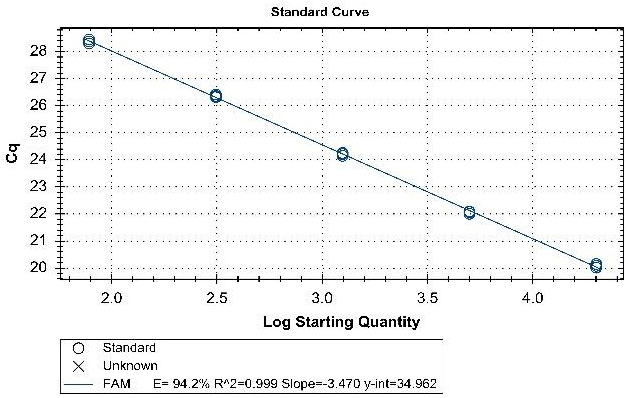
Appendix A: Standard- and melt curves of the twelve known fungal root and crown rot pathogens in South Africa indicating the efficiency and R^2 values.

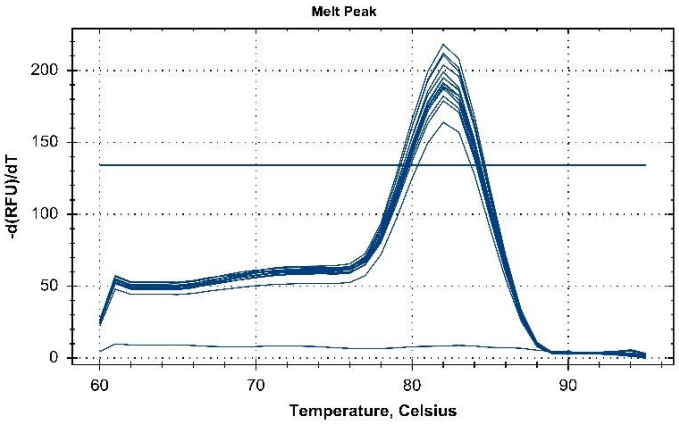


***C. eragostidis* standard curve**

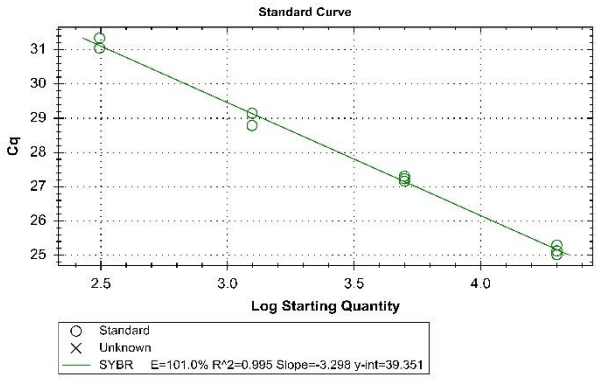
No melt curve due to the use of Taqman probe



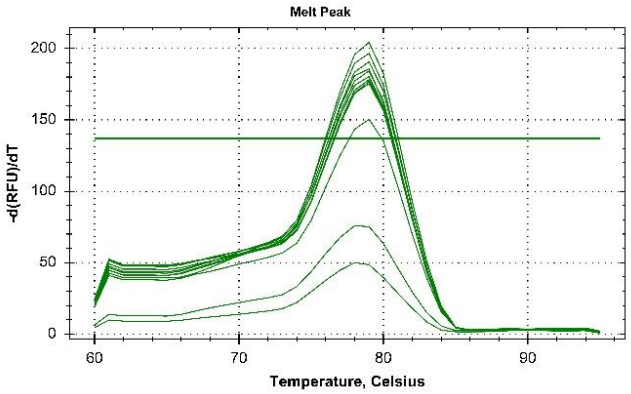
***E. pedicellatum* standard curve**



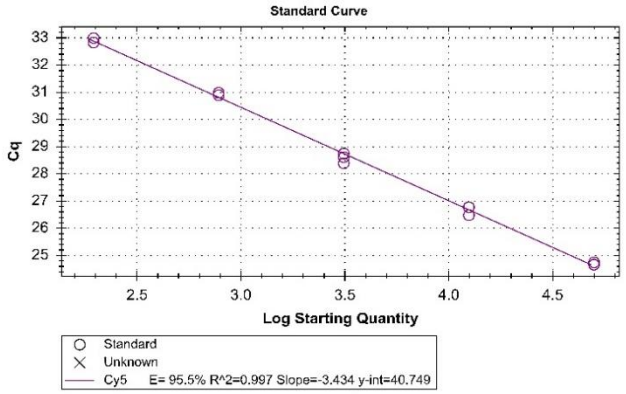
***E. pedicellatum* melt curve**



***F. chlamydosporum* standard curve**

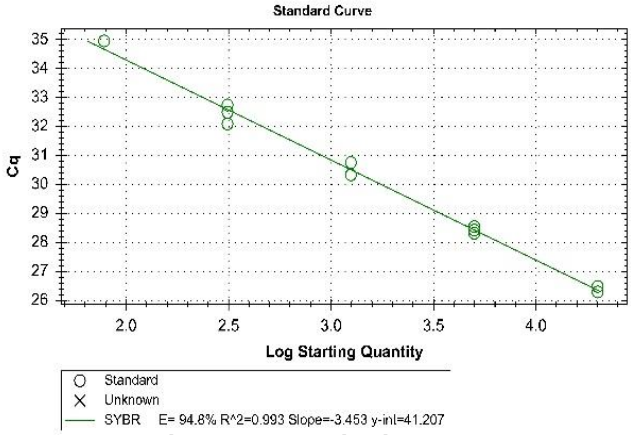


***F. chlamydosporum* melt curve**

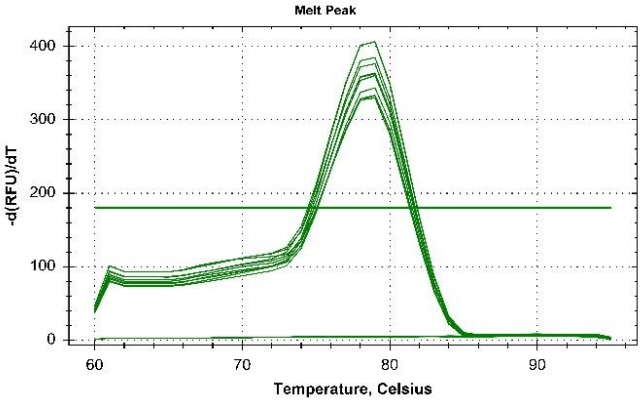


F. equiseti **standard curve**

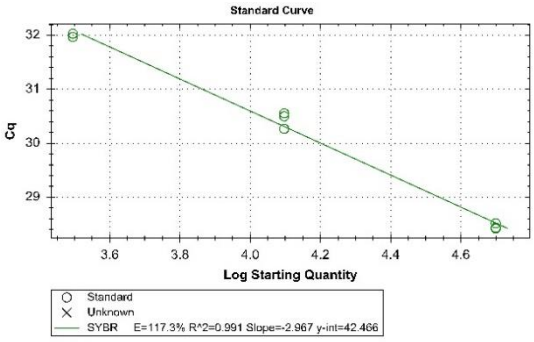
No melt curve due to the use of Taqman probe



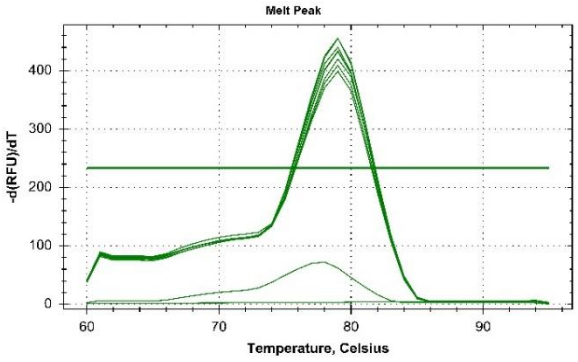
F. graminearum **standard curve**



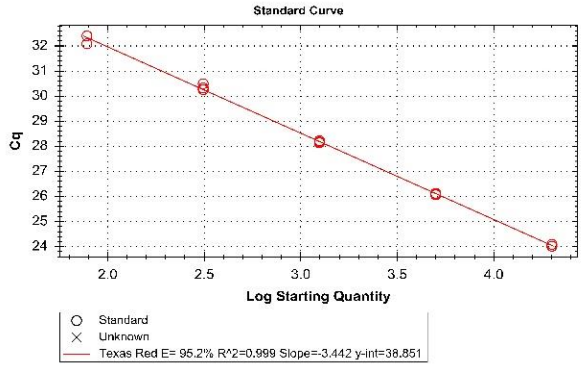
F. graminearum **melt curve**



F. oxysporum **standard curve**

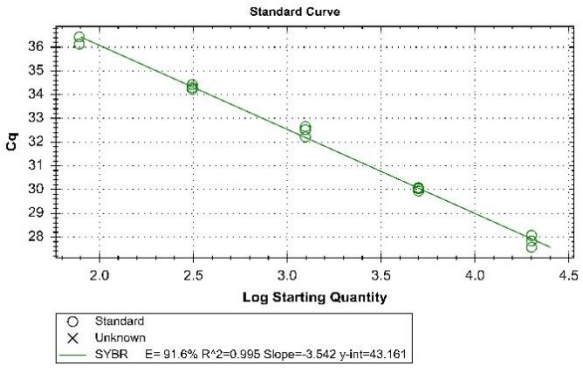


F. oxysporum **melt curve**

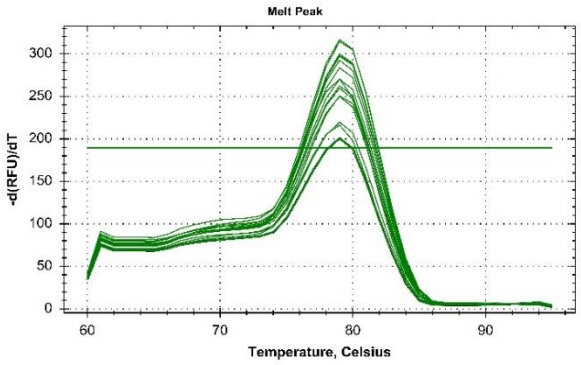


F. verticillioides **standard curve**

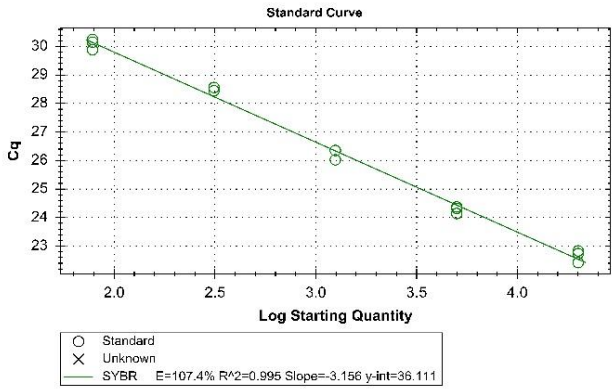
No melt curve due to the use of Taqman probe



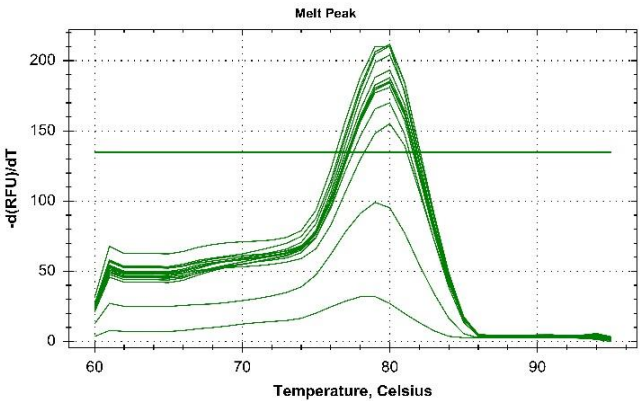
M. phaseolina **standard curve**



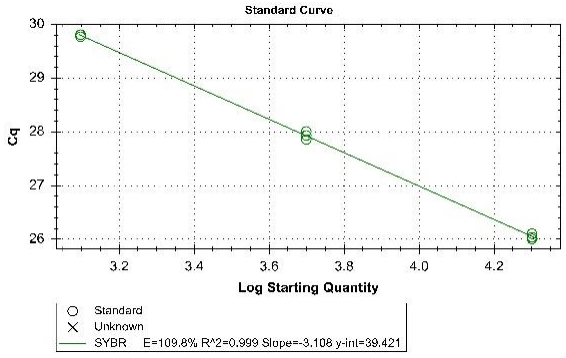
M. phaseolina **melt curve**



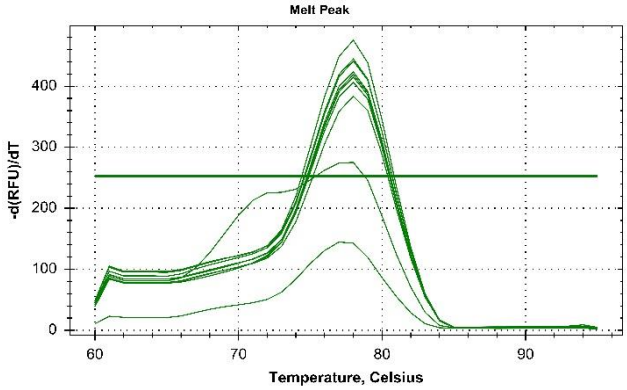
Phoma **spp. standard curve**



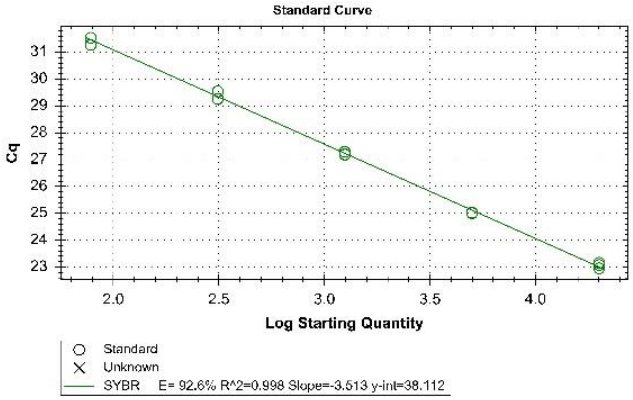
Phoma **spp. melt curve**



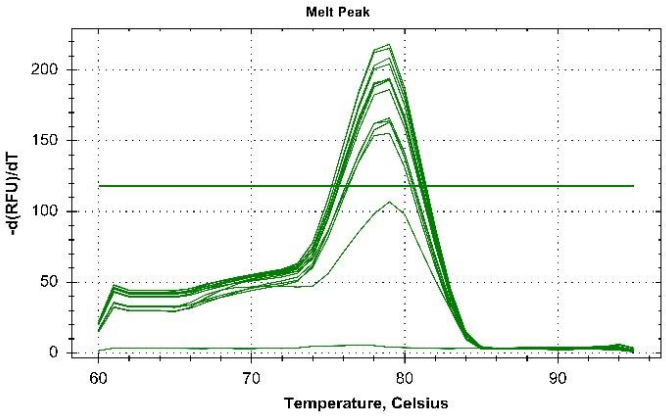
***Pythium* spp. standard curve**



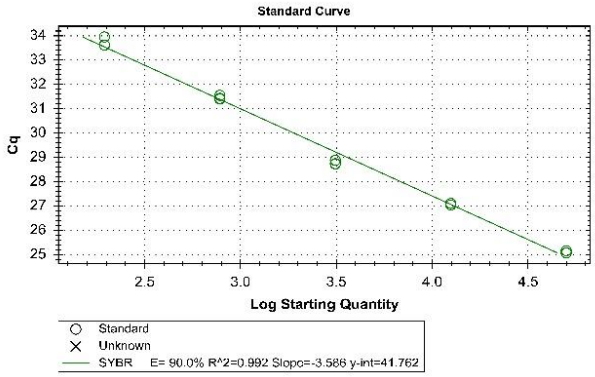
***Pythium* spp. melt curve**



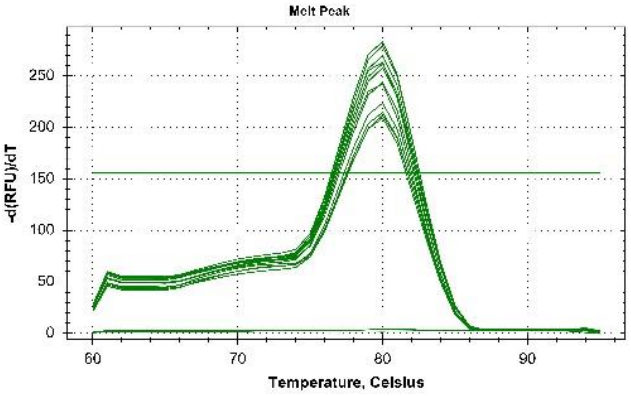
***R. solani* standard curve**



***R. solani* melt curve**



***Trichoderma* spp. standard curve**



***Trichoderma* spp. melt curve**

CHAPTER 3

INTERACTION OF FARMING PRACTICES AND THE COMPOSITION OF THE ROOT AND CROWN ROT COMPLEX IN DIFFERENT PROVINCES IN SOUTH AFRICA USING QPCR

3.1 Abstract

Maize cultivation in South Africa mainly takes place in three of the nine provinces, with differing environmental conditions and agricultural practices. The constant increase in food demands, growing populations and climate changes make it necessary to apply the best management practices in each province on the limited fertile soil available. The aim of this study was to determine if there were geographical areas with certain dominant soilborne fungal pathogens in South Africa under different cultivation practices. For two seasons (2014 and 2015) maize roots and crowns were sampled at fifteen localities in six provinces namely: Gauteng, North West, Free State, KwaZulu-Natal, Mpumalanga and the Northern Cape to determine whether specific root and crown rot fungi have specific geographical dominance. Twelve fungal isolates identified as most commonly occurring in South Africa were analysed through DNA extraction and qPCR (using hydrolysis probe or SYBR Green technology). Statistical analysis included ANOVA, Shapiro-Wilk tests and Fisher's t-tests. The qPCR results showed significant tillage x province interaction for *F. oxysporum*, irrigation x province interaction for *E. pedicellatum* and *R. solani. F. verticillioides* showed significant differences between different rotated crops. *R. solani* was also significantly higher in no-till fields compared to tilled fields, and between rotations with different crops. Some results showed that tillage would be a better strategic management implementation than no-till in certain parts of Mpumalanga and the Northern Cape against *F. oxysporum*. *E. pedicellatum* occurred more frequently on irrigated and dryland fields in the Free State and on dryland fields in the North West province compared to the other provinces. *R. solani* is also more problematic on irrigated fields in the Northern Cape and Free State and dryland fields in the North West province. The use of qPCR technology made it possible to perform an in-depth study of the composition of the root and crown rot fungal pathogens. Continued monitoring of fields using this technology will enable farmers to gather plant pathogenic data and empower them to formulate strategies to prevent these twelve known root and crown rot pathogens from causing devastating epidemics in maize crops.

Key words: Cultivation practices, disease, fungal pathogens, hydrolysis probes, management strategies, provinces, root and crown rot, SYBR Green

3.2 Introduction

The production of agricultural crops is significantly affected by water, air temperature and the photosynthetic radiation (climatic variables) as the driving forces of crop growth (Rosenzwieg *et al*., 1995) which differs for each of the provinces in South Africa. South Africa is the largest producer in the South African Development Community (SADC) region of maize, which is the most important staple food for animal and human consumption (Baleta & Pegram, 2014). Maize is mainly produced in the Free State, Mpumalanga and the North West province (Figure 3.1).

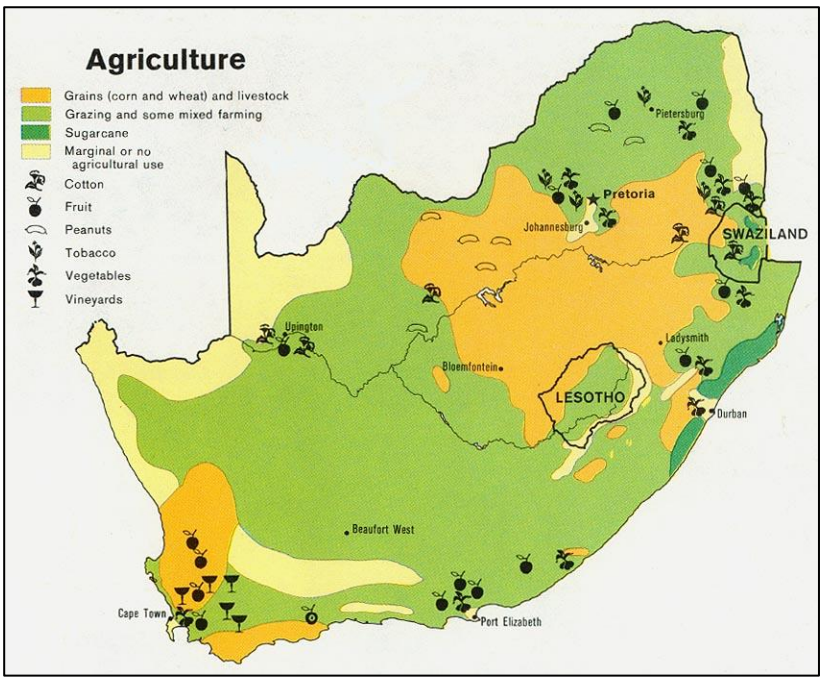


Figure 3.1: Main agricultural products and distribution across South Africa (NationMaster, 2013).

South Africa can be divided into the Highveld, Middleveld and Lowveld regions based on elevation, which contributes to certain regional properties like temperature and rainfall averages. For example, the soil in the Lowveld areas consists of larger varieties of soil types compared to the other regions (Coetzee, 2015). Deep, porous, acidic and well-drained soil can be found in the higher and wetter western parts, while in the lower, drier central and eastern part shallow fertile soils with good water retention prevails (Coetzee, 2015). The Highveld region of SA (parts of North West, Mpumalanga, Free State and the whole of Gauteng) as agro-ecosystem produces 70% of SA's commercially grown cereal of which 90% is maize (du Toit *et al.,* 2000). Between 2001 and 2014, South Africa produced an average of approximately 11.3 million tons of maize per year (JADAFA, 2014).

Environmental conditions and changing weather (extreme conditions) vary globally and regionally, influencing the maize yields and consequently the pathogen presence (Rosenzwieg *et al.,* 2001). In SA yields of 4.96 metric tons per hectare commercially and 1.1 metric tons per hectare for subsistence farms can be achieved under good climatic conditions, compared to the decreased 2.67- and 0.5 metric tons per hectare respectively when drought conditions occur (JADAFA, 2014). Wet and dry conditions contribute to ecological settings which either favour or hinder emergence or re-emergence of vectors and pathogens leading to disease. Temperatures and rainfall are critical factors in agriculture, greatly influencing crop yields, pests, pasture growth and the chance of fire outbreaks (Rosenzwieg *et al.*, 2001) and with these parameters certain regions can be grouped together. Heavy rainfall leads to crop infrastructure damage and erosion of productive topsoil, while drought directly influences commodity prices and yield success. All parts of maize plants (roots, crowns, stems, and ears) are susceptible to numerous diseases that influence plant growth resulting in yield losses (White, 1999).

