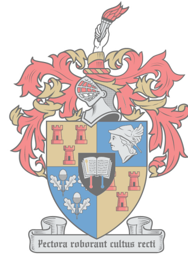
UNDERSTANDING CITRUS REPLANT DISEASE IN SOUTH AFRICA WITH THE AIM OF DEVELOPING A METHYL BROMIDE FREE MANAGEMENT STRATEGY

by

LAURIKA SWART



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Supervisor: Dr. Jan M. van Niekerk

Co-supervisor: Prof. Adéle McLeod

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DECLARATION

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SUMMARY

Citrus fruits are one of the most important fruit crops after deciduous fruit and vegetables cultivated in South Africa. The Citrus industry in South Africa, is seen as the third largest horticultural industry and is considered one of the most important horticultural crops due to its economic export value and local consumption. South Africa is currently the eleventh largest citrus producer in the world, and second largest in the Southern hemisphere. The growth of citrus production and the development of new cultivars in South Africa has become a priority that growers establish orchards on sites where citrus has been cultivated for many years. With the establishment of a new orchard symptoms associated with replant disease have been observed on these newly planted citrus trees like in many other parts of the world.

The casual agents associated with citrus replant disease in South Africa have been regarded as the citrus nematode, *Tylenchulus semipenetrans* and the soilborne pathogens, *Phytophthora nicotianae* and *Phytophthora citrophthora*. Symptoms can be characterised by the appearance of small leaves, the formation of gummosis and trees showing low vigour with short internodes. Previous studies conducted in South Africa on citrus replant disease did not focus on the characterisation of fungal organisms present in replant sites that may play a role in replant disease. Therefore, the aim of this study was determining whether the basis of citrus replant disease is biotic or abiotic. This study also aimed to identify the exact pathogens involved and investigating the molecular characterisation of oomycetes. Phylogenetic analyses of *Fusarium* spp. associated with replant soils was conducted to determine the diversity of Fusaria associated with citrus in South Africa.

Soil samples was collected from four orchards, aged between 37 and 47 in two major citrus producing areas. Representative soil samples from each orchard was used for nematode extraction and revealed that both juvenile and female citrus nematodes (*T. semipenetrans*) was present in the soil. The soil was subjected to six different treatments. The treatments included steam sterilisation, a 20% soil dilution, a mefenoxam, difenoconazole and cadusafos drench treatment and an untreated control. After treating the orchard soil two Carrizo citrange seedlings was planted per pot and left to grow for seven months in a glasshouse. Prior to planting and at trial evaluation the seedling length and weight was determined to compare the growth of the seedlings. Statistical analyses based on the growth response of citrus, revealed that all the treatments to some extent led to an increase in seedling growth. The steam sterilisation treatment showed to have the best effect on seedling growth eliminating pathogens in the soil, followed by the untreated control, mefenoxam, cadusafos, difenoconazole and soil dilution treatment.

None of the biocide treatments indicated the involvement of specific biological agents targeted by each biocide. Isolations from the roots of the control bioassay seedlings in all orchards showed that isolates belonging to the '*Fusarium solani'* species complex (FSSC)

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dominated in all orchards. *Fusarium oxysporum* species complex (FOSC) isolates and oomycetes (*Phytophthora nicotianae, Phytophthora citrophthora* and *Pythium irregulare*) were also associated with the citrus roots at more or less similar frequencies within each orchard. Identification of *Fusarium* spp. was based on the phylogenetic analyses of the translation elongation factor 1-alpha (TEF) and RNA polymerase II second largest subunit RPB2 gene region.

Phylogeny of the FSSC isolates showed that the citrus isolates grouped into four clades including a *Neocosmospora solani* clade (25 isolates), *Neocosmospora croci* clade (one isolate), an unnamed *Fusarium* spp. clade (13 Isolates) with *F. falciformis* as the most related known *Fusarium* spp., and another clade (one isolate) containing an unnamed *Fusarium* species. The citrus FOSC isolates grouped within the *F. oxysporum* phylogenetic species II. The FOSC citrus isolates were furthermore polyphyletic and distributed among two subclades, previously designated as Clade 3 (11 isolates) and Clade 4 (two Isolates).

This study showed that the growth response (weight and length increases) of the seedlings in the bioassay are biological in nature. *Phytophthora nicotianae*, *Phytophthora citrophthora*, *Pythium irregulare*, *Fusarium* spp. within the FSSC and FOSC as well as the citrus nematode *Tylenchulus semipenetrans* are all shown to be associated with citrus tree roots and replant soil. The management strategies indicate that the applications could be possible substitutes for methyl bromide soil fumigation in orchards earmarked for replant. But ultimately the effective prevention of citrus replant disease using non-methyl bromide fumigation is dependent on knowing what is present in the soil and making the correct decisions based on this knowledge.

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OPSOMMING

Sitrus word gesien as een van die belangrikste vrugte naas sagtevrugte en groente wat in Suid-Afrika verbou word. Die Sitrusbedryf in Suid-Afrika word beskou as die derde grootste hortologie bedryf en word beskou as een van die belangrikste hortologiese gewasse, weens die ekonomiese uitvoerwaarde en plaaslike verbruik. Suid-Afrika is tans die elfde grootste sitrusprodusent ter wêreld en die tweede grootste in die Suidelike Halfrond. Die groei van sitrusproduksie en die ontwikkeling van nuwe kultivars in Suid-Afrika het 'n prioriteit geword dat produsente nuwe boorde op vooraf gevestigde sitrus boorde herbou en hervestig. Met die vestiging van 'n nuwe boord word simptome waargeneem wat algemeen geassosieer word met herplant siekte op nuut geplante sitrus bome.