Root and crown rot is a complex disease that involves more than one fungus and includes interactions with bacteria, insects and nematodes. The fungi present in the soil and on the roots will differ in different maize growth stages, different environments and climatic conditions, on different maize genotypes and due to different management practices applied. In sandy soil *Rhizoctonia* crown and brace root rot is most likely to prevail (Anees *et al*., 2010), while wet weather conditions (humidity, dew and free moisture) favours *Pythium* root and stalk rot and seedling blight as well as *Rhizoctonia* crown and brace rot. Where waterlogging occurs in the maize field, *Fusarium* root and crown rot will occur together with *Pythium* and *Rhizoctonia* (Wise *et al*., 2016). Cool climatic conditions will favour *Pythium* root rot, while warmer conditions will increase *Pythium* and *F. graminearum* stem rot (Wise *et al.,* 2016). Root rot and diseases of different species have similar symptoms (rotted roots, reduced roots and lesions) making diagnostic ability based only on symptoms difficult and requires further molecular diagnostic identification (Wise *et al*., 2016). With the use of Real-time or quantitative PCR (qPCR) the detection and quantification of the twelve most commonly occurring pathogens of maize root and crown rot can be determined. This technique uses enzymatic reactions that amplify a specific region of the DNA of a target species (fungal pathogen) after which the presence of the fungal pathogen in the sample is quantified (Chaplin *et al*., 1999). It is a highly sensitive, specific and fast method for quantification to replace the classical identification (morphologically based) which is time consuming, requires experts in taxonomy and has limited biomass quantification (Niessen, 2007).

The occurrence of the root rot fungal complexes in each region and province will differ, due to different field and weather conditions and different soil types, in terms of diseases present and

the severity, while some similarities can also occur. The aim of this study was to determine the composition of the root and crown rot fungal complex occurring throughout maize production areas of South Africa using qPCR technology.

3.3 Materials and methods

3.3.1 Locality and plots sampled

During the 2014 and 2015 seasons, sampling of maize roots and crowns were done at fifteen localities in six provinces namely: Gauteng, North West, Free State, KwaZulu-Natal, Mpumalanga and the Northern Cape (Figure 3.2).

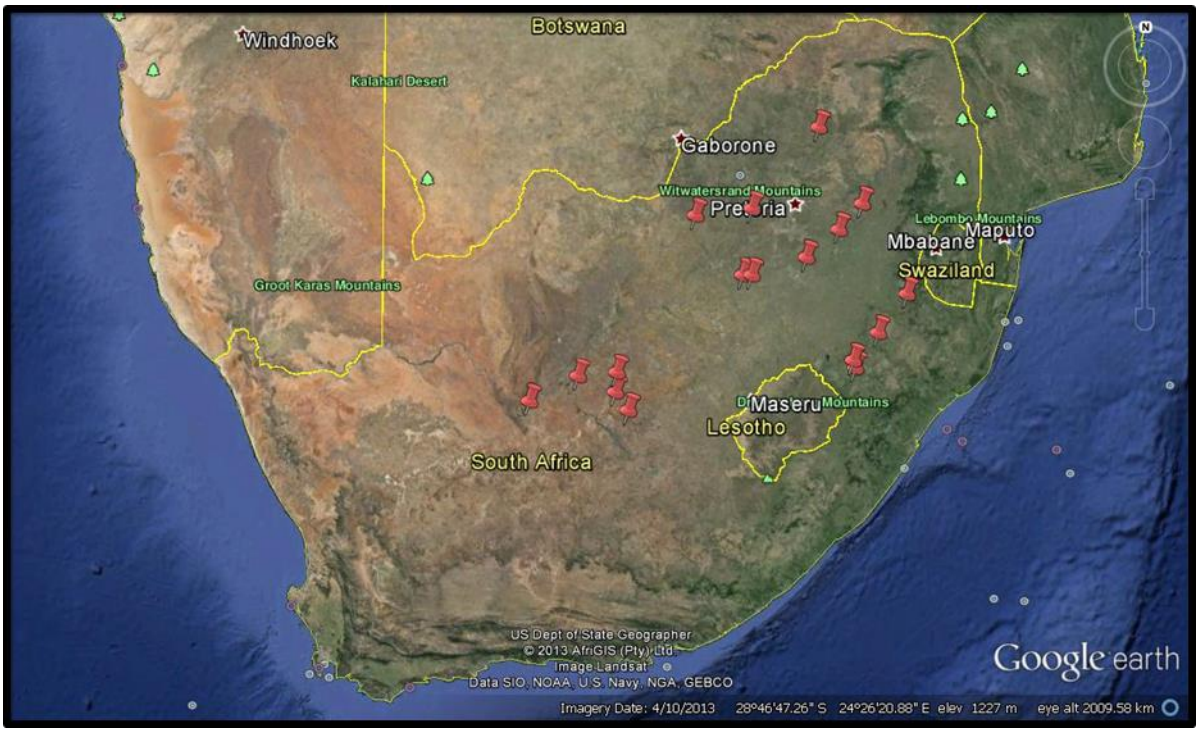


Figure 3.2: The 15 localities that were sampled across the different provinces in South Africa during seasons 2014 and 2015.

In these provinces both irrigated and dryland fields were used to sample from. In the first season, 38 fields were sampled and in the second season 29 fields, depending on farmer cooperation. The province, locality and crop information are summarized in Appendix B.

3.3.2 Sampling

Ten plants showing symptoms of disease or stress were sampled at a physiologically mature stage, approximately 100 days after planting, on the various farms. Sampling needed to be done when the growth stopped and before drying off (for optimal above ground / visual symptoms). The

roots were washed and separated from the crowns, cut into small pieces and stored at -8°C until DNA extraction was done.

3.3.3 Crop rotations, cultivation and irrigation

Fields that were deeply ploughed to prepare the seedbeds and for weed control were compared to fields where no tillage practices were applied. No-till conserves previous crop residue, soil moisture, reduse soil erosion and leaves a higher organic matter and carbon content in the soil. Some fields were completely dependent on rainfall, while others used for comparison were subjected to pivot irrigation. Monoculture maize as the main crop of the study were compared to maize fields that were rotated with soybeans, sunflowers, wheat, potatoes and white beans.

3.3.4 Molecular analysis of fungal pathogen complex

3.3.4.1 DNA extraction

The root and crown samples were crushed separately using liquid nitrogen with a mortar and pestle. DNA was extracted using the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Möller *et al,* 1992). Approximately 0.25 ml ground material was added to 2-ml centrifuge tubes (Eppendorf, Hamburg, Germany) together with 1 ml DNA extraction buffer (DEB: 0.2 M Tris HCl (Tris(hydroxymethyl)aminomethane hydrochloride), 0.15 M NaCl (sodium chloride), 0.025 M EDTA (ethylenediamine tetra-acetic acid), 0.5% SDS (sodium dodecyl sulfate), the plant material mixture frozen in the -80°C freezer for 1 hour and put into boiling water for 5 minutes. In the following step 600 µl phenol:chloroform:isoamylalcohol (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes. The top aqueous layer was removed and added into a new tube. Immediately after 200 µl 2xCTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris pH 8, 20 mM EDTA, 0.2% β-mercaptoethanol pH 8.0) and 400 µl chloroform:IAA (24:1) were added to the tube and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes, the top aqueous layer removed and added to a new tube. In the following step 60 µl 3 M Sodium acetate and 800 µl 100% ice cold ethanol were added to the supernatant, inverted and centrifuged for 10 minutes at 14 000 rpm. The supernatant was then discarded and 500 µl of 70% ethanol added to wash the DNA pellet. The samples were then centrifuged for 5 minutes at 14 000 rpm. The pellet was left to dry in the laminar flow (1 hour) and resuspended in 50 µl Low TE buffer (10 mM Tris pH 8 (T8443), 1 mM EDTA (AppliChem, Germany). The samples were then stored at -80°C until the DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and the quality determined with the A_{260}/A_{280} ratio.

3.3.4.2 Quantification of fungal species

The DNA samples were diluted to 10 ng/µl for further use. qPCR was performed with a Bio-Rad CFX 96 thermal cycler (United States, Hercules, USA) using hydrolysis probes (Taqman® probe) and/or SYBR Green protocols and the fungal mass was quantified as pg/µl for the following species: *F. oxysporum*, *F. verticillioides*, *F. equiseti*, *F. graminearum*, *F. chlamydosporum*, *C. eragostidis*, *M. phaseolina*, *Trichoderma* spp., *Pythium* spp., *Phoma* spp., *R. solani* and *E. pedicellatum* (Schoeman, 2016).

The primers and probes used were designed to target the translocation elongation factor 1α (*TEF*1) gene of *F. equiseti*, *F. graminearum*, *F. verticillioides*, *F. oxypsorum* and *T. longibrachiatum*. The internal transcribed spacer region (ITS) of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *M. phaseolina*, *P. sorghina*, *P. periilum* and *R. solani* was used for primer and probe design. (Schoeman, 2016). The amplification product of SYBR Green and Hydrolysis probe protocols was sequenced in order to confirm the specificity of the amplified product (amplicon) and to ensure that the correct fungal species are identified (Schoeman, 2016).

3.3.4.3 SYBR Green protocol

The SYBR Green protocol was carried out in 25 µl reaction volumes containing 10 µl Bio-Rad iTaq Universal SYBR Green supermix (Hercules, USA), 0.4 µM of the forward and reverse primers, 0.8 ng/µl DNA and RNase/DNase free water (Biolab Diagnostics, Wadeville, S.A.). The PCR cycles consisted of denaturation 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, 95°C for 30 sec and 40°C for 30 seconds. A melt curve was done at 60°C to 95°C, increment of 1.0°C for 10 sec followed by the plate read. The SYBR Green protocol was used for the quantification of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *F. graminearum*, *F. oxysporum*, *Pythium* spp., *Phoma* spp., *R. solani, Trichoderma* spp. and *M. phaseolina* as the SYBR Green bind to double stranded DNA, generate detectable fluorescence, and as result the amount of signal is proportional to the amount of double stranded DNA present . The primer sequences are summarized in Table 3.1.

3.3.4.4 Hydrolysis probe protocol

The hydrolysis probe protocol was carried out in 20 µl reaction volumes containing 10 µl Bio-Rad iQ, 0.3 µM of the forward and reverse primers, 0.2 µM of the probe, 0.8 ng/µl of DNA) and RNase/DNase free water (Biolab Diagnostics, Wadeviile, S.A.) The denaturation took place at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and annealing at 60°C for 1 min (with plate read). The hydrolysis probe protocol was used for *F. verticillioides* and *F. equiseti* for

optimum quantification results (with Taqman probes). The probe sequences can also be found in Table 3.1.

Table 3.1: Twelve fungal pathogens, separate primer and probe sets and melt temperatures.

Fungal pathogen	Forward primer	Reverse primer	qPCR technique (probe if applicable)	Melt temperature	Culture	References
C. eragostidis	CurASCF 5'-GCC CAA AGA CTC GCC TTA AA-3'	CurASCR 5'- GAT GGA TTG CTG GCC TCT TTA G-3'	SYBR	82 °C	PPRI 5447	Schoeman (2016)
E. pedicellatum	ExPBASF 5'- AGC CGG CCT ACT GGT TTC -3'	ExPBASR 5'- CCT ACC TGA TCC GAG GTC AA-3'	SYBR	82 °C	PPRI 10037	Schoeman (2016)
F. chlamydosporum	F.chl ASCF 5'- CAC ATA TTC AAC GCC AAG ACA C-3'	F.chl ASCR 5'- TGT ATC TTC TTC TCT TCA CCC TTC -3'	SYBR	78 °C	PPRI 4580	Schoeman (2016)
F. equiseti	Feqi ASDF 5'- TTA CAC TCA TAA CCT TCT CAT GC-3'	Feqi ASDR 5'- CAA TGA TGA GAA TAG CGC AAT CG-3'	Taqman probe Fequi probe 5'- /5Cy5/CA TGT ATT CCA GAC GCT CCC GGT C/3IAbRQSp/ Cy5	-	PPRI 7735	Schoeman (2016)
F. graminearum	Gram2F 5'- CCC TCT TCC CAC AAA CCA TT-3'	Gram2R 5'- GCT TCC TAT TGA CAG GTG GTT A -3'	SYBR	77 °C	M13.08	Schoeman (2016)
F. oxysporum	FoxyASCF 5'- CTC TCC TCG ACA ATG AGC AT -3'	FoxyASCR 5'- GGT CTG TGA AAC GAT GTC AGT A -3	SYBR	78 °C	PPRI 7729	Schoeman (2016)
F. verticillioides	Vert1F 5'- CGC GTT TCT GCC CTC TC -3'	Vert1R 5'- TCG GAT GGT TAG TGA CTG CT -3'	Taqman probe Verti probe 5'- /5TEX615/CC ACA ACC TCA CTG AGC TCA TCG T/3IAbRQSp/-3' Texas Red	-	MRC 826	Schoeman (2016)
M. phaseolina	MacPASCF 5'- GCA ATC CTG TCG GAC TGT T-3'	MacPASCR 5'- GCG ATG CCG ATA CCA AGA T-3'	SYBR	79 °C	PPRI 1051	Schoeman (2016)
Phoma spp.	PhoASCF 5'- GCT CTG GTG TCT ACA ATG G -3'	PhoASCR 5'- GTC AGT TCT AGT ACC TCG TTG AAG -3'	SYBR	78 °C	PPRI 10098	Schoeman (2016)
Pythium spp.	PytASDF 5'- GGT TAC GCC TGG AAG TAT GT -3'	PytASDR 5'- CAA TCA AAC AAC CGA CGA CTA C -3'	SYBR	78 °C	PPRI 20772	Schoeman (2016)
R. solani	RhizASCF 5'- TGT TAT GCT TGG TTC CAC TCG-3'	RhizASCR 5'- GGA CTA TTG GAA GCG GTT CAT C -3'	SYBR	77 °C	PPRI 10376	Schoeman (2016)
Trichoderma spp.	TriASDF 5'-GGG TGC GTA TTC CAT CAA TCA -3'	TriASDR 5'- CAC GGT GGT CGA CTT TCC-3'	SYBR	80 °C	PPRI 9138	Schoeman (2016)

3.3.4.5 Data analysis

Standard curves were generated for each fungal pathogen by diluting each species' DNA, obtained from the national databank PPRI (Plant Protection Research Institute), (approximately 10 ng/µl) 4x, 16x, 64x, 256x and 1024x, and the extracted maize DNA (target DNA) were compared to these standard curves. Three independent qPCR runs were done for each sample. Using the standard curve, the C_t value was transformed into DNA concentration for each fungal pathogen in each trial analysed. To ensure that the unknown samples fall in the acceptable range compared to the standard curve of the known fungal pathogen, the efficiency was between 90-110% (optimal), the R^2 >0.95 (optimal) and the slope -3.32 (optimal). Melt curve values of the unknown samples that differed more than ± 1°C, were not used in the analysis (Appendix B).

3.3.5 Statistical analysis

A combined, factorial analysis of variance (ANOVA) was performed with significance at P=0.05, using years and localities (respectively) as repetitions, comparing the factors; irrigation-systems, tillage- and crops and their interaction effects. The standardized residuals were normaly distributed (Shapiro-Wilks test) and therefore the means of the significant effects were separated using Fisher's t-test (least significant difference – LSD) tested at the 5% level of significance (Douglas C. M., 1984). All data analyses were performed using SAS v9.3 (SAS, 1999).

3.4 Results

The results of the qPCR (Appendix B) indicated that the unknown samples gathered over the two seasons were consistant with the standard curves of each fungal pathogen (separately) using species specific primers. The target DNA data for the crowns were insufficient for comparison to data of the roots. No distinction could be made between interactions of the different parameters (year x tillage x irrigation x crop x province) based on the crowns and roots. The total fungal mass (60 samples) of the presence of the fungal pathogens over the two seasons showed that *F. graminearum* was the most abundant with 1 389 622 pg/µl, followed by *F. equiseti* with 872 533 pg/µl, *R. solani* with 747 699 pg/µl and *F. oxysporum* 108 525 pg/µl. *C. eragostidis* had the lowest fungal mass of only 3 pg/µl (Table 3.2). The extremely high values found need to be confirmed or proven wrong based on further similar studies.

Table 3.2: The total fungal pathogen mass (pg/µl) over two seasons, in descending order.

Fungal pathogens	Total fungal pathogen mass (pg/µl)
F. graminearum	1 389 622
F. equiseti	872 833
R. solani	747 699
F. oxysporum	108 252
E. pedicellatum	56 056
Trichoderma spp.	30 144
F. verticillioides	11 222
Phoma spp.	6 864
F. chlamydosporum	3 360
Pythium spp.	3 037
M. phaseolina	1 099
C. eragostidis	3

Table 3.3 Analysis of variance of the impact of irrigation, tillage, crop choice, province and the interaction of the variables on *F. oxysporum.*

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Irrigation	1	65 791 503.6	65 791 503.6	1.75	0.20
Tillage	1	40 705 108.9	40 705 108.9	1.07	0.31
Irrigation x Tillage	1	12 687 491.5	12 687 491.5	0.33	0.57
Crop	5	250 747 435.2	50 149 487.0	1.31	0.29
Irrigation x Crop	1	1 977 065.7	1 977 065.7	0.05	0.82
Province	4	310 856 446.5	77 714 111.6	2.04	0.12
Irrigation x Province	1	6 005 175.8	6 005 175.8	0.16	0.70
Tillage x Province	1	383 950 884.1	383 950 884.1	10.06	**0.00**

From the twelve pathogens tested using qPCR, significant tillage x province interaction (Table 3.3) for *F. oxysporum* occured. Overall had the highest species complex target DNA per kapita over the two years were found in North West, while the Northern Cape had the lowest total concentration of target DNA. *F. oxysporum* were not further influenced significantly by crop rotation or irrigation practices. In KwaZulu-Natal, tillage practices had a significant effect (P≤0.05) on *F. oxysporum* presence (24 185 pg/µl) (Table 3.4). Mpumalanga had very low *F. oxysporum*

presence in tilled fields (123 pg/µl) (Table 3.4). In the Northern Cape *F. oxysporum* had the lowest presence (84 pg/µl) in tilled fields compared to all the other provinces (Table 3.4). However, there was not a significant difference between tilling practices in the provinces, except for KwaZulu Natal.

Table 3.4: T-test of the maize roots and crown samples across South Africa for *F. oxysporum* showing a significant tillage x province interaction (Significance P≤0.05).

Tillage x Province	Mean
Till x KwaZulu-Natal	24185 a
Till x Gauteng	5320 b
No-till x Mpumalanga	3628 b
No-till x Free State	2010 b
Till x Free State	1914 b
No-till x North West	1649 b
No-till x KwaZulu-Natal	1537 b
No-till x Northern Cape	136 b
Till x Mpumalanga	123 b
Till x Northern Cape	84 b

The irrigation x province interaction significantly effected *E. pedicellatum* infection (P= 0.02) (Table 3.5).

Table 3.5: Analysis of variance of the impact of irrigation, tillage, crop choice, province and the interaction of the variables on *E. pedicellatum.*

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Irrigation	1	20189528.1	20189528.1	1.87	0.18
Tillage	1	165099.5	165099.5	0.02	0.90
Irrigation x Tillage	1	1901987.8	1901987.8	0.18	0.68
Crop	5	147088470.2	29417694	2.73	**0.04**
Irrigation x Crop	1	258529633.0	25852963.3	2.40	0.13
Province	4	28641690.8	7160422.7	0.66	0.62
Irrigation x Province	1	70151662.7	70151662.7	6.51	**0.02**
Tillage x Province	1	153824.1	153824.1	0.01	0.91

In the Free State *E. pedicellatum* had the highest presence on dryland fields (5 032 pg/µl) (Table 3.6) and was the only significant difference compared to the other provinces and irrigated fields. *E. pedicellatum* had the lowest presence (218 pg/µl) on irrigated fields in KwaZulu-Natal and on dryland fields (127 pg/µl) in Mpumalanga (Table 3.6).

Table 3.6: T-test of the maize roots and crown samples across South Africa for *E. pedicellatum* showing significant irrigation x province interaction (Significance P≤0.05).

Irrigation x Province	Mean
Dryland x Free State	5032 a
Dryland x North West	4128 ab
Irrigation x Free State	1510 ab
Irrigation x Northern Cape	500 b
Dryland x KwaZulu-Natal	348 b
Irrigation x Gauteng	239 b
Irrigation x KwaZulu-Natal	218 b
Dryland x Mpumalanga	127 b

Table 3.7: Analysis of variance of the impact of irrigation, tillage, crop choice, province and the interaction of the variables on *R. solani.*

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Irrigation	1	313646547	313646547	0.54	0.47
Tillage	1	4604139419	4604139419	7.98	**0.01**
Irrigation x Tillage	1	1617003	1617003	0.00	0.96
Crop	5	35535528359	7107105672	12.32	**<0.00**
Irrigation x Crop	1	9906672	9906472	0.02	0.90
Province	4	4342567093	1085641773	1.88	0.15
Irrigation x Province	1	2721414294	2721414194	4.72	**0.04**
Tillage x Province	1	1524324724	1524324724	2.64	0.12

The irrigation x province interaction significantly influenced *R. solani* infection (P= 0.04). *R solani* were further influenced significantly by tillage (P= 0.01) and crop rotation (P< 0.00) (Table 3.7). Tillage had a significantly higher infection than no-till, while infection in maize were also significantly higher compared to all the other rotated crops, with potatoes having the lowest infection.

R. solani infection were the lowest on irrigated (2 230 pg/µl) and dryland (2 320 pg/µl) fields in KwaZulu-Natal compared to all the other provinces, while *R. solani* were the highest on irrigated fields (40 755 pg/µl). No definite preference for irrigated or dryland fields can be seen for or *R. solani* (Table 3.8).

Table 3.8: T-test of maize roots and crown samples across South Africa for *R. solani* showing significant irrigation x province interaction (Significance P≤0.05).

Irrigation x Province	**Mean**
Irrigation x Northen Cape	40755 a
Dryland x North West	37040 a
Irrigation x Free State	22184 ab
Dryland x Mpumalanga	19244 ab
Dryland x Free State	8360 ab
Irrigation x Gauteng	3084 b
Dryland x KwaZulu-Natal	2320 b
Irrigation x KwaZulu-Natal	2230 b

R. solani's interaction with tillage practices resulted in significantly higher occurrence when no-till (29 085 pg/µl) was applied compared to tilled (8 300 pg/µl) fields (P=0.01) (Figure 3.3).

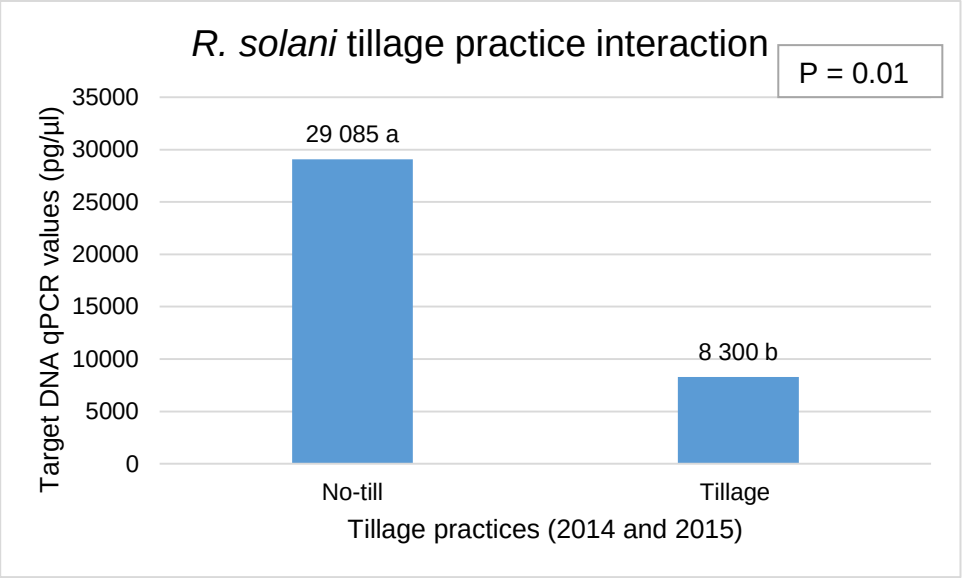


Figure 3.3: *R. solani* target DNA of the maize root and crown samples across South Africa after tillage and no-till practices (Significance P⩽0.05).

There were also significant differences (P<0.00) where potato was used as a rotated crop (206 211 pg/µl) compared to the other crops (sunflower, wheat, soybean, dry bean and maize in decreasing order) for *R. solani*, with maize having the lowest concentration (3 822 pg/µl) (Figure 3.4). However, the differences in occurrence of *R. solani* between sunflower, wheat, soybeans, white beans and maize were not significant.

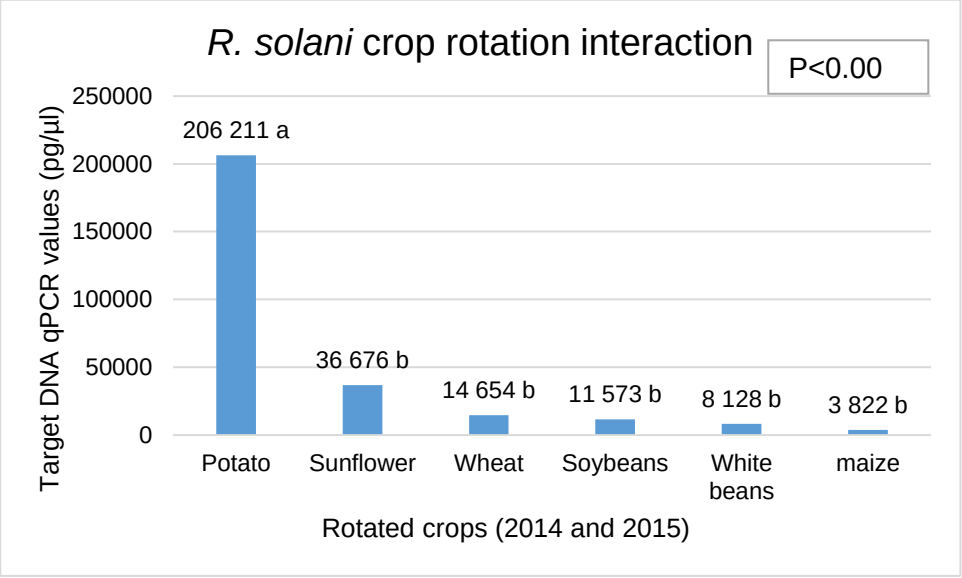


Figure 3.4: *R. solani* target DNA of maize root and crown samples across South Africa after rotating maize with maize, potato, soybean, sunflower, wheat and white beans (Significance P⩽0.05).

Significant differences occurred between the crop rotation (P= 0.00) with soybean and potato or white beans for *F. verticillioides* (Table 3.9), with the highest occurrence of the pathogen target DNA on soybean (576.6 pg/µl) and after the rotation of soybean with maize (291.2 pg/µl), white bean maize rotation had the lowest pathogen (11.2 pg/µl) value (Figure 3.5). *F. verticillioides* had only been significantly influenced by the different rotated crops used, and not by tillage practices, irrigation or by the different provinces (Table 3.9). There was also a significant irrigation x crop rotation interaction for *F. verticillioides* (P=0.05), where soybean on irrigated fields had the highest interaction (201,6 pg/µl) and white beans on dryland fields the lowest interaction (11.2 pg/µl) (Table 3.10).

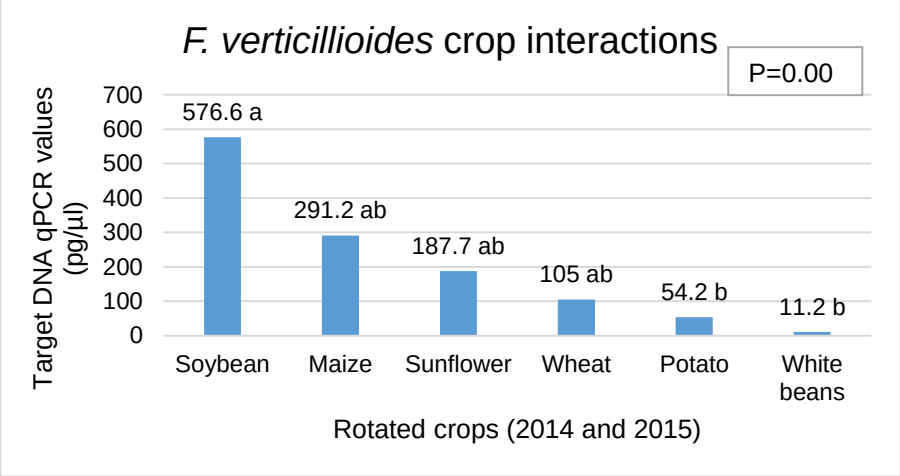


Figure 3.5*: F. verticillioides* target DNA of the maize root and crown samples across South Africa after rotating maize with maize, potato, soybean, sunflower, wheat and white beans (Significance P≤0.05).

Table 3.9: Analysis of variance of the impact of irrigation, tillage, crop choice, province and the interaction of the variables on *F. verticillioides.*

Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
Irrigation	1	330335.105	330335.015	2.54	0.12
Tillage	1	97678.060	97678.060	0.75	0.39
Irrigation x Tillage	1	568962.955	658962.955	4.37	0.06
Crop	5	3933163.679	786632.736	6.05	**0.00**
Irrigation x Crop	1	532310.616	532310.616	4.09	**0.05**

Province	4	822389.091	205597.273	1.58	0.21
Irrigation x Province	1	66825.856	66825.856	0.51	0.48
Tillage x Province	1	99868.608	99868.608	0.77	0.39

Table 3.10: T-test of the maize roots and crown samples across South Africa for *F. verticillioides* showing significant irrigation x crop rotation interaction (Significance P⩽ 0.05).

Irrigation x Crop rotation	Mean
Irrigation x Soybean	706 a
Dryland x Soybean	548 a
Dryland x Maize	345 a
Irrigation x Maize	202 b
Dryland x Sunflower	188 b
Irrigation x Wheat	105 b
Irrigation x Potato	54 c
Dryland x White bean	11 c

It was noticeable, but not significant that between the twelve species tested for in 2014, *F. graminearum* were the most abundant in the roots and crowns in North West (crowns 77%, roots 57.1%) and Gauteng (crowns 68.4%, roots 54%), while only on the roots for Free State (86.1%) and KwaZulu-Natal (89%) (Figure 3.6 and 3.7). *Fusarium* spp. is known to be found under a wide range of soil temperature and moisture levels and it correlates with its presence in all the provinces over the two seasons. *F. equiseti* were the most abundant in the crowns in the Northern Cape (40.1%), Free State (61.3%) and KwaZulu-Natal (91.3%) and for the roots in Mpumalanga (64.1%) for the 2014 season. *Trichoderma* spp. occurred most in the crowns in Mpumalanga (87.7%) (Figure 3.6 and 3.7).

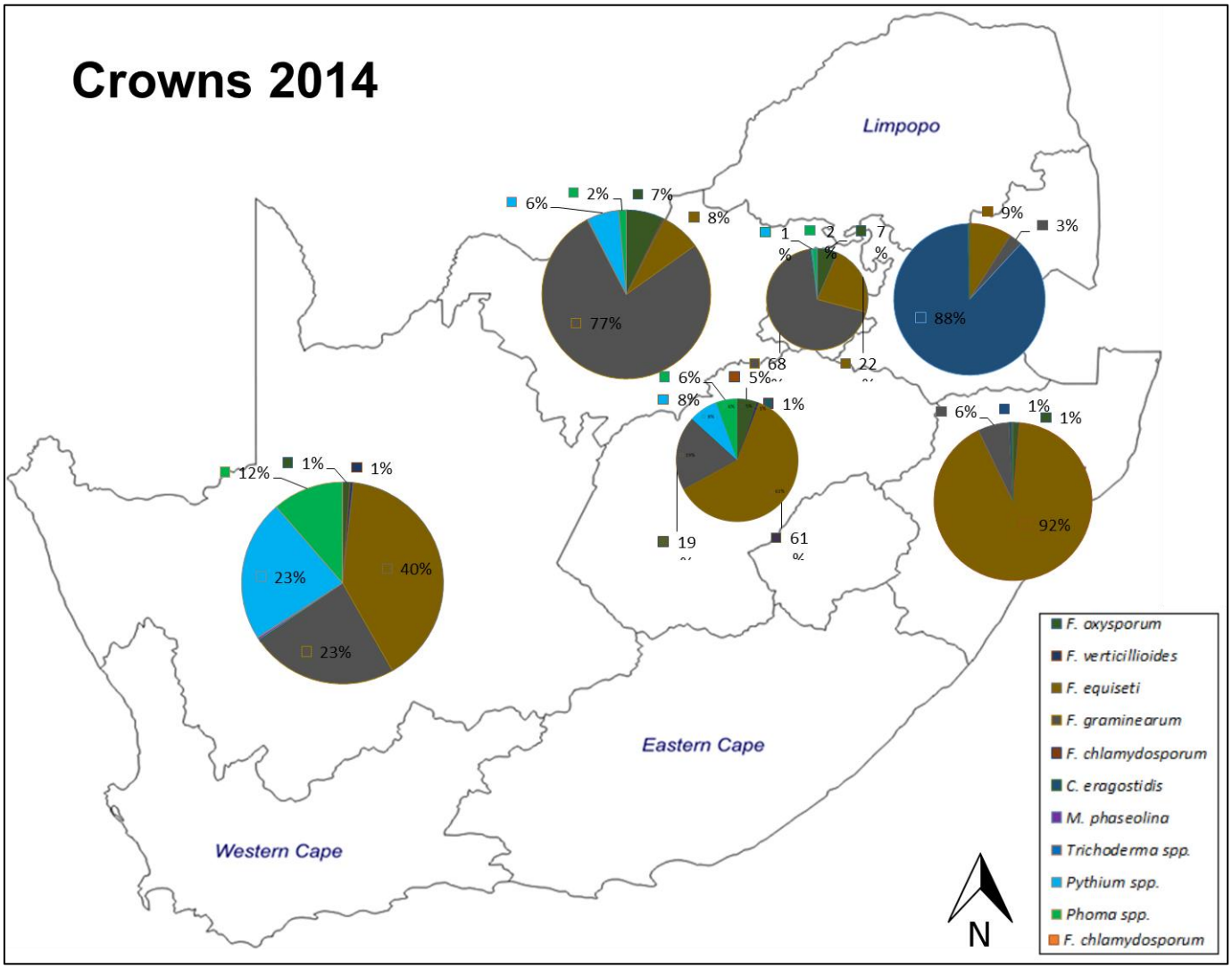


Figure 3.6: Circle graph in percentage of fungal pathogen presence per province, during the 2014 growing season in the crowns.

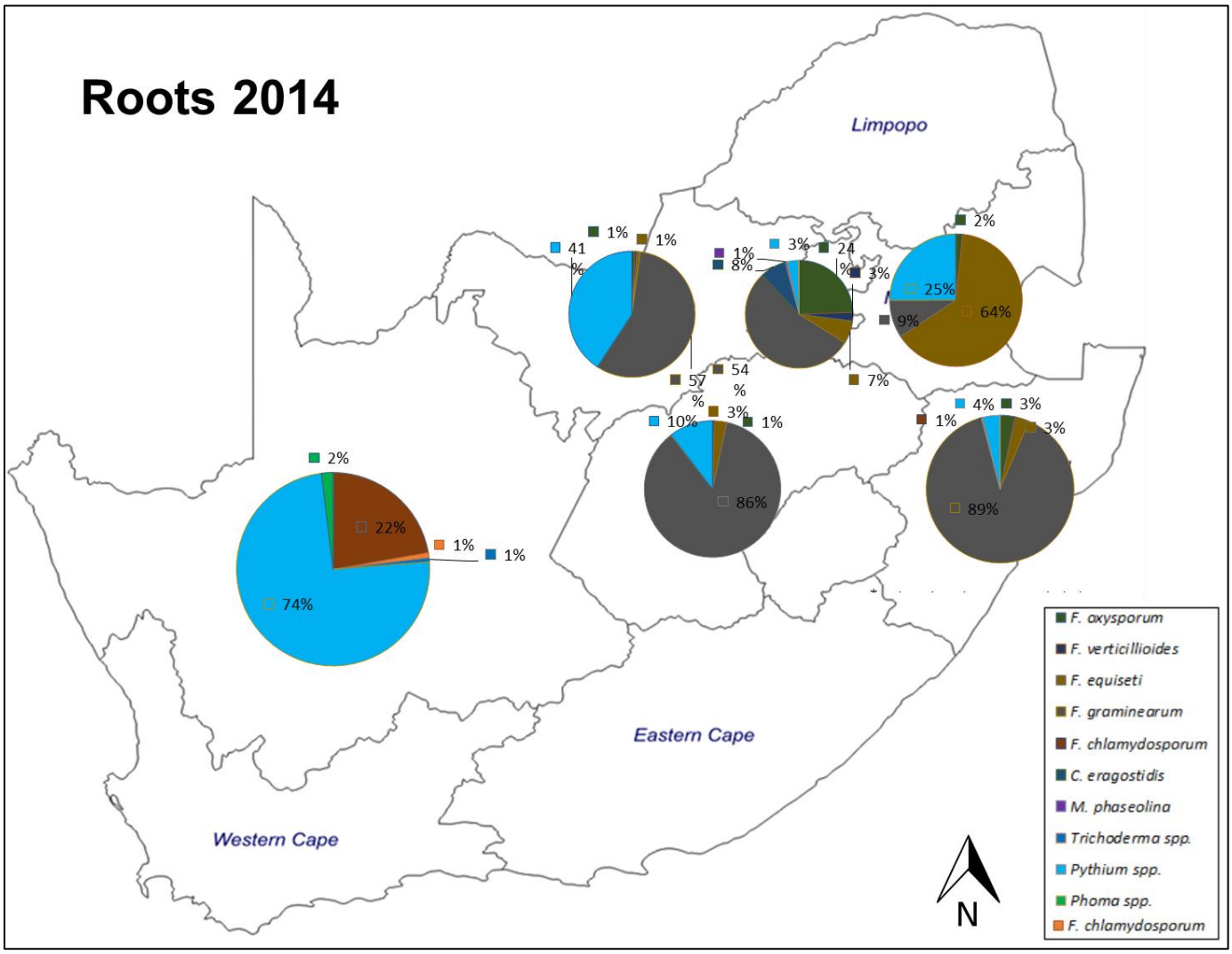


Figure 3.7: Circle graph in percentage of fungal pathogen presence per province, during the 2014 growing season in the roots.

In the 2015 season, *F. verticillioides* were the most abundant in the roots (90.8%) and crowns (63.2%) of the Free State, and the roots of North West (88.1%), Mpumalanga (95.7%), Gauteng (96.3%) and KwaZulu-Natal (89.5%). *F. equiseti* were the most abundant in the crowns of North West (81%) and Gauteng (99.1%). *F. verticillioides* occurred mostly in the crowns in KwaZulu-Natal (75.9%) (Figure 3.8 and 3.9).

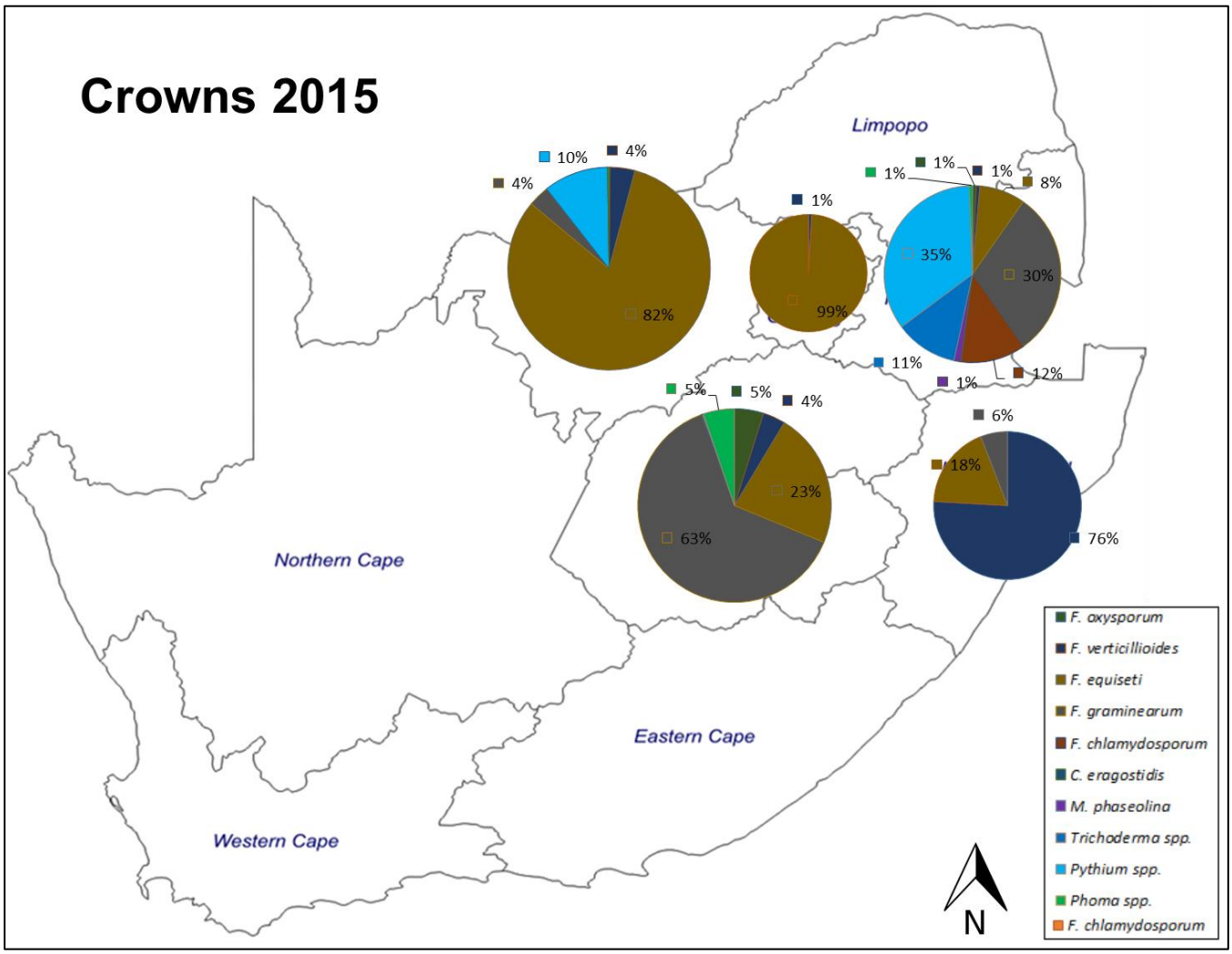


Figure 3.8: Circle graph in percentage of fungal pathogen presence per province, during the 2015 growing season in the crowns.

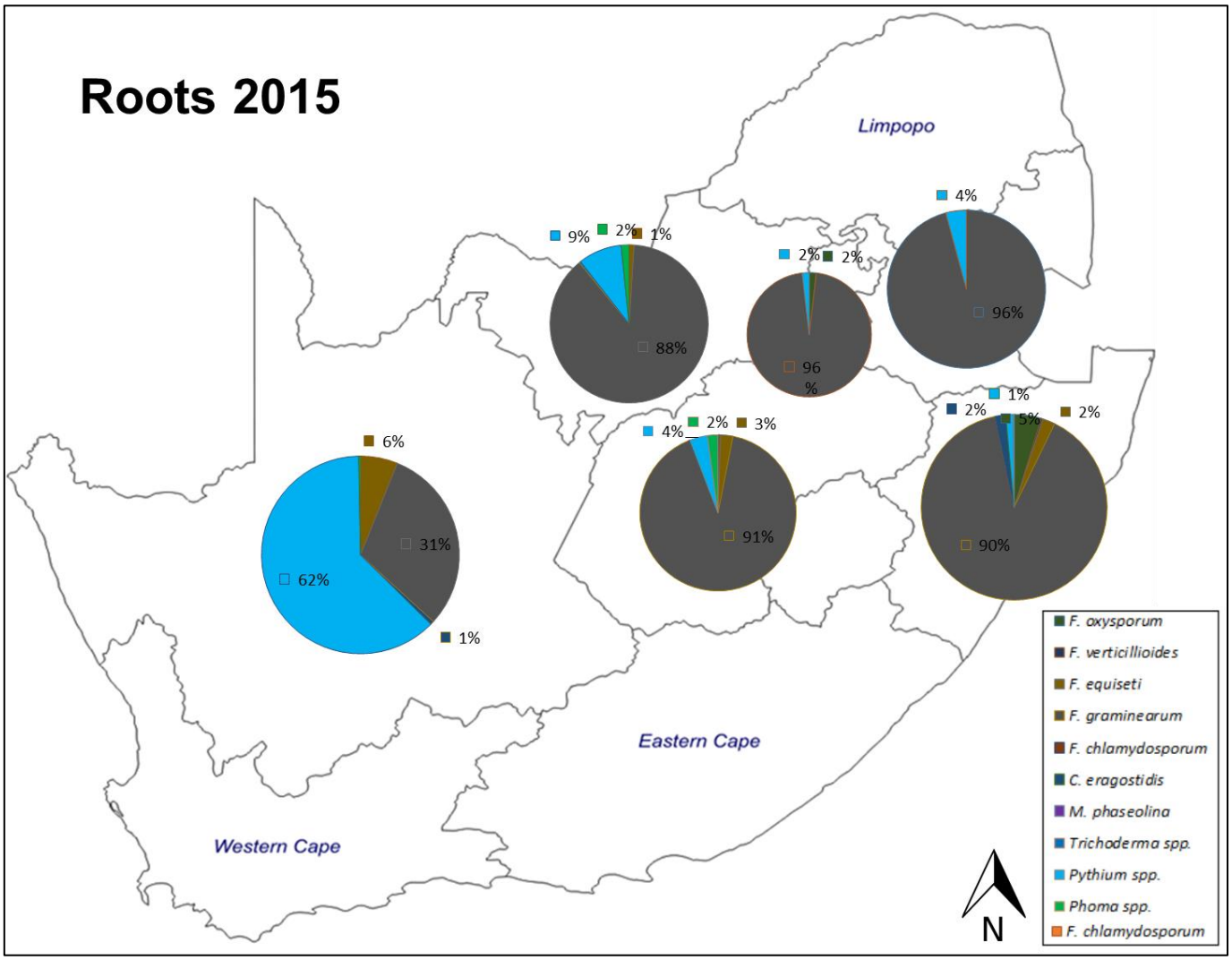


Figure 3.9: Circle graph in percentage of fungal pathogen presence per province, during the 2015 growing season in the roots.

3.5 Discussion and conclusion

The use of qPCR technology offers a unique way in studying different variables contributing to the occurrence and severity of root and crown rot diseases. In this study it was possible to include different geographic regions, cultivation practices as well as seasons. The contribution of the variable climatic conditions in South Africa across the provinces influence the pathogen species presence and the fungal complex changes over seasons (years) and differs in plant parts within a season leading to different fungi being important in root and crown rot diseases (Wise *et al.,* 2016).

The results of this study indicated that the qPCR methods could successfully be applied to identify and quantify different root and crown rot fungal isolates. The complex of twelve commonly occurring fungal pathogens investigated across South Africa, shows the dominance and persistence of *F. graminearum* in KwaZulu-Natal, North West and Gauteng. In contrast Boutigny *et al.* (2012) that did a study on maize grain, found *F. graminearum* commonly and dominantly occurred in the western maize production area (Western Free State) with some similarities to this study on the roots and crowns, as both found North West to have high *F. graminearum* presence. Pathogens in the grain can differ from the pathogens found in the roots and crowns and could explain the variance.

Environmental conditions influence the dominance of fungal species in South Africa: over two seasons with different rainfall and temperatures the species complex found and the abundance of each will differ. According to Wise *et al.* (2016) wet weather conditions (high humidity, waterlogged soils) that commonly occur under irrigation and in high rainfall areas in KwaZulu-Natal will be likely to favour *Pythium*, *Rhizoctonia* and *Fusarium* crown and root rot. A maize plant is most susceptible to fungal pathogen infection under stress conditions: too wet or too dry conditions, compacted soils, cold temperatures and extreme warm temperatures or due to herbicide injury and when plant fertility problems occur (Wise *et al.,* 2016).

Pythium spp. presence in the Northern Cape where hot and dry conditions prevail, which doesn't normally favour *Pythium* (Agrios, 2005) can be explained by irrigation on all these fields. Tissue specificity throughout the trial favoured the roots, but *Phoma* spp. occurred more in the crowns than the roots. *Phoma* spp. primarily infect host plants through wounds caused by cultivation practices, interactions with other organisms or favourable weather conditions (Aveskamp *et al.*, 2008), which explain the high percentage of *Phoma* spp. in the crowns for the 2014 season where tillage was applied on al the fields in the Northern Cape. The application of different cultivation practices contributed to the root and crown rot fungal composition. From the results in certain

parts of Mpumalanga and the Northern Cape Province, *F*. *oxysporum* presence can be reduced or tend to be low under tillage practices, this could be because of soil disturbance or direct sun exposure (Summerell *et al.*, 2011). *E. pedicellatum* is most likely to be problematic on dryland and irrigated fields in the Free State and on dryland fields in North West. *R. solani* is most likely to be problematic on irrigated fields in the Northern Cape and Free State and dryland fields in the North West Province but is not restricted to specific soil temperatures and moisture levels. This corresponds with the findings of Wise *et al.* 2016, which states that sandy soils (as occurring in some parts of the Northern Cape, Free State and North West provinces) are ideal conditions for *Rhizoctonia* crown and root rot. The development of *Rhizoctonia* can be enhanced by aerated soils (Abbas *et al*., 2014), and can be the reason for higher pathogen numbers or more severe disease where tilling is implemented. Further deductions that could be made from the results of this study are that *F. verticillioides* presence can most likely be lowered by crop rotation with dry beans and potatoes. It seems that *R. solani* inoculum can be reduced when tillage is applied and crop rotation with soybean, dry beans or monoculture maize are implemented. These conditions might favour another soil borne pathogen within the same niche, reducing *R. solani* increase.

The information gathered and presented makes it possible to develop more focused integrated disease management strategies, especially for root and crown rot on maize and in particular against *E. pedicellatum, F. oxysporum, F. verticillioides* and *R. solani*. Further and continual research like this will broaden and consistently improve the possible methods of disease management in South Africa, specific to fungal pathogens, different climatic regions (provinces) and under various cultivation practices.

3.6 References

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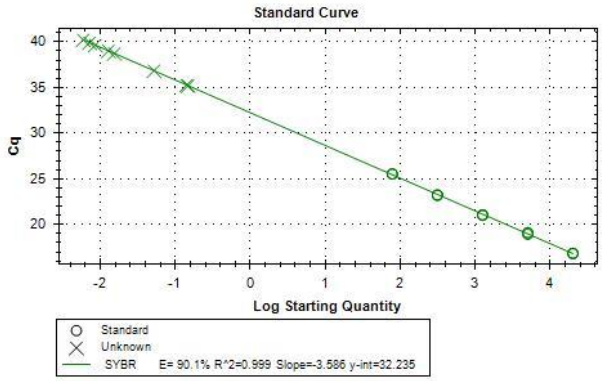
Appendix B: The province, locality, cultivar and planting conditions of the 2013/14 and 2014/15 seasons.

Province	Locality	Maize Cultivar	2013/14 rotations	2014/15 rotations	Dryland/ Irrigated	Plant density of maize	Till/No-till
Free State	Koppies	PHI 30y82BR	Maize/Maize	Maize/Maize	Dryland	16 000	No-till
Free State	Viljoenskroon 1	Dekalb 73-74 BT	Maize/Maize	Maize/Maize	Dryland	28 000	Till
Free State	Vijoenskroon 2	PAN 5Q-649 BR	Soybean/Maize	Maize/Maize	Dryland	24 000	No-till
Free State	Koffiefontein	PHI 31D24B	Wheat/Maize	Wheat/Maize	Irrigated	85 000 to 90 000	Till
Northern Cape	Richie	PAN 6236	Wheat/Maize	Wheat/Maize	Irrigated	85 000 to 90 000	Till
Free State	Jacobsdal 1	PAN 3D736BR	Wheat/Maize	Wheat/Maize	Irrigated	75 000 to 85 000	No-till
Free State	Jacobsdal 2	PAN 6126	Wheat/Maize	Wheat/Maize	Irrigated	75 000 to 85 000	Till
Free State	Jacobsdal 3	P31D22BR	Wheat/Maize	Wheat/Maize	Irrigated	85 000 to 90 000	Till
Northern Cape	Douglas 1	PHI 16-15R	Wheat/Maize	Wheat/Maize	Irrigated	72 000	Till
Northern Cape	Douglas 2	DKC 6478BR	Wheat/Maize	Wheat/Maize	Irrigated	92 000	Till
Northern Cape	Douglas 3	PAN 6236	Wheat/Maize	Wheat/Maize	Irrigated	85 000	Till
Northern Cape	Douglas 4	PAN 6236	Wheat/Maize	X	Irrigated	85 000	Till
Northern Cape	Douglas 5	DKC 6478BR	Wheat/Maize	X	Irrigated	85 000	Till
Northern Cape	Prieska 1	PHB31D22	Wheat/Maize	Wheat/Maize	Irrigated	61 000	Till
Northern Cape	Prieska 2	PHB33y74	Wheat/Maize	Wheat/Maize	Irrigated	65 000	Till
Northern Cape	Prieska 3	PHB33y74	Wheat/Maize	Wheat/Maize	Irrigated	75 000	Till
KwaZulu-Natal	Paulpietersburg 1	PHB30Y83	Soybean/Maize	Soybean/Maize	Dryland	60 000	No-till
KwaZulu-Natal	Paulpietersburg 2	PHB2369	Soybean/Maize	Soybean/Maize	Irrigated	52 000	Till
KwaZulu-Natal	Paulpietersburg 3	PHB32Y87B	X	Soybean/Maize	Irrigated	60 000	No-till
KwaZulu-Natal	Paulspietersburg 4	PAN3Q740BR	Maize/Maize	Soybean/Maize	Dryland	55 000	No-till
KwaZulu-Natal	Paulspietersburg 5	PHB32y87BT	Soybean/Maize	X	Dryland	55 000	No-till
KwaZulu-Natal	Paulpietersburg 6	Mixture of PHB 1615 DKC7374 8524	Soybean/Maize	X	Dryland	55 000	No-till
KwaZulu-Natal	Paulpietersburg 7	DKC 73-70 BGEN	Soybean/Maize	Soybean/Maize	Dryland	38 000	No-till
KwaZulu-Natal	Paulpietersburg 8	PHI33H52	Maize/Maize	X	Dryland	50 000	No-till
KwaZulu-Natal	Paulpietersburg 9	BT maize dekalb	Maize/Maize	X	Dryland	38 000	No-till
KwaZulu-Natal	Paulpietersburg 10	Carnia 7372	X	X	Dryland	55 000	No-till
KwaZulu-Natal	Dundee 1	Pioneer 3 D99	Maize/Maize	X	Dryland	53 000	Till
KwaZulu-Natal	Dundee 2	PHI 30Y83	Maize/Maize	Maize/Maize	Irrigated	60 000	Till
KwaZulu-Natal	Bergville 1	PHI 32Y85	Soybean/Maize	X	Irrigated	85 000	No-till
KwaZulu-Natal	Bergville 2	PHI 32Y85	X	Soybean/Maize	Irrigated	65 000	Till
KwaZulu-Natal	Bergville 3	PHI 32Y85	Wheat/Maize	Wheat/Maize	Irrigated	65 000	No-till
KwaZulu-Natal	Bergville 4	PAN 3Q222	Wheat/Maize	Wheat/Maize	Irrigated	70 000	No-till
KwaZulu-Natal	Winterton 1	P1184	Soybean/Maize	Maize/Maize	Irrigated	80 000	No-till
KwaZulu-Natal	Winterton 2	Pioneer 31M84	Soybean/Maize	Soybean/Maize	Irrigated	58 000	No-till
North West	Lichtenburg1	DKC 78-15	Maize/Maize	Maize/Maize	Dryland	30 000	No-till
North West	Lichtenburg 2	DKC 77-85	Maize/Maize	Maize/Maize	Dryland	24 000	No-till
North West	Lichtenburg 3	DKC 77-85	Maize/Maize	Maize/Maize	Dryland	24 000	No-till
North West	Lichtenburg 4	DKC 77-85	Maize/Maize	Maize/Maize	Dryland	30 000	Till
Gauteng	Carletonville 1	DKC7815BT	Maize/Maize	Maize/Maize	Dryland	35 000	Till
Gauteng	Carletonville 2	DKC7845BR	Maize/Maize	Maize/Maize	Irrigated	65 000	Till
Mpumalanga	Middelburg 1	PAN6Q445	Maize/Maize	Soybean/Maize	Dryland	42 000	Till
Mpumalanga	Delmas 1	PANnar 6q308BT	Maize/Maize	Maize/Maize	Dryland	42 000	Till
Mpumalanga	Ogies 1	Pioneer BT	Maize/Maize	X	Dryland	52 000	No-till

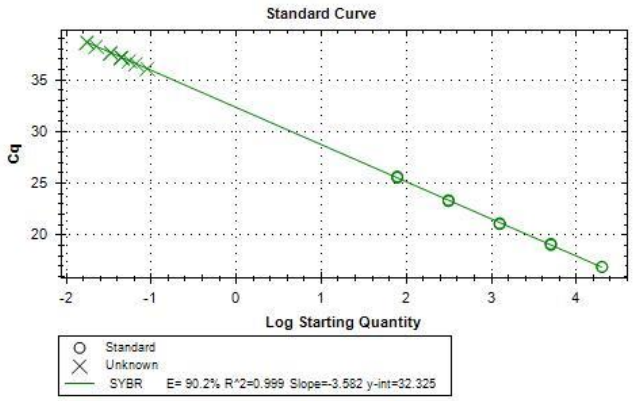
*X- fields left unplanted

Appendix C: Standard curves of the twelve known fungal root and crown rot pathogens in South Africa including the efficiency and R^2 values

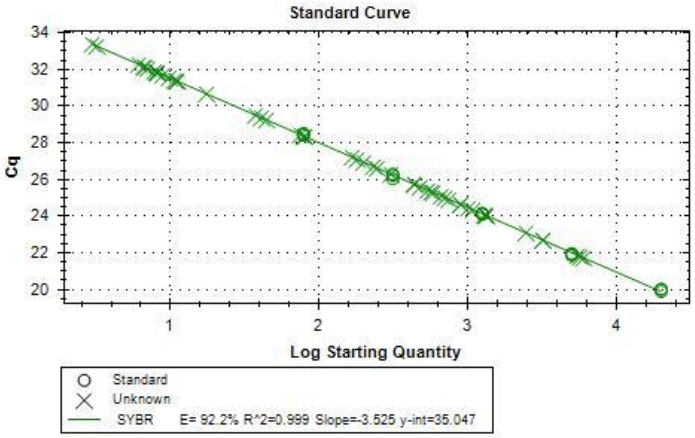
C. eragostidis 2014



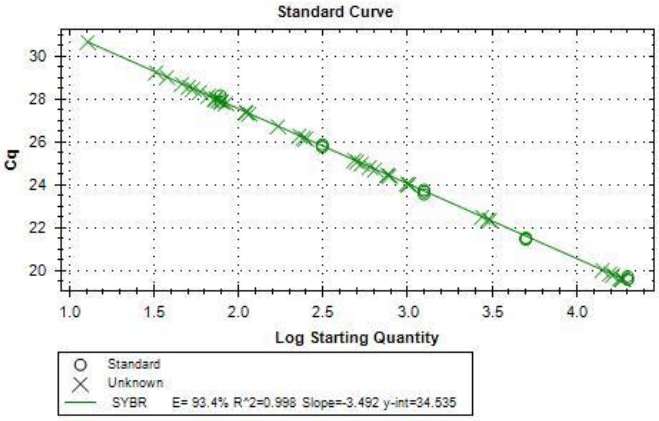
C. eragostidis 2015



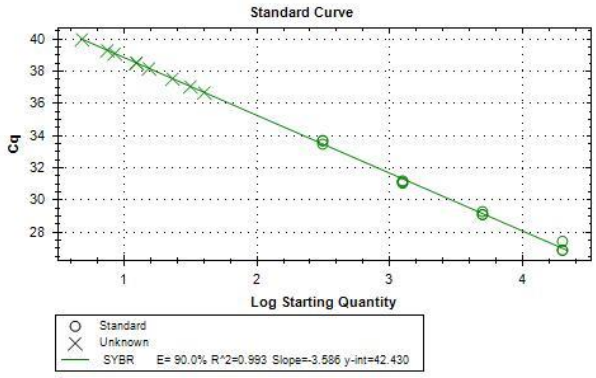
E. pedicellatum 2014



E. pedicellatum 2015



F. chlamydosporum 2014



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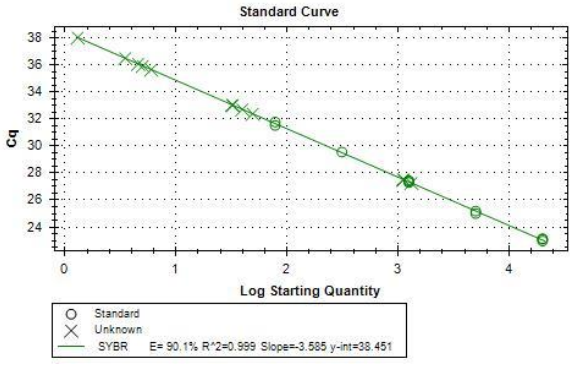
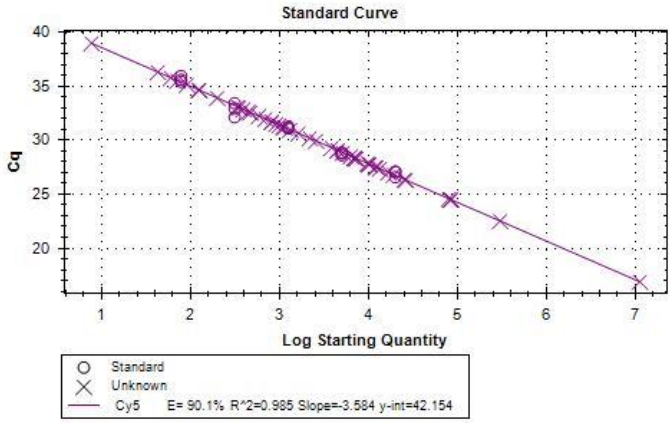
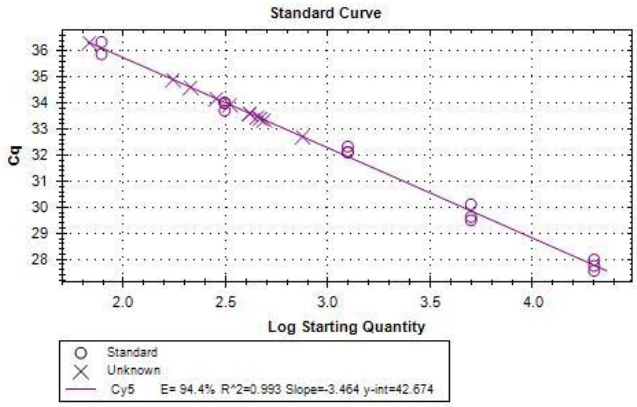


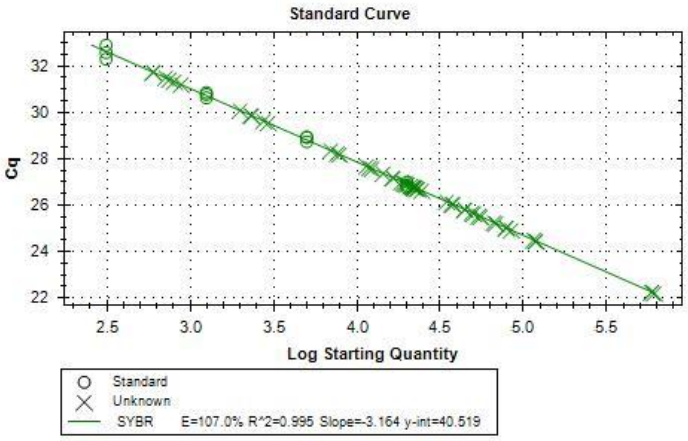
F. equiseti 2014



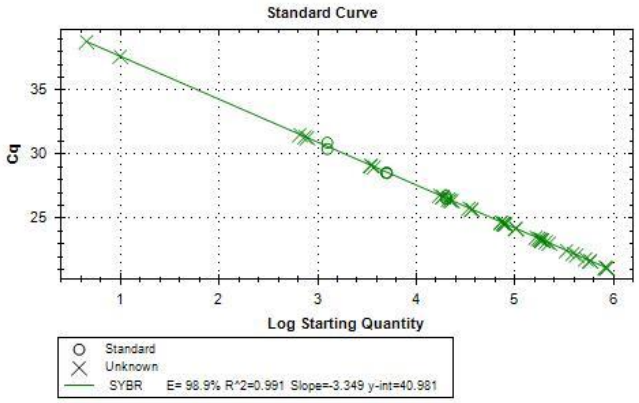
F. equiseti 2015



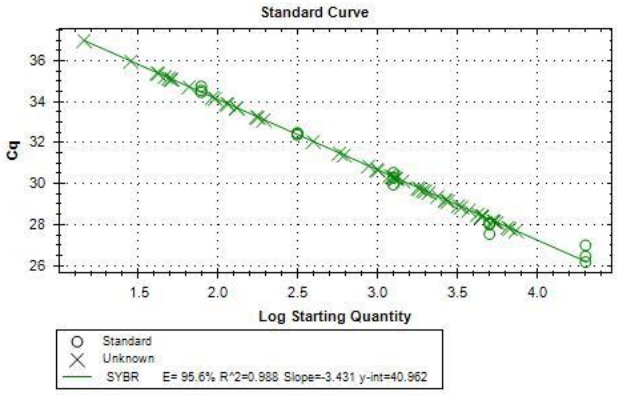
F. graminearum 2014



F. graminearum 2015



F. oxysporum 2014



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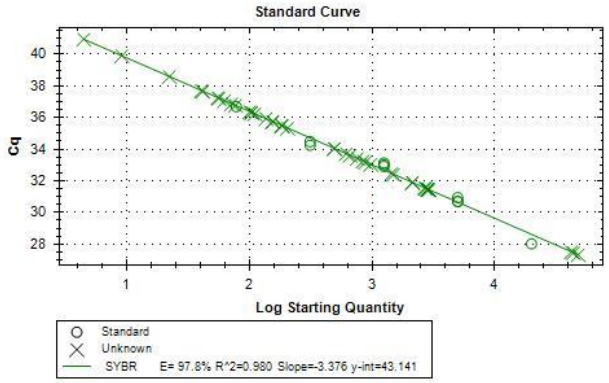
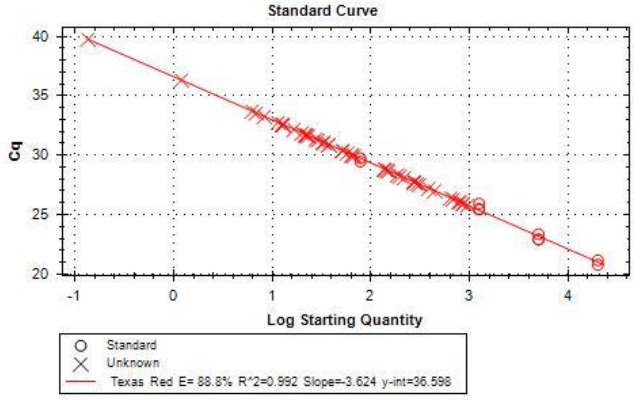
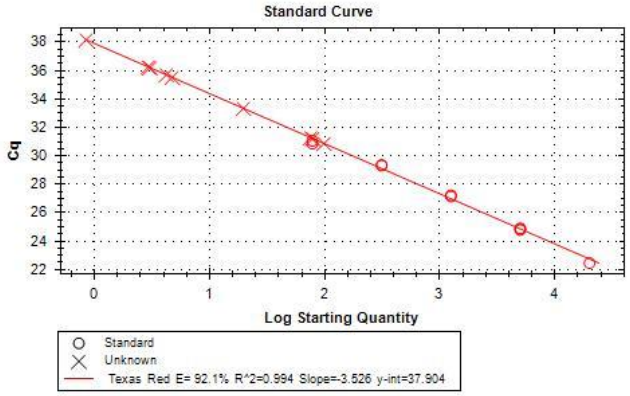


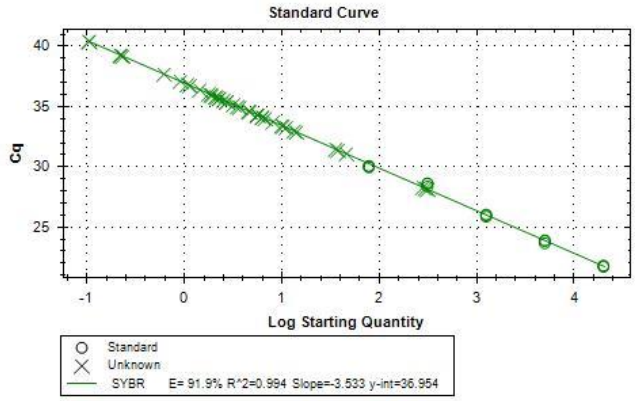
F. verticillioides 2014



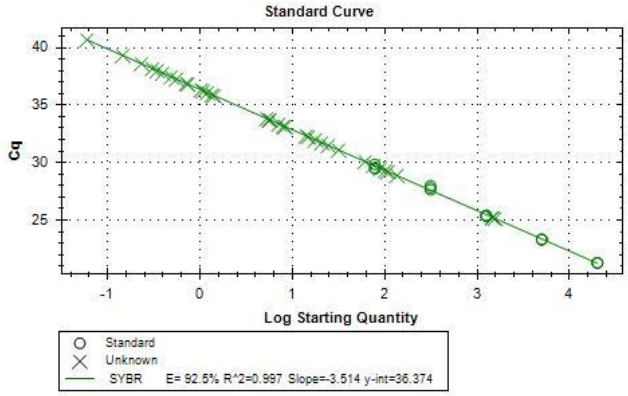
F. verticillioides 2015



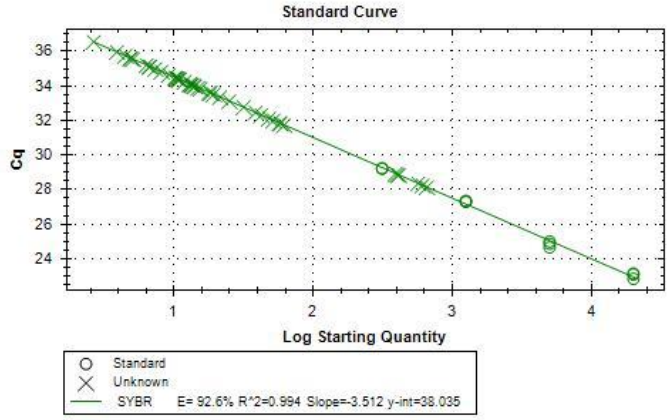
M. phaseolina 2014



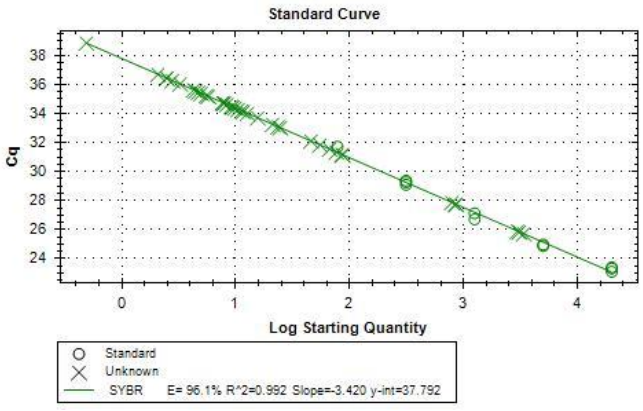
M. phaseolina 2015



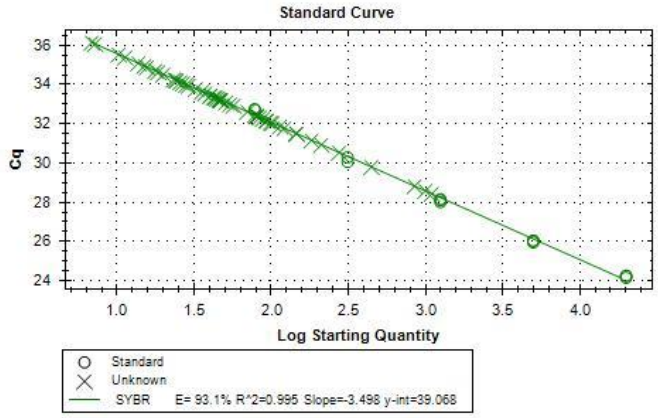
Phoma spp. 2014



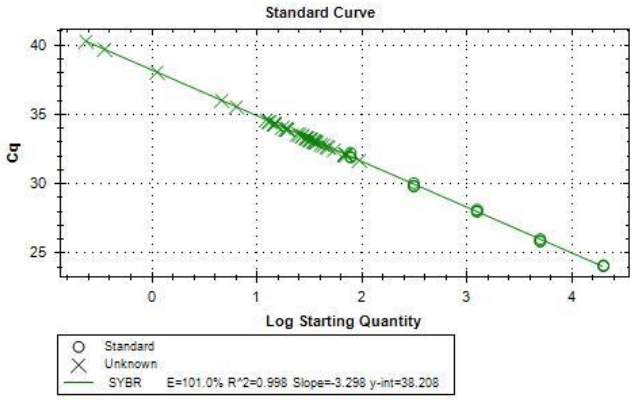
Phoma spp. 2015



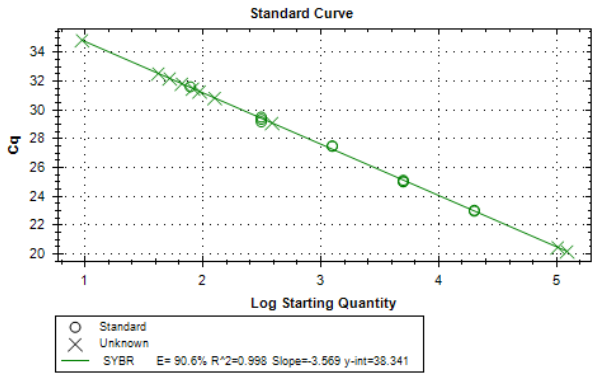
Pythium spp. 2014



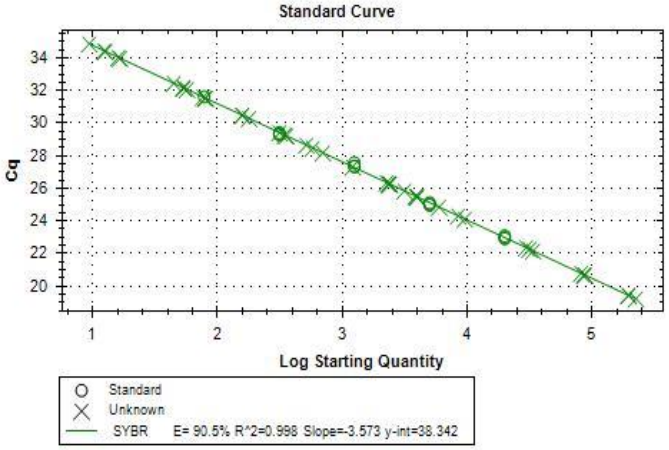
Pythium spp. 2015



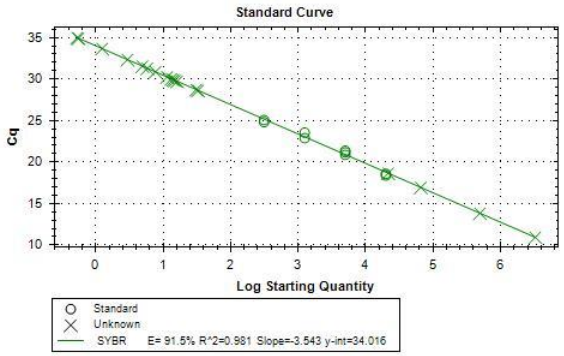
R. solani 2014



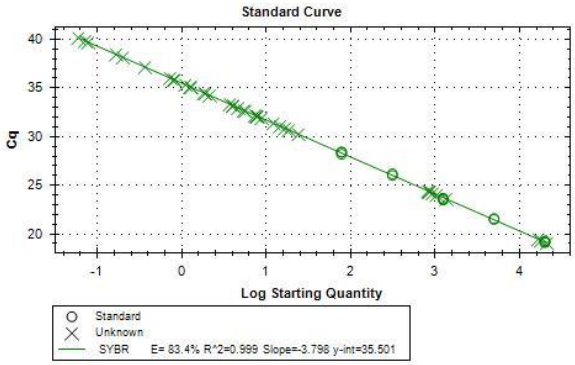
R. solani 2015



Trichoderma spp. 2014



Trichoderma spp. 2015



CHAPTER 4

EVALUATION OF THE SUCCESSION OF MAIZE SOIL BORNE FUNGAL COMPLEX CAUSING ROOT AND CROWN ROT USING DIFFERENT MAIZE CULTIVARS

4.1 Abstract

Soil borne pathogens threaten successful cultivation of maize and the choice of maize cultivar is an important factor contributing to integrated disease management in different environments. Soilborne diseases are caused by different pathogens that change during the season as the maize plant grows. Thus, maize plants become more or less susceptible to these diseases depending on growth stages and other management strategies applied. The succession of the pathogens and evaluation of cultivars readily used can assist in possible management strategies to reduce soil borne disease occurrence and inoculum presence. The aim of this study was to evaluate the succession of the soil borne fungal pathogen complex for the first time during a maize growing season and to understanding the influence of different localities and cultivars on the fungal complex and disease symptom development in maize roots and crowns. Trials were conducted in Potchefstroom and Vaalharts, where three cultivars (BG 3292, IMP 50-10 B and DKC 61-94 BR) were planted and the roots and crowns sampled six times at different developmental stages (seedling, vegetative stage (V-stages: 11 leaf/ tassel and beard appearance), flowering or reproductive (R-stage), blistering, soft dough and dent stage) of the maize plant. The root and crown biomass and root and crown visual disease severity were determined for the samples. qPCR was performed using hydrolysis probe or SYBR Green technology to quantify the target DNA for 12 most commonly occurring fungal pathogens. Statistical analysis was performed using ANOVA, Levene's and the Shapiro-Wilk test. The target DNA showed trends of increase as the plants grow and decrease at the end of the season as the maize dries off for all the cultivars. Cultivar BG 3292 had significantly higher plant biomass for the 70, 80, 90 and 100 DAP sampling dates compared to the other two cultivars. Disease severity showed significant preference for roots rather than the crowns throughout all the growth stages. Cultivar BG 3292 had the lowest severity root and crown rot in both localities compared to the other two cultivars. The qPCR analysis showed significant locality x sampling date interactions for *C. eragostidis* and *E. pedicellatum*. *F. oxysporum* had significant cultivar x plant part x locality interactions*. Phoma* spp. were significantly affected by the sampling date x plant part interaction (pathogen presence increased in the roots with time and decreased in the crowns) and *Pythium* spp. with the sampling date x plant part x locality interaction (pathogen presence increased in the

roots of samples taken from Vaalharts over time and decreased in the crowns of samples taken from Vaalharts and Potchefstroom over time). The sampling date x plant part x cultivar x locality interactions had a significant effect on *Trichoderma* spp. This was the first study to successfully determine the succession and composition of pathogen fungal species in root and crown tissue. For the first time four different variables were evaluated and monitored during a growing season for root and crown rot diseases caused by twelve commonly occurring plant pathogens.

Key words: Cultivars, disease, fungal pathogens, management strategies, root and crown rot, succession.

4.2 Introduction

Various variables affecting the occurrence and severity of root and crown rots make it difficult to do in-depth studies on the topic. The impact of soil borne pathogens on maize production varies every year due to variable weather conditions, hybrid selection, production practices, susceptibility to disease, management practices and geography (Wise *et al.,* 2016). Paulitz *et al*. (2002) states that dominant pathogens forming disease complexes, which cause root rot, differ between different areas and during successive growing seasons, which is one of the many challenges root health associated research is encountering. A maize plant has different developmental stages at which it may or may not be more susceptible to particular diseases. The growth stages can be divided into the vegetative (based on the leaf collar method) and reproductive (based on kernel development) stages (Figure 4.1).

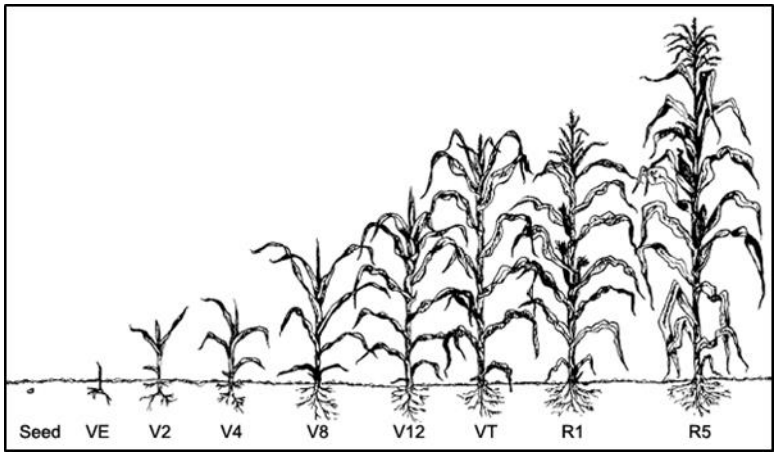


Figure 4.1: Stages of maize development (V=vegetative, R=reproductive) (Wise *et al.,* 2016).

Fusarium root and crown rot is known to occur from the mid-season until late season (VT-R6) (Wise *et al.,* 2016). *Pythium* spp. causing seedling blight and root rot occur prevalently in the early season (VE –V4), while *Rhizoctonia* crown and brace root rot occur in the early and late season (VE-V4, R4-R6) (Wise *et al.,* 2016). Thus knowing the developmental stage and most likely associated disease (pathogens present) will influence the choice of management strategy (Wise *et al.,* 2016).

As maize grows during the season the different growth stages are susceptible to different fungal pathogens. Failure to germinate or seedling blight can be due to *F. graminearum* (White, 1999), *E. pedicellatum* (Shurtleff *et al.,* 1993) *Pythium* spp. and *R. solani* (White 1999). During flowering stage *Fusarium* infections can be more prominent and commonly act as secondary invaders that colonise necrotic roots (Summerell *et al.,* 2011). As the seed starts to senesce *M. phaseolina*, some *Trichoderma* spp., *Pythium* spp. and *R. solani* (White 1999) can be problematic. White

(1999) and Smit *et al*. (1997) stated that certain fungi are favoured by certain environmental conditions (Whitney & Mortimore, 1961). Dodd (1980) stating that after flowering, plants become more subjected to root and stalk rots. At that stage, carbohydrate deficiencies start to occur, making them more susceptible to soil borne pathogens as defence mechanisms fail and at this plant stage environmental stress factors (which include leaf diseases, hail and insect damage) become more prominent (Dodd, 1980).

Plant disease laboratory studies mostly consisted of single microbial strains, lacking the possible interspecies pathogen interactions known as complex communities (Lamichhane & Venturi, 2015). For more effective control measures against plant diseases this complexity of a disease needs to be taken into consideration, hence the improved methods used. In maize diseases (ear, root, crown and stalk rot) pathogen-pathogen synergistic interactions that lead to disease occurrence and increased severity has the following causal pathogens: *F. meridionale* and *F. boothii* for ear rot, *Trichoderma* spp., *Penicillium* spp*., Pyrenochaeta indica, F. verticillioides* (*F. moniliforme*)*, F. graminearum* and *F. oxysporum* for root and stalk rot and *F. boothii, F. graminearum* and *F. meridionale* for crown and root rot (Lamichhane & Venturi, 2015). Each pathogen's succession of host infection in a complex disease and the trophic level at which it takes place can affect the interactions between the pathogens that lead to diseases. The outcome of co-occurrence of pathogens on the same host plant can be antagonistic and/or synergistic which seems to be influenced by the order of their association and occurrence throughout the season (Lamichhane & Venturi, 2015). The risk of certain diseases changes as pathogens continually evolve and change.

Maize hybrid genes also change (through breeding practices) impacting the susceptibility or resistance to diseases (Wise *et al.,* 2016). Information regarding resistance of maize cultivars to root and crown rot pathogens are limited, general treatment for protection is incorporated into seed treatments and cultivars, mostly improving the plant health that makes it less susceptible to certain diseases (Pannar, 2017).

Known anti-microbial strategies currently used in agriculture are mostly specific to control a single pathogen and chemical control becomes limited when a complex of pathogens causes a certain disease, because of the ineffective control of all the pathogens at the same time (Lamichhane & Venturi, 2015). Valuable information was gathered regarding the soil borne pathogens occurring in South Africa when maize root and crown pieces were plated out and each fungal growth morphologically identified (Craven & Nel, 2016; Smit, 1998) but understanding the disease complex as well as cultivar resistance completely is not yet possible. The lack of information of the succession and complex structure of the root and crown rot soil borne pathogens and the resistance against them, throughout the growing season might be due to previous studies that

failed to include these possible interactions. Now qPCR strategies can be used specifically to facilitate the understanding of the species complex causing the disease by tracking and comparing their presence over the entire maize growing season for different maize cultivars.

The aim of this study was to investigate the succession of the soil borne fungal pathogen complex present in maize roots and crowns (*C. eragostidis, E. pedicellatum, F. chlamydosporum, F. equiseti*, *F. graminearum, F. oxysporum, F. verticillioides*, *M. phaseolina*, *Pythium* spp., *Phoma* spp., *R. solani* and *Trichoderma* spp.*)* causing rot during a season (at six-time intervals) in two localities using three maize cultivars, which will be done for the first time using qPCR techniques.

4.3 Materials and methods

4.3.1 Localities, cultivars and plots sampled

Two localities were used with the following coordinates: Potchefstroom (26° 74' 50''S, 27° 07'45.1''E) and Vaalharts (27° 56' 37.7''S, 24° 50' 28.7''E) both under irrigation. Three maize cultivars were used at both trials and replicated three times. Cultivar 1: BG 3292 (Pannar yellow maize, GMO free, control), Cultivar 2: IMP 50-10 B (Agricol yellow maize, Bt-gene), Cultivar 3: DKC 61-94 BR (Monsanto yellow maize Bt-genes), in natural field conditions under irrigation. BG 3293 is a Pannar seed, ultra-yellow BioGene hybrid that has very good stability and standability (the ability of a plant to stand unsupported). This hybrid is agronomically well balanced with good tolerance to *Puccinia sorgi* (rust), *Stenocarpella maydis* and *S. macrospore* (Diplodia ear rot) (Pannar, 2013). DKC 61-94 BR with Genuity Yield Guard II protection yields well under irrigation, has an ultra-short growth season with good yield and standability. It should be planted under high densities and delivers high quality grain (Agricol, 2017). The IMP 50-10 B hybrid offers potentially high yields with single stemmed and mainly single eared plants. It has excellent standing ability planted under irrigation and offers technical advantages of protection against stalk borers and tolerance against glyphosate herbicides (AgriEco, 2013). The border rows were planted with IMP 582/11R at the ends of the field and between the replicates. No herbicides or insecticides were applied after plant.

4.3.2 Sampling and biomass of plant material

Five plants were sampled per replicate per cultivar, six times throughout the season (21, 55, 70, 80, 86 and 100 days after planting (DAP)). The 21 DAP sampling represented the seedling stage, 55 DAP the V-stages at 11 leaf or tassel and beard appearance, 70 DAP the flowering stage (R-stage), 80 and 90 DAP the blistering and soft dough stage and 100 DAP the dent stage. The roots and crowns were washed and weighed as the mass for each sampling stage using a tripod scale.

The samples were cut into smaller pieces and stored at -80˚C until DNA extractions could be done.

4.3.3 Disease ratings

Disease incidence was established using the percentage of plants sampled for each treatment and replicates that demonstrated visual discolouration (some degree of rot) for the roots and crowns. The root and crown rot severity ratings were done seperately in percentage from the plants sampled, by using the root and crown disease index (RDI, CDI) that is based on the adjusted scale ranging from 0-5 (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = <25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, and 5 = dead or totally wilted) The disease severity was calculated as the product of disease incidence x RDI or CDI (Soonthornpoct *et al*., 2001).

4.3.4 Molecular analysis of fungal pathogen complex

4.3.4.1 DNA extraction

Simultaniously visual screenings were done, representative samples of diseased roots and crown material were collected from each locality, six times throughout the season. The samples were crushed using liquid nitrogen with mortar and pestle. Representative amounts (approximately 0.25 ml ground material) were taken from the roots and crowns of the 5 plants sampled per cultivar and for the replicates. DNA was extracted using the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Möller *et al*, 1992), approximately 0.25 ml ground material was added to 2-ml centrifuge tubes (Eppendorf, Hamburg, Germany) together with 1 ml DNA extraction buffer (DEB: 0.2 M Tris HCl (Tris(hydroxymethyl)aminomethane hydrochloride), 0.15 M NaCl (sodium chloride), 0.025 M EDTA (ethylenediamine tetra-acetic acid), 0.5% SDS (sodium dodecyl sulfate), the plant material mixture frozen in the -80°C freezer for 1 hour and put into boiling water for 5 minutes. In the following step 600 µl phenol:chloroform:isoamylalcohol (25:24:1) (Merck, Germany) was added and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes. The top aqueous layer was removed and added into a new tube. Immediately after 200 µl 2xCTAB buffer (2% CTAB, 1.4 M NaCl, 0.1 M Tris pH 8, 20 mM EDTA, 0.2% β-mercaptoethanol pH 8.0) and 400 µl chloroform:IAA (24:1) were added to the tube and mixed by inversion. The samples were then centrifuged at 14 000 rpm for 15 minutes, the top aqueous layer removed and added to a new tube. In the following step 60 µl 3 M Sodium acetate and 800 µl 100% ice cold ethanol was added to the supernatant, inverted and centrifuged for 10 minutes at 14 000 rpm. The supernatant was then discarded and 500 µl of 70% ethanol added to wash the DNA pellet. The samples were then centrifuged for 5 minutes at 14 000 rpm. The pellet was

left to dry in the laminar flow (1 hour) and resuspended in 50 µl Low TE buffer (10 mM Tris pH 8 (T8443), 1 mM EDTA) (AppliChem, Germany). The samples were then stored at -80°C until the DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and the quality determined with the A_{260}/A_{280} ratio.

4.3.4.2 Quantification of fungal species

The DNA samples were diluted to 10 ng/µl for further use. qPCR was performed with Bio-Rad CFX 96 thermal cycler (United States, Hercules, USA) using hydrolysis probes (Taqman® probe) or SYBR Green protocols and the target DNA was quantified as pg/µl for the following species: *F. oxysporum*, *F. verticillioides*, *F. equiseti*, *F. graminearum*, *F. chlamydosporum*, *C. eragostidis*, *M. phaseolina*, *Trichoderma* spp., *Pythium* spp., *Phoma* spp., *R. solani* and *E. pedicellatum*. The primers and probes used were designed to target the translocation elongation factor 1α (*TEF*1) gene of *F. equiseti*, *F. graminearum*, *F. verticillioides*, *F. oxypsorum* and *T. longibrachiatum*. The internal transcribed spacer region (ITS) of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *M. phaseolina*, *P. sorghina*, *P. periilum* and *R. solani* was used for primer and probe design (Schoeman, 2016). The amplification product of SYBR Green and Hydrolysis probe protocols were sequenced in order to confirm the specificity of the amplified product (amplicon) and to ensure that the correct fungal species are identified (Schoeman, 2016).

4.3.4.3 SYBR Green protocol

The SYBR Green protocol was carried out in 25 µl reaction volumes containing 10 µl Bio-Rad iTaq Universal SYBR Green supermix (Hercules, USA), 0.4 µM of the forward and reverse primers, 0.8 ng/µl DNA and RNase/DNase free water (Biolab Diagnostics, Wadeville, S.A.). The PCR cycles consisted of denaturation 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, 95°C for 30 sec and 40°C for 30 seconds. A melt curve was done at 60°C to 95°C, increment of 1.0°C for 10 sec, followed by the plate read. The SYBR Green protocol was used for the quantification of *C. eragostidis*, *E. pedicellatum*, *F. chlamydosporum*, *F. graminearum*, *F. oxysporum*, *Pythium* spp., *Phoma* spp., *R. solani, Trichoderma* spp. and *M. phaseolina* as the SYBR Green bind to double stranded DNA, generate detectable fluorescence, and as result the amount of signal is proportional to the amount of double stranded DNA present. The primer sequences are summarized in Table 4.1.

4.3.4.4 Hydrolysis probe protocol

The hydrolysis probe protocol was carried out in 20 µl reaction volumes containing 10 µl Bio-Rad iQ, 0.3 µM of the forward and reverse primers, 0.2 µM of the probe, 0.8 ng/µl of DNA) and RNase/DNase free water (Biolab Diagnostics, Wadeviile, S.A.) The denaturation took place at

95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and annealing at 60°C for 1 min (with plate read). The hydrolysis probe protocol was used for *F. verticillioides* and *F. equiseti* for optimum quantification results (with Taqman probes). The probe sequences can also be found in Table 4.1.

Table 4.1: Twelve fungal pathogens, separate primer and probe sets and melt temperatures.

Fungal pathogen	Forward primer	Reverse primer	qPCR technique (probe if applicable)	Melt temperature	Culture	References
C. eragostidis	CurASCF 5'-GCC CAA AGA CTC GCC TTA AA-3'	CurASCR 5'- GAT GGA TTG CTG GCC TCT TTA G-3'	SYBR	82 °C	PPRI 5447	Schoeman (2016)
E. pedicellatum	ExPBASF 5'- AGC CGG CCT ACT GGT TTC -3'	ExPBASR 5'- CCT ACC TGA TCC GAG GTC AA-3'	SYBR	82 °C	PPRI 10037	Schoeman (2016)
F. chlamydosporum	F.chl ASCF 5'- CAC ATA TTC AAC GCC AAG ACA C-3'	F.chl ASCR 5'- TGT ATC TTC TTC TCT TCA CCC TTC -3'	SYBR	78 °C	PPRI 4580	Schoeman (2016)
F. equiseti	Feqi ASDF 5'- TTA CAC TCA TAA CCT TCT CAT GC-3'	Feqi ASDR 5'- CAA TGA TGA GAA TAG CGC AAT CG-3'	Taqman probe Fequi probe 5'- /5Cy5/CA TGT ATT CCA GAC GCT CCC GGT C/3IAbRQSp/ Cy5	-	PPRI 7735	Schoeman (2016)
F. graminearum	Gram2F 5'- CCC TCT TCC CAC AAA CCA TT-3'	Gram2R 5'- GCT TCC TAT TGA CAG GTG GTT A -3'	SYBR	77 °C	M13.08	Schoeman (2016)
F. oxysporum	FoxyASCF 5'- CTC TCC TCG ACA ATG AGC AT -3'	FoxyASCR 5'- GGT CTG TGA AAC GAT GTC AGT A -3	SYBR	78 °C	PPRI 7729	Schoeman (2016)
F. verticillioides	Vert1F 5'- CGC GTT TCT GCC CTC TC -3'	Vert1R 5'- TCG GAT GGT TAG TGA CTG CT -3'	Taqman probe Verti probe 5'- /5TEX615/CC ACA ACC TCA CTG AGC TCA TCG T/3IAbRQSp/-3' Taxas Red	-	MRC 826	Schoeman (2016)
M. phaseolina	MacPASCF 5'- GCA ATC CTG TCG GAC TGT T-3'	MacPASCR 5'- GCG ATG CCG ATA CCA AGA T-3'	SYBR	79 °C	PPRI 1051	Schoeman (2016)
Phoma spp.	PhoASCF 5'- GCT CTG GTG TCT ACA ATG G -3'	PhoASCR 5'- GTC AGT TCT AGT ACC TCG TTG AAG -3'	SYBR	78 °C	PPRI 10098	Schoeman (2016)
Pythium spp.	PytASDF 5'- GGT TAC GCC TGG AAG TAT GT -3'	PytASDR 5'- CAA TCA AAC AAC CGA CGA CTA C -3'	SYBR	78 °C	PPRI 20772	Schoeman (2016)
R. solani	RhizASCF 5'- TGT TAT GCT TGG TTC CAC TCG-3'	RhizASCR 5'- GGA CTA TTG GAA GCG GTT CAT C -3'	SYBR	77 °C	PPRI 10376	Schoeman (2016)
Trichoderma spp.	TriASDF 5'-GGG TGC GTA TTC CAT CAA TCA -3'	TriASDR 5'- CAC GGT GGT CGA CTT TCC-3'	SYBR	80 °C	PPRI 9138	Schoeman (2016)

4.3.4.5 Data analysis

Standard curves were generated for each fungal pathogen by diluting each species' DNA, obtained from the nasional databank PPRI (Plant Protection Research Institute), (approximately 10ng/µl) 4x, 16x, 64x, 256x and 1024x, and the extracted maize DNA (target DNA) were compared to these standerd curves. Three independent qPCR runs were done for each sample. Using the standard curve, the C_t value was transformed into DNA concentration for each fungal pathogen in each trial analysed. To ensure that the unknown samples fall in the acceptable range compared to the standard curve of the known fungal pathogen, the efficiency was between 90-110% (optimal), the R^2 >0.95 (optimal) and the slope -3.32 (optimal). Melt curve values for the SYBR Green protocol of the unknown samples that differed more than ± 1 °C, were not used in the analysis (Appendix C).

4.3.5 Statistical analysis

A combined, factorial analysis of variance (ANOVA) was performed with significance at P=0.05, using three repetitions, comparing the factors; localities, cultivars, plant parts, sampling dates and interaction effects. The standardized residuals were acceptable, normally distributed (Shapiro-Wilks test) (Shapiro & Wilk, 1965) and therefore the means of the significant effects were separated using Fisher's Unprotected t-test (least significant difference – LSD) tested at the 5% level of significance (Montgomery, 1984). All data analysis was performed using SAS v9.2 (SAS, 1999).

4.4 Results

4.4.1 Plant biomass

Plant biomass increased in the roots and crown parts for all the cultivars over time in Potchefstroom and Vaalharts. Towards the end of the season the maize plants dry off and the decrease of biomass was evident for all the cultivars. However, cultivar BG 3292 had significantly higher biomass for the 70, 80 and 90 DAP sampling dates (Figure 4.2) compared to the other two cultivars (IMP 50-10 B and DKC 61-94 BR) at Potchefstroom.

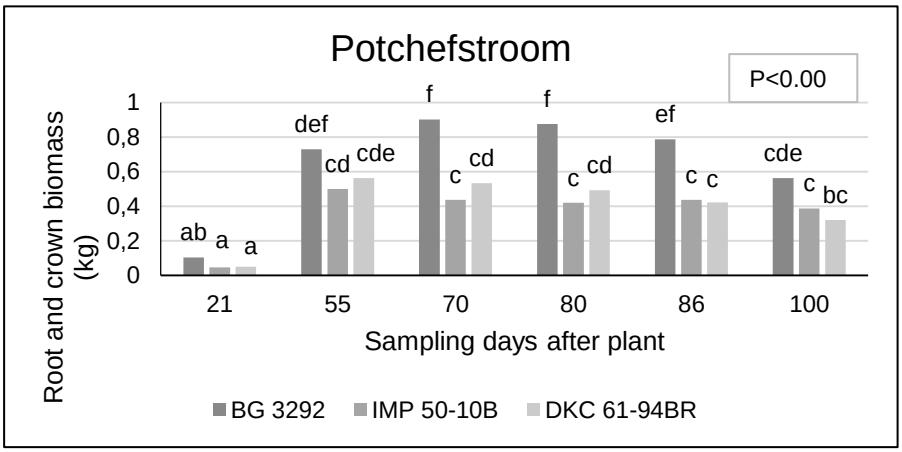


Figure 4.2: Root and crown biomass of maize planted in Potchefstroom, sampled six times throughout the season (Significance P≤0.05).

Cultivar BG 3292 also had significantly higher biomass compared to the other two cultivars (IMP 50-10 B and DKC 61-94 BR) for the 70, 80, 90 and 100 sampling date (Figure 4.3) at Vaalharts.

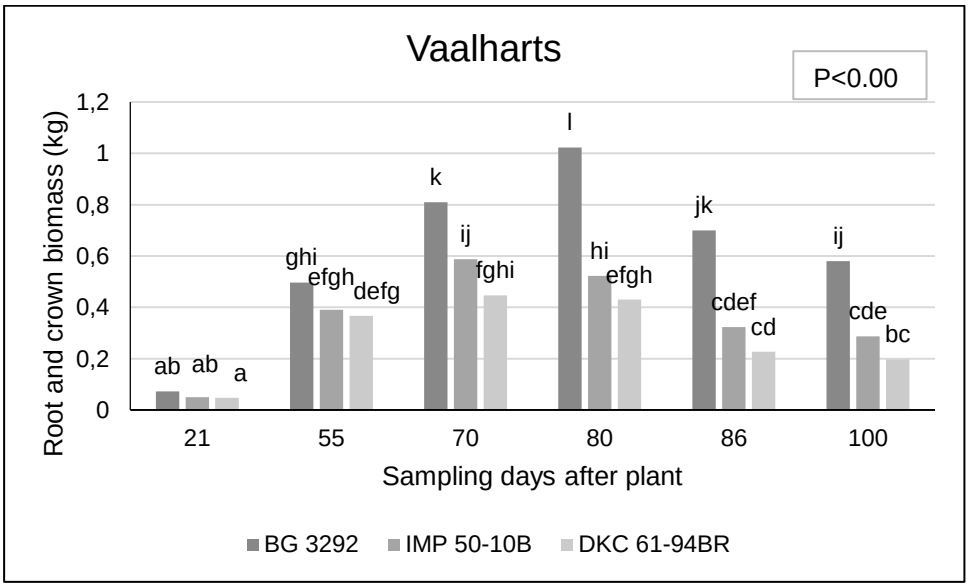


Figure 4.3: Root and crown biomass of maize planted in Vaalharts, sampled six times throughout the season (Significance P≤0.05).

4.4.2 Root and crown disease severity

The disease severity trend of the roots (visual rot ratings) was higher ratings than the disease severity trend of the crowns sampled in both Potchefstroom and Vaalharts and increased towards the end of the season. The cultivar BG 3292 exhibited the lowest disease severity trend in both localities in the roots (Potchefstroom) and crowns (Vaalharts). Cultivar DKC 61-94BR showed the highest disease severity ratings and trend in Potchefstroom (Figure 4.4 and 4.5) and for Vaalharts.

Values of 0 disease severity indicate that no visual disease (discoloration or rot) could be seen on the sampled plant parts for that sampling date. Below-ground symptoms can only be seen after the roots were washed and crowns cut open, making it possible to sample plant with no visual disease severity.

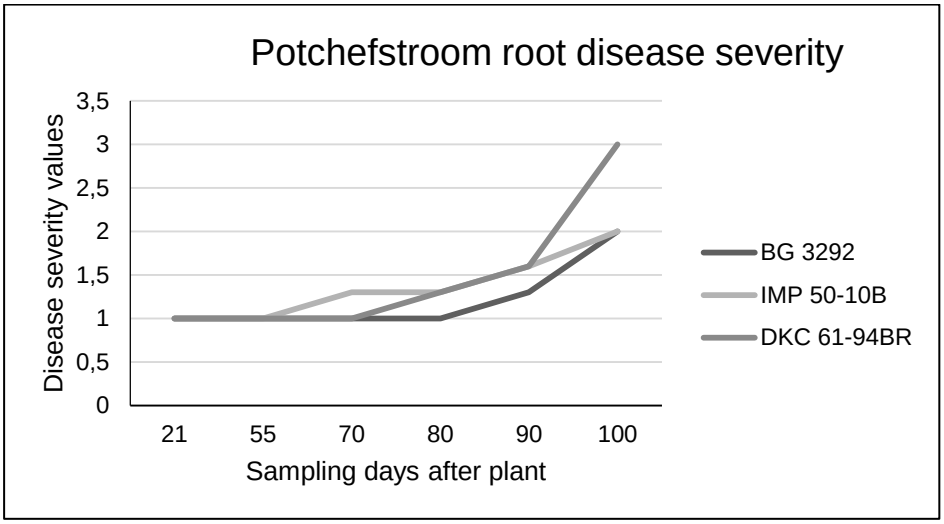


Figure 4.4: Disease severity value for the roots of maize planted in Potchefstroom, sampled six times throughout the season (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = < 25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, 5 = dead, totally wilted).

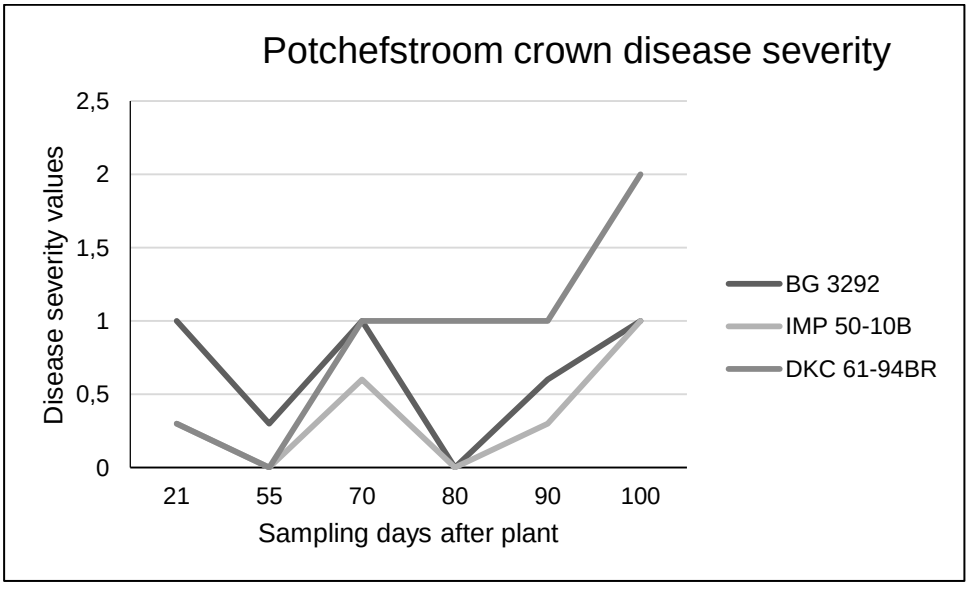


Figure 4.5: Disease severity value for the crowns of maize planted in Potchefstroom, sampled six times throughout the season (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = < 25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, 5 = dead, totally wilted).

DKC 61-94BR and IMP 50-10B both had the highest severity score of 2 and trend of increase in the roots and crowns (Figure 4.6 and 4.7).

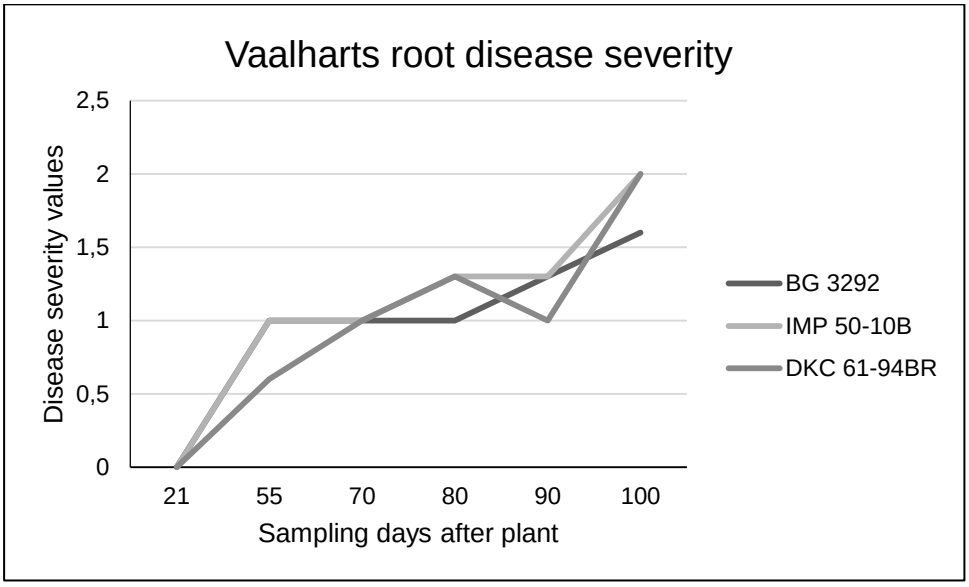


Figure 4.6: Disease severity value for the roots of maize planted in Vaalharts, sampled six times throughout the season (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = < 25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, 5 = dead, totally wilted).

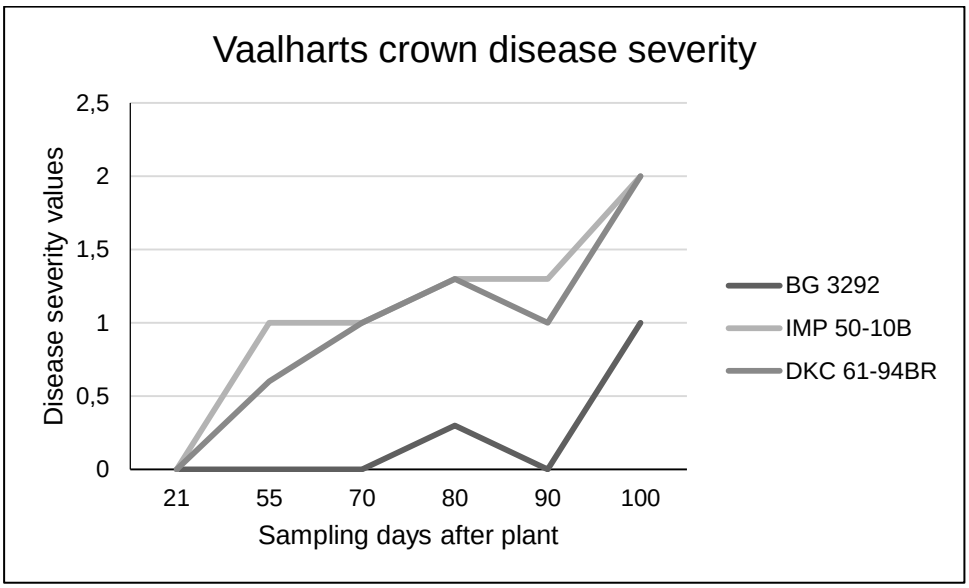


Figure 4.7: Disease severity value for the crowns of maize planted in Vaalharts, sampled six times throughout the season (Soonthornpoct *et al.,* 2001; 0 = no symptoms, 1 = < 25% rot, 2 = 25-49% rot, 3 = 50-74% rot, 4 = 75% rot or greater, 5 = dead, totally wilted).

4.4.3 Fungal pathogen complex

Concentration of the fungal target DNA of the samples, gathered over the two seasons, was determined using standard curves of each pathogen generated by species specific primers. *C. eragostidis* qPCR's efficiency did not completely stay in range (90-110%), but could still be used for identification of *C. eragostidis*. The total concentration (216 samples) of the fungal pathogens for six sampling dates in the season, showed that *M. phaseolina* was the most abundant with 357 237 223 pg/µl, followed by *F. equiseti* with 186 377 320 pg/µl and *F. graminearum* with 81 825 876 pg/µl. *R. solani* had the lowest concentration of only 320 pg/µl (Table 4.2).

Table 4.2: The total fungal pathogen concentration (pg/µl) over two seasons, in descending order.

Fungal pathogens	**Total fungal pathogen mass (pg/µl)**
M. phaseolina	357 237 223
F. equiseti	186 377 320
F. graminearum	81 825 876
Pythium spp.	1 078 987
F. chlamydosporum	880 208
Trichoderma spp.	580 750
Phoma spp.	62 833
F. oxysporum	26 078
F. verticillioides	11 762
E. pedicellatum	8 850
C. eragostidis	5 440
R. solani	320

*Total fungal pathogen count of the whole season (6 sampling dates)

For the twelve species tested, only *C. eragostidis, E. pedicellatum, F. oxysporum, Phoma* spp*., Pythium* spp*. and Trichoderma* spp*.* showed significant interactions between two or more of the following variables: localities (Potchefstroom and Vaalharts), sampling dates (21, 55, 70, 80, 90 and 100 DAP), cultivars (BG 3292, DKC 61-94 BR and IMP 50-10 B) and plant parts (roots and crowns).

Table 4.3: Analysis of variance of the impact of interaction between locality, sampling date, plant part and cultivar respectively on *C. eragostidis*, *E. pedicellatum*, *F. oxysporum, Phoma* spp*., Pythium* spp*. and Trichoderma* spp.

Species	Source	Degrees of Freedom	Sum of Squares	Mean Square	F value	Pr>F
C. eragostidis	**Locality x Sampling date**	5	22060.99364	4412.19873	5.03	**0.00**
E. pedicellatum	**Locality x Sampling date**	5	370905.0393	74181.0079	2.55	**0.03**
F. oxysporum	**Locality x Plant part x Cultivar**	2	184478.582	92239.291	3.23	**0.04**
Phoma spp.	**Sampling date x Plant part**	5	3080556.4	616111.28	15.04	**<0.00**
Pythium spp.	**Locality x Sampling date x Plant part**	5	296287928	59257586	6.8	**<0.00**
Trichoderma spp.	**Locality x Sampling date x Plant part x Cultivar**	10	312711155.6	312711115.6	2.58	**0.01**

The locality x sampling date interaction for *C. eragostidis* had a significantly higher effect in Potchefstroom compared to Vaalharts at 80, 90 and 100 DAP (P=0.00) (Figure 4.8). Between the 90 and 100 DAP sampling dates the qPCR values also differed significantly within each locality. At 21, 55 and 70 DAP no significant differences occurred between the two localities or the sampling dates. The presence of this fungal species throughout the season increased until 80 DAP and then decreased in Potchefstroom, while it stayed relatively the same for Vaalharts but also decreased at the end of the season (Figure 4.8).

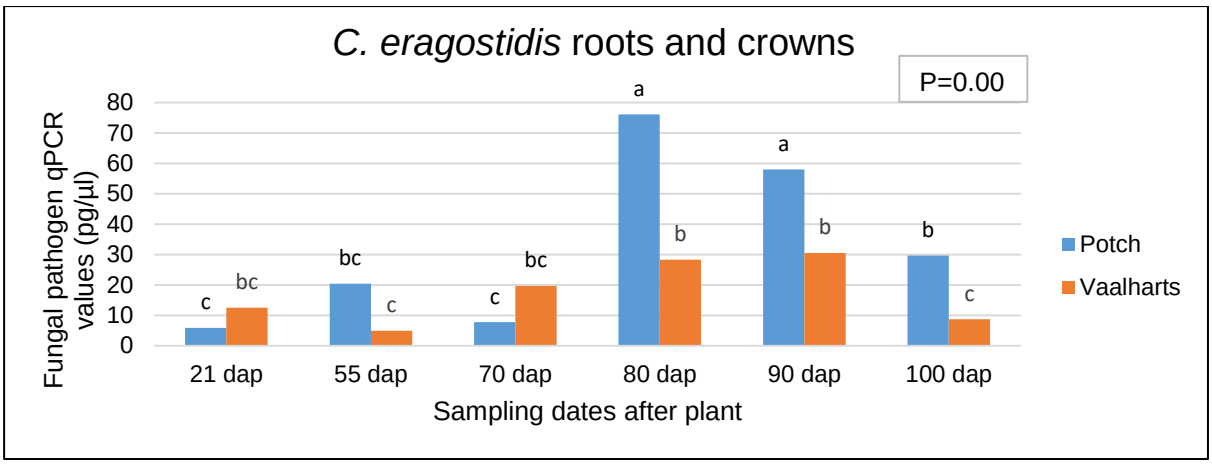


Figure 4.8: C. eragostidis target DNA of the roots and crowns measured in Potchefstroom and Vaalharts separately, at 6 sampling dates (Significance P≤0.05).

The locality x sampling date interaction had a significant effect on *E. pedicellatum* colonization where it was the highest at Potchefstroom at 100 DAP compared to the other sampling dates of Potchefstroom and for all the sampling dates of Vaalharts (P=0.03) (Figure 4.9).

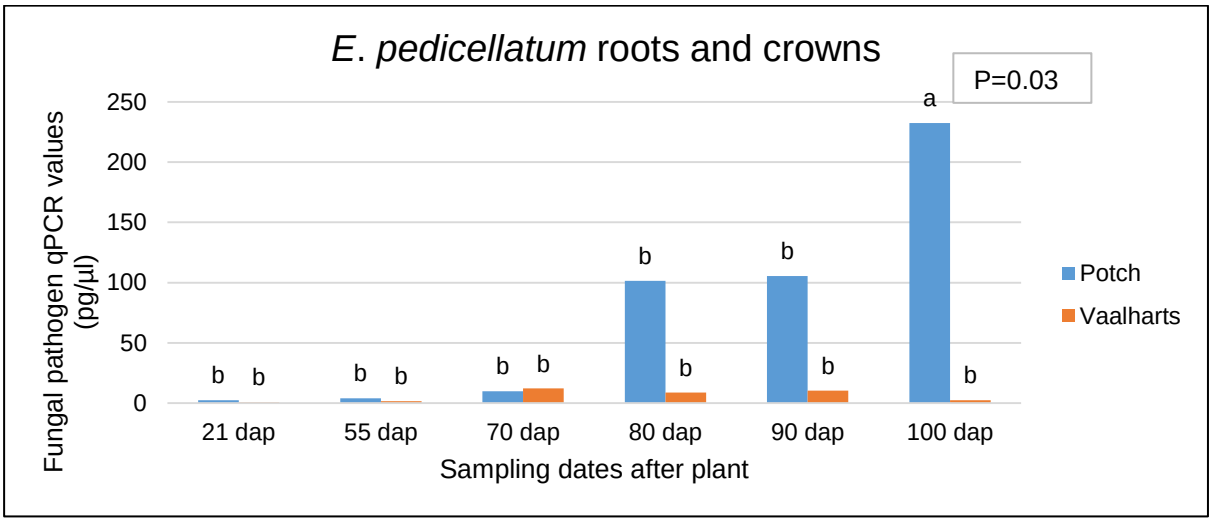


Figure 4.9: *E. pedicellatum* target DNA of the roots and crowns measured in Potchefstroom and Vaalharts separately, at 6 sampling dates (Significance P≤0.05).

The locality x plant part x cultivar interaction had a significant effect on *F. oxysporum* colonization, being significantly lower for cultivar DKC 61-94BR in the crowns compared to BG 3292 and IMP 50-10B (P=0.04) (Figure 4.10). None of the other cultivars differed significantly between each other for the plant parts separately, but within each cultivar significant differences occurred for the same plant part between localities. For BG 3292 the roots and crowns had significantly higher target DNA in Potchefstroom compared to Vaalharts. For IMP 50-10B the roots of Potchefstroom had significantly higher target DNA compared to the roots of Vaalharts (P=0.04) (Figure 4.10).

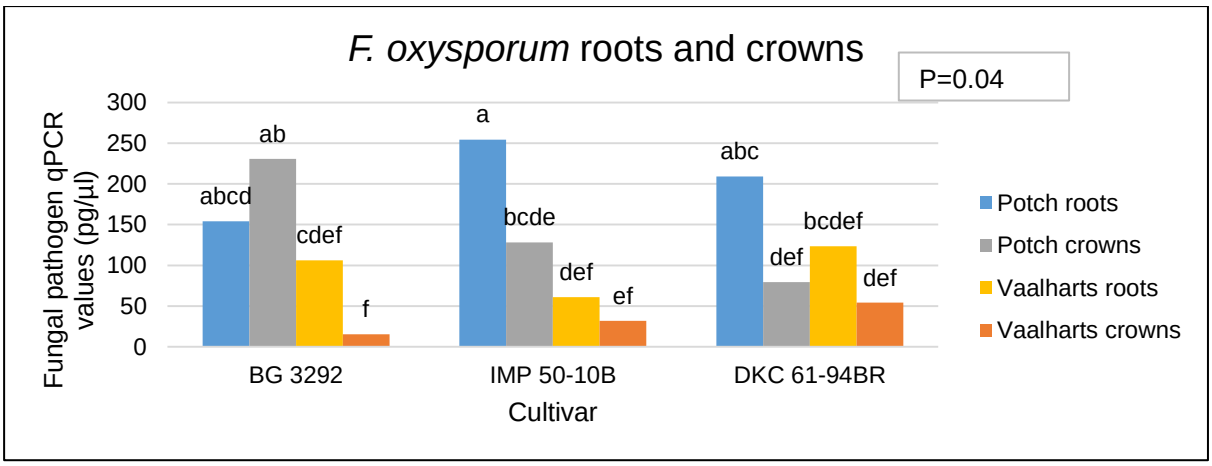


Figure 4.10: *F. oxysporum* target DNA of the roots and crowns separately in Potchefstroom and Vaalharts, measured for the three cultivars (Significance P≤0.05).

The sampling date x plant part interaction had a significantly effect on Phoma spp. colonization, being significantly higher in the roots compared to the crowns for all the sampling dates (throughout the season) (P=0.00). For the crowns the presence of *Phoma* spp. significantly decreased from the 55 DAP sampling and the quantity did not change significantly further in the season. For the roots significant differences throughout the season were observed (increasing or decreasing between every sampling date) (Figure 4.11).

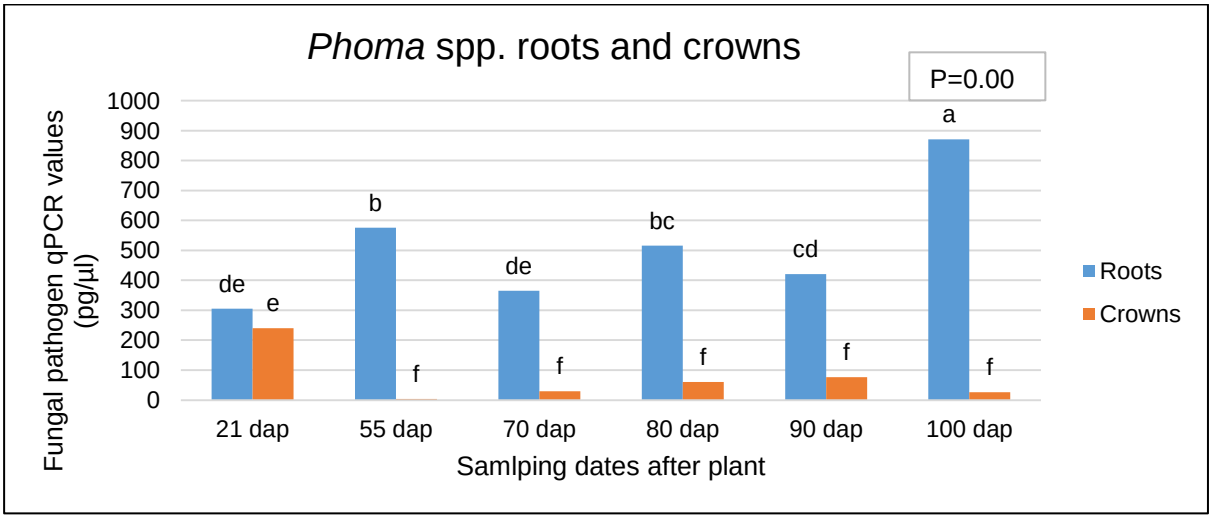


Figure 4.11: *Phoma* spp. target DNA of the roots and crowns over both localities, at 6 sampling dates (Significance P≤0.05).

The locality x sampling date and plant part interaction had a significant effect on *Pythium* spp. colonization, being significantly higher in the roots compared to the crowns for most sampling dates, except at 21 DAP where the results were more or less the same and 80 DAP where

Potchefstroom crowns did not differ from roots from the same locality. At 80 DAP the *Pythium* spp. incidence was significantly higher in the crowns in Potchefstroom than Vaalharts. The roots of the two localities had significantly higher fungal colonization at 70, 80, 90 and 100 DAP in Potchefstroom and Vaalharts. Potchefstroom also had significantly higher fungal colonization for the roots at these dates compared to Vaalharts. No definite pattern could be observed at both localities and for both plant parts for the occurrence of *Pythium* spp. and how it changes over the season (P=0.00) (Figure 4.12).

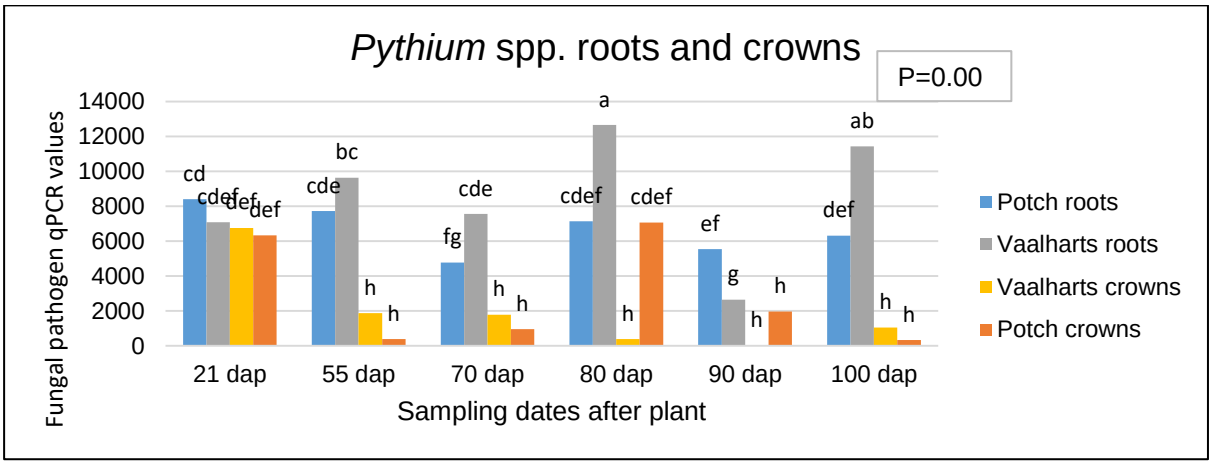


Figure 4.12: *Pythium* spp. target DNA of the roots and crowns measured in Potchefstroom and Vaalharts, at 6 sampling dates (Significance P≤0.05).

The locality x sampling date x cultivar interactions had a significant effect on *Trichoderma* spp. colonization, being significantly higher in the roots of the maize plants at 100 DAP for BG 3292 and DKC 61-94B in Vaalharts compared to the other sampling dates, as well as between these two cultivars and between the different localities of the other sampling dates (P=0.01) (Figure 4.13).

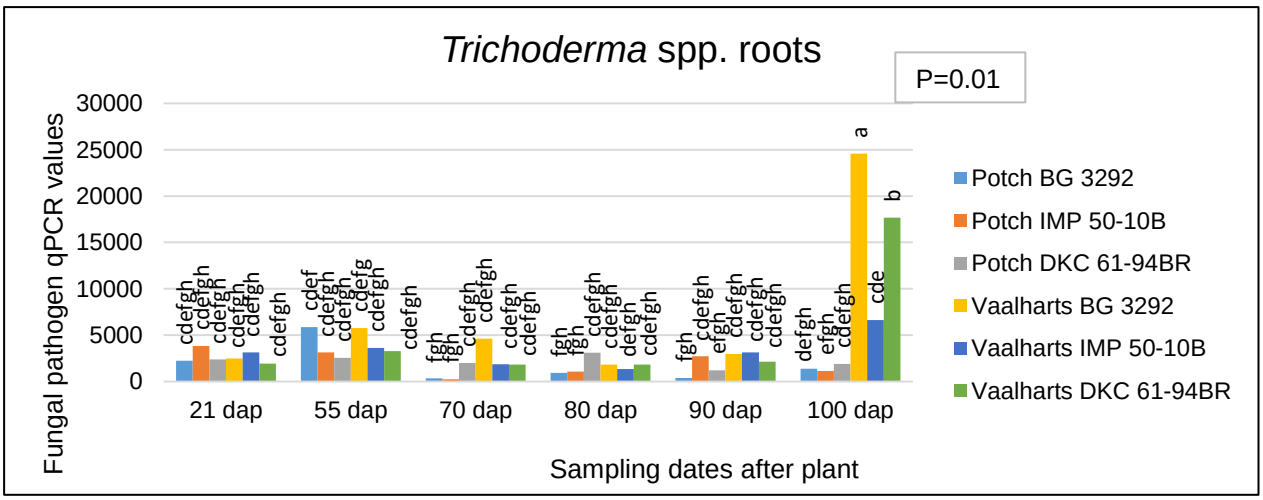


Figure 4.13: *Trichoderma* spp. target DNA of the roots measured in Potchefstroom and Vaalharts for three cultivars, at 6 sampling dates (Significance P≤0.05).

The locality x sampling date x cultivar interactions had a significant effect on *Trichoderma* spp. colonization, being significantly higher in the crowns of maize plants in Potchefstroom at 21 DAP for DKC 61-94BR compared to the other sampling dates, the different localities, the other cultivar at this date and between these two cultivars at 21 DAP (Figure 4.14). *Trichoderma* spp. target DNA in cultivar DKC 61-94BR was significantly higher in Vaalharts at 100 DAP compared to most other cultivars and localities at the 55, 70, 80 and 90 DAP (P=0.01) (Figure 4.14).

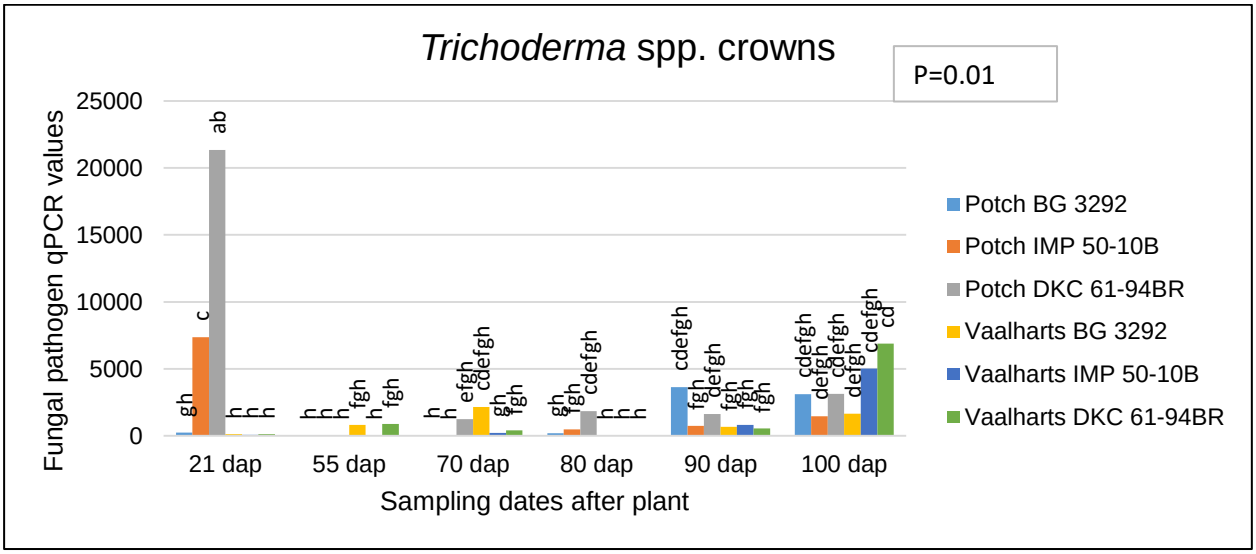


Figure 4.14: *Trichoderma* spp. target DNA of the crowns measured in Potchefstroom and Vaalharts for three cultivars, at 6 sampling dates (Significance P≤0.05).

4.5 Discussion and conclusion

This study was the first to monitor twelve commonly occurring fungal pathogens during the maize growing season. Using qPCR technology that provides the concentration of each pathogen separately in the sample makes it possible to study different variables contributing to root and crown rot. Three different maize cultivars were included as a factor in this chapter together with developmental stages of maize plants, cultivar selection and different localities.The factors influencing the fungal pathogens (*C. eragostidis, E. pedicellatum, F. oxysporum, Phoma* spp*., Pythium* spp*. and Trichoderma* spp.) can be used in an integrated management strategy in order to minimise the effect of these pathogens. None of the factors tested had a significant effect on *M. phaseolina, F. graminearum* or *R. solani,* even with high fungal colonization.

F. oxysporum and *Trichoderma* spp. were significantly influenced by cultivar. In this study, BG 3292 which showed the least root and crown rot diseases, was also the cultivar with the highest plant biomass. The roots of maize plants are essential for the acquisition of water and minerals from the soil and will give a rough indication of the health of the entire maize plant (Meister *et al*.,

2014). Diseased roots will be under more pressure to acquire the same amount of water and nutrients compared to healthy roots, the maize plant with diseased roots will consequently not be provided with adequate water and nutrients and will be more susceptible to secondary diseases and environmental stress. Diseased roots restrict plant growth and greatly decrease yield. Increased root biomass (healthy roots for example) will reduce wilting under water stress conditions as more roots are available to secure the most water and nutrient uptake possible (Meister *et al*., 2014).

Localities significantly influenced the presence and abundance of pathogens. The interaction with sampling date for *C. eragostidis* increased and then decreased at 80 DAP in Potchefstroom and Vaalharts, and *E. pedicellatum* increased with time in Potchefstroom. *Pythium* showed variable significant increased and decreases in the roots of Vaalharts. Potchefstroom and Vaalharts differ in environmental conditions, with Vaalharts having some drought stress, because of interrupted irrigation (due to cable theft) which would have influenced fungal infections. *C. eragostidis* at both localities increased through the season up until more or less 80 days and tend to decrease toward the end of the season and as the maize dries off. *E. pedicellatum* was more abundant at the end of the season in the presence of the other complex pathogens, which seems to have enable it to better compete saprophytically.

Pythium spp. were also more abundant in the roots and prevailed in the roots whilst the presence in the crowns decreased towards the end of the season, except for Potchefstroom crowns which indicated a spike in concentration at 80 DAP (indicating a possible later flowering occurance with high infection, at the usual blistering date) . According to the SAWS (South African Weather Service, 2017), based on nearby weather stations in Potchefstroom and Vaalharts, from January till May 2017, the average minimum temperatures were lower (11.64°C vs. 11.94°C), the average maximum temperatures higher (28.62°C vs. 27.62°C) and the total rainfall in mm higher in Potchefstroom compared to Vaalharts (403.6 mm vs. 292 mm). This is an additional moisture reference, since the maize was planted under irrigation. These climatic conditions in Vaalharts seemed to favour *Pythium,* taking less irrigation water into account, due to a technical difficulty (cable theft).

Phoma increased and decreased in the roots and crowns. *F. oxysporum*'s presence in the roots and crowns were favoured by locality and occurred at a higher concentration in Potchefstroom that had higher rainfall figures for the season (higher humidity). Wise *et al.* (2016) stated that *F. oxysporum* or *F. graminearum* root and crown rot may occur through the whole growing season. Lipps & Deep (1991) found that the presence of *Fusarium* spp. in the crowns and stalks of maize (Crows brand 444, Crown Hybrid Corn Co., Milford, IL) increased as time increased over the

season. They also found *F. graminearum* and *F. verticillioides* to be the most abundant of the different species. Kommedahl *et al*. (1979) also found that *Fusarium* spp. incidence in maize roots and stalk increases with time and especially after tasselling occured where three maize cultivars were planted in Minnesota (Minhybrid 5302, 508 and 511).

Phoma spp. has a definite root preference compared to crowns at both localities and significantly increased from the beginning of the season towards the end. *Phoma* spp. as endophyte has benefits such as promoting plant health, improving growth of plants and acting as a potential bio-control agent (Sinha *et al*., 2012). Endophytes (*Phoma* spp.) can inhibit fungal pathogen presence in the rhizospheres by means of competition for the available nutrients, for space and oxygen, parasitism and by physical damage to the fungal cell walls through the production of hydrolytic enzymes (Taechowisan *et al.,* 2009). *Rhizoctonia* was found by Wise *et al.* (2016) to have early and late season occurrence, but in this study very low levels were observed throughout the season (data not shown).

Seemingly, *Trichoderma* spp. occurred more frequently in Vaalharts compared to Potchefstroom, at the end of the season in the roots for BG 3292 and DKC 61-94BR. Towards the end of the season the temperatures at both locatities decreased and as mentioned above Vaalharts had less rain with higher maximum and lower minimum temperatures, possibly showing temperature preferences of *Trichoderma* spp. for these two cultivars. From grain fill onwards in the plant growth stages the plant extracts more sugars from the stalk to the grain and could also possibly explain why the roots, crowns and stalks are more susceptible to certain fungi at the end of the season. BG 3292 again showed the lowest target DNA values for all the sampling dates except at 100 DAP. *Trichoderma* spp. in the crowns were significantly higher at 21 DAP for two cultivars in Potchefstroom, remained very low for the other sampling dates and at 100 DAP increased in all the cultivars and at both localities. *Trichoderma* spp. which is known as a secondary invader remained relatively high and constant in concentration in a study throughout the season carried out by Lipps & Deep (1991). It occurred in the plant parts (subcrown mesocotyl, crowns and stalks) that indicated a possible parasitic relationship with the maize after invasion and not a pathogenic relationship where increased numbers of *Trichoderma* spp. would occur. Some increases in pathogen presence in this study can then be because of a parasitic relation. Liu & Baker (1980) stated that studies where *Trichoderma* spp. parasitized pathogenic fungi, the pathogen populations decreased as the *Trichoderma* spp. increased. Three *Trichoderma* species have been identified in South Africa namely *T. asperrellum, T. afro-harzianum* and *T. gamsii* (Viviers, 2014). Viviers (2014) found that from these three species, a positive property to their presence is that *T. gamsii* and *T. afro-harziarum* commonly colonize maize roots and promote seedling growth.

Cultivar DKC 61-94 BR had the highest disease severity for the roots and crowns in Potchefstroom and cultivar IMP 50-10B in Vaalharts at the end of the sampling dates. Cultivar BG 3292 showed the lowest disease severity measurements in this study. BG 3292 also was also the cultivar with the highest plant biomass and lowest fungal colonization. Cultivar choice is therefore very important in disease management and Smit & McLaren (1997) confirmed that the use of a different maize cultivar in their study planted in the 1993/1994 season attributed to the lower isolation frequencies of *E. pedicellatum.* However, in this study no significant differences occurred for *E. pedicellatum* between the three cultivars used.

There was an insignificant correlation between the fungal colonization and disease severity ratings in Potchefstroom and Vaalharts. *C.eragostidis, E. pedicellatum* and *F. oxysporum* were favoured by the environmental conditions in Potchefstroom, while *Pythium* and *Trichoderma* spp. were more abundant in Vaalharts. An overall increase in disease severity over time suggests that fungicides can in future be applied just before the time/growth phase where exponential increases of the pathogens were observed as ground application (systemic mode of action). This management strategy can only be economically justifiable if spot treatment application is used in areas that over time showed to be the disease prone. Most available fungicides are broad spectrum seedcoat treatments for which the specific action on a target pathogen is rarely determined and limited research has been done on species complexes causing diseases, instead these seed treatments are known to form protective barriers around the germinating seed, reduce seed decay, damage and entry of any possible pathogen present in the soil (Rodrigues-Brljevich, 2008). Systemic fungicide treatments penetrate the seedcoat, translocate to the endosperm, embryo and eventually reach to the growing points of the maize plant (Rodrigues-Brljevich, 2008), but the protection is only at seedling stage.

This study showed the value of using qPCR to study different variables influencing soil borne fungal pathogens. Four different variables were evaluated in a more time efficient and less laborious manner. The fungal pathogen occurring in the highest levels was *M. phaseolina* followed by *F*. *graminearum* and *R*. *solani* which are some of the major pathogens occurring on maize. Interestingly these fungal pathogens were not significantly affected by the different variables during this study. It was clear that other fungal pathogens were significantly affected by variables and management strategies might be more easily adapted to these pathogens in order to minimize infection and eventual severity of root and crown rots. During the study, it was found that there is a lack of thorough studies done on fungicide treatments and that a limited amount of products are available for use later than seedling stage. Future studies should include the development and testing of fungicide treatments at different development stages of maize plants and fungal specific treatments.

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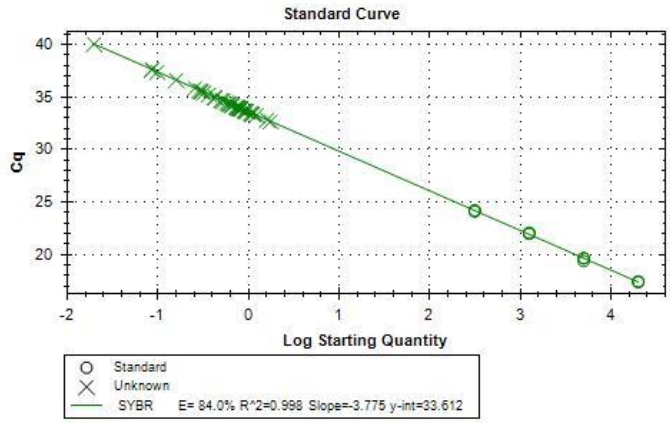
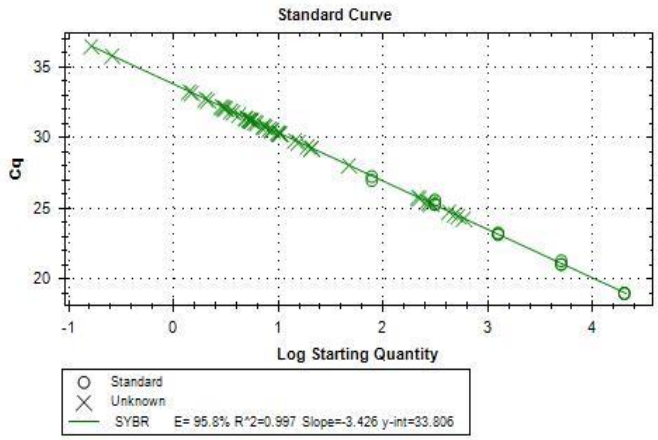
Appendix D: Standard curves of the twelve known fungal root and crown rot pathogens in South Africa including the efficiency and R^2 values

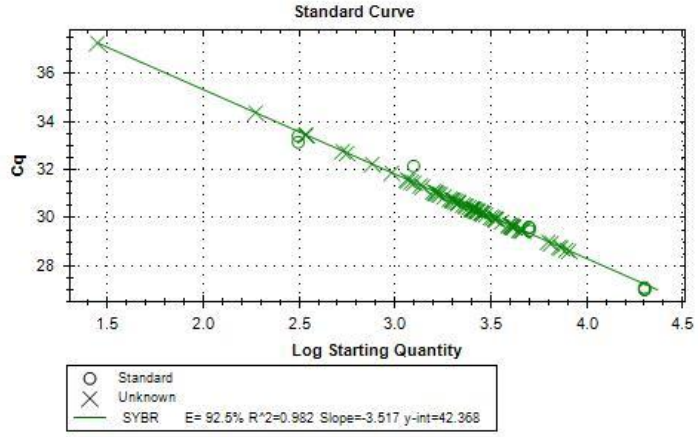
C. eragostidis



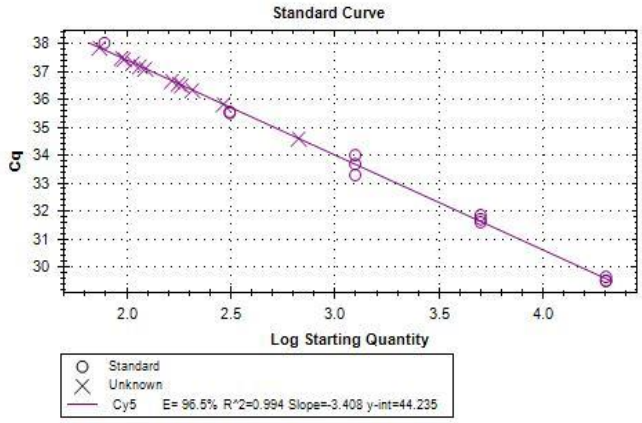
E. pedicellatum



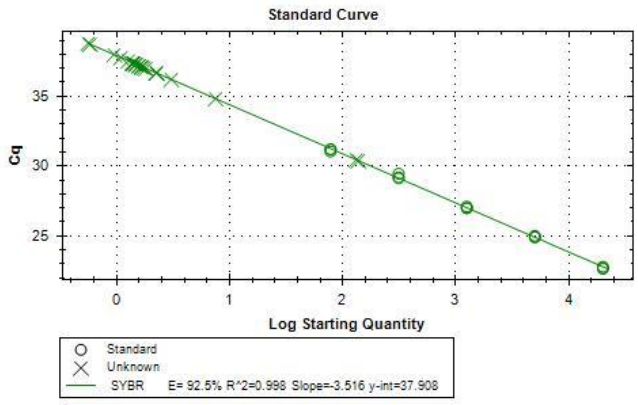
F. chlamydosporum



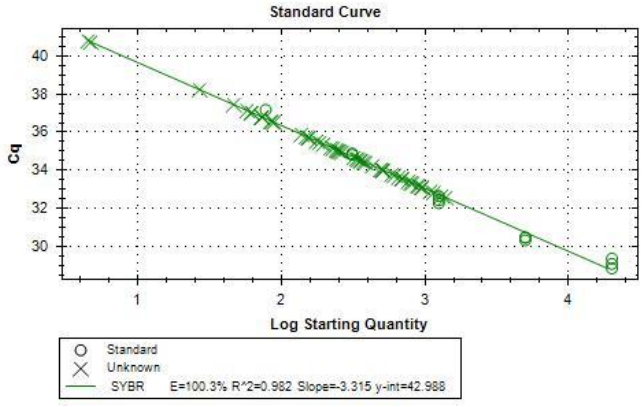
F. equiseti



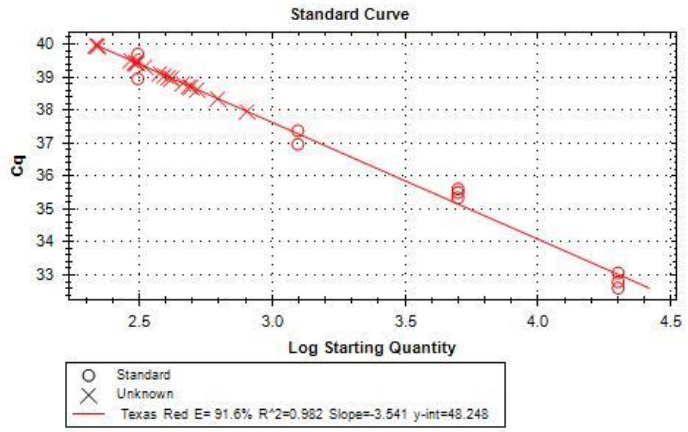
F. graminearum



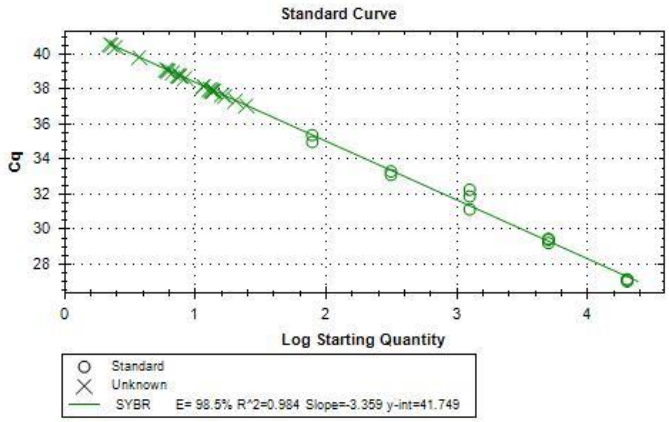
F. oxysporum



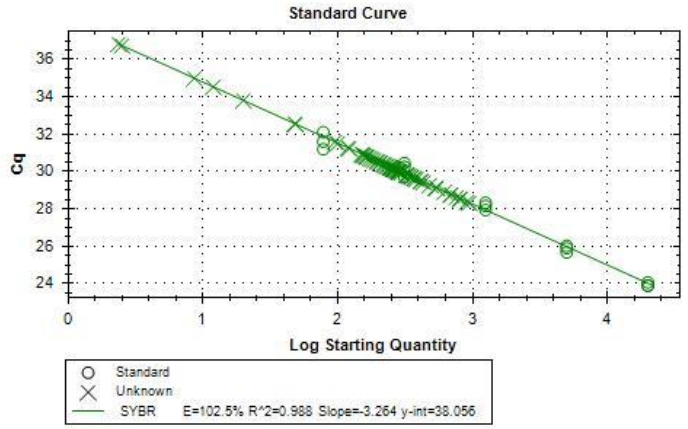
F. verticillioides



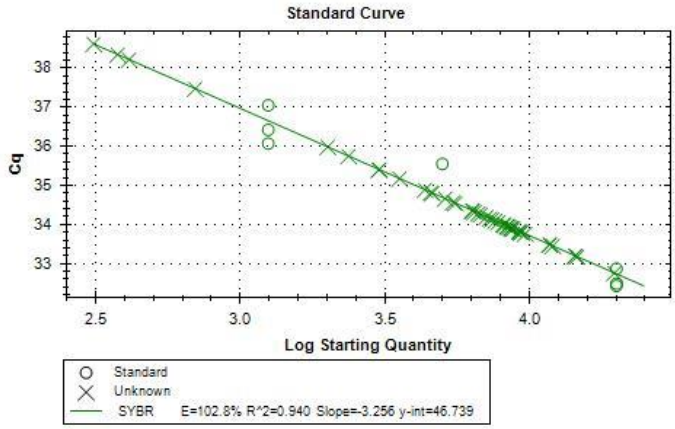
M. phaseolina



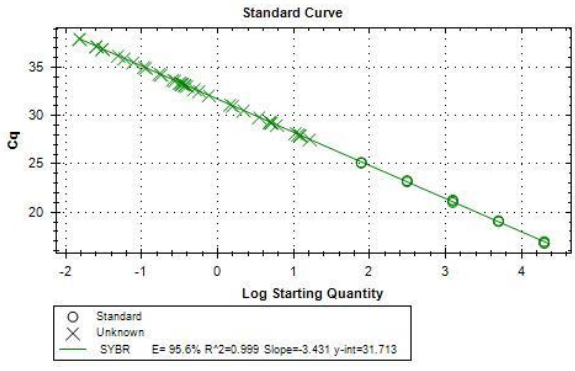
Phoma spp.



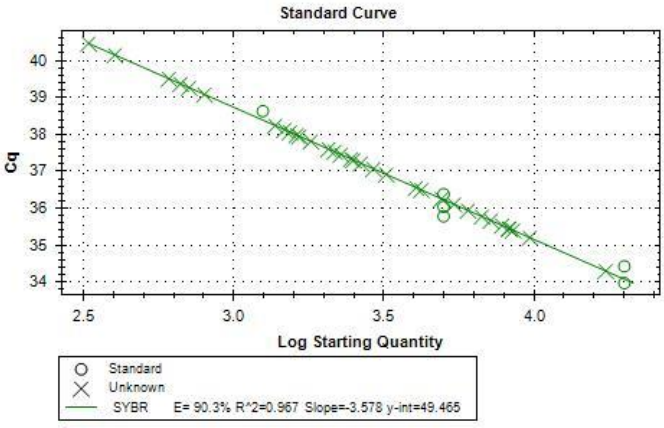
Pythium spp.



R. solani



Trichoderma spp.



CHAPTER 5

DISCUSSION AND CONCLUSION

Plant diseases are known by the abnormalities that arise physically and change functions of a plant over some time. Maize (*Zea mays*) is a staple food crop in South Africa (produced at an average of 11.3 million tons per year between 2001 and 2014) and forms part of the agricultural sector that made a 2.6% contribution to the GDP in 2013. Plant diseases threaten yield production and seed quality of maize. Specifically, root and crown rot (fungal diseases) occur due to a complex of fungal pathogens' presence and synergisms between these pathogens, soil micro-biota and the environment. The impact of root and crown rot varies from year to year as it is dependent on weather conditions, crop production practices applied, and maize cultivar selections. The overall aims of this study were to improve the understanding of disease complexes and the successions, to quantify the disease incidence and severity and help formulate management strategies for root and crown rots. This knowledge would then be used to optimize maize production in South Africa and limit economic losses due to root and crown rot.

Disease severity ratings, DNA extractions and qPCR methods were used in all the chapters and proved to be an efficient way of achieving the aims of this study and it will be useful for future studies to include these methods. Using qPCR made it possible to evaluate more variables and gave highly specific, sensitive and fast results. The aims of the second chapter were to determine the effect of different cultivation practices on twelve commonly known root and crown rot fungal pathogens of maize in South Africa and to determine tissue specificity between roots and crowns for the rot/pathogens. Two trials were planted during the 2015 and 2016 seasons in the Free State province. The plant biomass and disease severity results showed no significant differences in the conventional CA practices in the different seasons. Definite disease severity (rot) was found to occur more in the roots than the crowns of maize plants. The qPCR analyses for the conventional and CA practices showed that *Phoma* spp*., Pythium* spp., *F. oxysporum*, *F. chlamydosporum* and *F. graminearum* were the most prominent of the fungi tested. Different fungal species showed significant interaction between seasons, tillage practices applied and plant parts, however none of the significance obtained pointed to a viable management strategy for the fungi analysed in this chapter. The importance of multiple seasons' data and more similar trials are essential in order to obtain better insight into the effect of cultivation practices and crop rotation on soilborne pathogens and the impact they have on root and crown rot development.

The aim of the third chapter was to determine if there were geographical areas with certain dominant soilborne fungal pathogens in South Africa under different cultivation practices. In 2014

and 2015 maize roots and crowns were sampled at fifteen localities in six provinces namely: Gauteng, North West, Free State, KwaZulu-Natal, Mpumalanga and the Northern Cape to determine the dominance and cultivation preferences in these areas. The qPCR results indicated significant tillage x province interaction for *F. oxysporum*, irrigation x province interaction for *E. pedicellatum and R. solani. F. verticillioides* showed significant differences between different rotated crops. *R. solani* was found significantly more in no-till fields compared to tilled fields, and between rotations with different crops. Some strategic management practices could be derived from the results: in certain parts of Mpumalanga and the Northern Cape tillage practices rather than no-till will keep *F. oxysporum* numbers as low as possible. *E. pedicellatum* had a preference to irrigated and dryland fields in the Free State and dryland fields in the North West province. *R. solani* is also more problematic on irrigated fields in the Northern Cape and Free State and dryland fields in the North West province which should influence the farmer's choice in how the maize would be planted.

The fourth chapter aimed to evaluate the succession of the fungal pathogen complex for the first time during a maize growing season and to understanding the influence of different localities and cultivars on the fungal complex and disease symptom development.Two trials were planted (Potchefstroom and Vaalharts), with three cultivars (BG 3292, IMP 50-10 B and DKC 61-94 BR) and the roots and crowns sampled six times at different developmental stages (seedling, 21 DAP, V-stages (11 leaf /tassel and beard appearance, 55 DAP), R-stages (flowering, 70 DAP), blistering (80 DAP), soft dough (90 DAP) and dent stage (100 DAP)) of the maize plant. The fungal biomass increased as the plants grew and decreased at the end of the season as the maize dried off for all three cultivars, cultivar BG 3292 had significantly higher plant biomass for the 70, 80, 90 and 100 sampling dates compared to the other two cultivars. Cultivar BG 3292 had the lowest severity root and crown rot in both localities and the overall disease severity showed significant preference for the roots compared to the crowns. The qPCR analysis showed significant locality x sampling date interactions for *C. eragostidis* and *E. pedicellatum*. *F. oxysporum* had significant cultivar x plant part x locality interactions. *Phoma* spp. were significantly affected by the sampling date and plant part interaction (pathogen presence increased in the roots with time and decreased in the crowns) and *Pythium* spp. with the sampling date x plant part x locality interaction (pathogen presence increased in the roots of Vaalharts with time and decreased in the crowns of Vaalharts and Potchefstroom). *Trichoderma* spp. showed the highest order interaction that differed significantly: sampling date x plant part x cultivar x locality. This was the first study to successfully determine the succession and composition of pathogen fungal species in root and crown tissue for different cultivars and at two different localities.

To conclude: the hypotheses that (1) certain cultivation practices increase the occurrence of specific fungal pathogens, (2) abiotic factors influence the occurrence of specific fungal pathogens, (3) different area preferences for the fungal pathogens exist and (4) tissue specificity that occurs were all proven to a certain point. The use of qPCR technology made it possible to perform an in-depth study of the composition of the root and crown rot fungal pathogens under different variables that included: till and no-till practices, crop rotation, dryland and irrigated fields, developmental stages of maize plants, cultivar selection, locality and environmental conditions. The results obtained were complex and highly variable, and continued monitoring of fields using this technology will be essential. Knowledge gathered will enable farmers to gather plant pathogenic data and empower them to formulate strategies to prevent these twelve known root and crown rot pathogens from causing devastating epidemics in maize crops.