Die patogene wat algemeen geassosieer word met sitrus herplant in Suid-Afrika word beskou as die sitrus nematode, *Tylenchulus semipenetrans* en die grond gedraagde patogene, *Phytophthora nicotianae* en *Phytophthora citrophthora*. Simptome word gekenmerk deur die voorkoms van klein blare, die teenwoordigheid van gom op die bas en lote asook lae groeikrag met kort internodes. Vorige herplant studies was onvolledig in die karakterisering van herplant patogene teenwoordig in die grond. Hierdie studie het ook daarop gemik om die presiese patogene betrokke by herplant te identifiseer en die molekulêre karakterisering van oomycete te ondersoek. Filogenetiese analise van *Fusarium* spp. is uitgevoer om die diversiteit van Fusaria wat met sitrus in Suid-Africa geassosieer word te bepaal.

Grond- en wortel monsters is van vier boorde in twee sitrus produserende gebiede, tussen die ouderdom van 37 en 47 versamel. Verteenwoordigende grondmonsters van elke boord is gebruik vir nematode-ekstraksie en het aan die lig gebring dat beide onvolwasse en vroulike volwasse sitrus nematodes (*T. semipenetrans*) in die grond teenwoordig was. Die grond is verder gebruik waar ses verskillende behandelings toegedien is. Die behandelings sluit in stroomsterilisasie, 'n 20% grondverdunning, en 'n mefenoxam-, difenoconasol- en cadusafos drenkbehandeling en ‘n onbehandelde beheer. Na die behandeling van die grond is dit in potte geplaas, waar twee Carrizo citrange saailinge per pot geplant is en vir sewe maande gelaat is om te groei in ‘n glashuis. Voor plant en tydens proefevaluering is die saailinglengte en gewig bepaal om die groei van die saailinge oor die tydperk van sewe maande te vergelyk. Statistiese ontledings gebaseer op die groeireaksie van sitrusplantjies, het aangetoon dat al die behandelings tot 'n mate gelei het tot 'n toename in saailinggroei. Die stoom behandeling het getoon om die beste uitwerking op saailingsgroei te hê wat patogene in die grond uitskakel, gevolg deur die onbehandelde beheer, mefenoxam, cadusafos, difenoconasol en grondverdunning.

Geen biologiese agente is deur die teenwoordigheid van chemiese middel aangeteken nie. Wortel-isolasie vanuit die onbehandelde grond het aan die lig gebring dat oomycete (*Phytophthora nicotianae, Phytophthora citrophthora* and *Pythium irregulare*) en *Fusarium*

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spesies wat deel uitmaak van die *Fusarium solani* spesies kompleks (FSSK) en *Fusarium oxysporum* spesies kompleks (FOSK) geassosieer word met herplant grond- en sitruswortels. Identifikasie van *Fusarium* spp. was gebaseer op die filogenetiese ontledings van die translasie verlenging fakor 1-alfa (TEF) en RNA polimerase II tweede grootste subeenheid (RPB2) geen streek.

Filogenetiese analise van die FSSK isolate het getoon dat sitrus isolate in vier afsondelike klade groepeer. Hierdie klade verteenwoordig die, *Neososmospora solani* (25 isolate), *Neocosmospora croci* (een isolaat), 'n onbenoemde *Fusarium* spp. (13 isolate) met *F. falciformis* as die mees naverwante spesie en 'n ander naamlose *Fusarium* klade (een isolaat). Die FOSC sitrus-isolate was verder polifileties en versprei onder twee subklades, voorheen aangewys as Klade 3 (11 isolate) en Klade 4 (two Isolates).

Hierdie studie het geoon dat die groeireaksie van die saalinge biologies van aard is. *Phytophthora nicotianae*, *Phytophthora citrophthora*, *Pythium irregulare* en *Fusarium* spp. binne die FSSK en FOSK sowel as die sitrus aalwurm *Tylenchulus semipenetrans* word almal geassosieer met sitrus wortels en herplant grond. Die bestuurstrategieë in hierdie studie dui daarop dat die toepassings moontlike plaasvervangers vir metiel bromied grondberoking kan wees. Effektiewe voorkoming van sitrus herplant siektes wat van nie-metiel bromied beroking gebruik maak, is afhanklik daarvan om te weet wat in die grond teenwoordig is en om die korrekte besluit te neem op grond van hierdie kennis.

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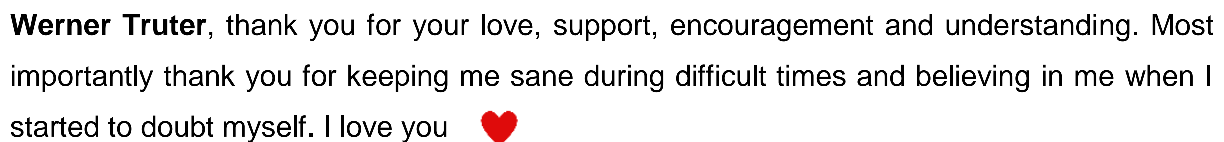
Werner Truter, thank you for your love, support, encouragement and understanding. Most importantly thank you for keeping me sane during difficult times and believing in me when I started to doubt myself. I love you 

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CHAPTER 1

Citrus replant associated pathogens and the management options of these pathogens

INTRODUCTION

Replant problems or -disease are terminologies used when describing a condition where newly planted, healthy nursery trees die after being planted into old orchard soils previously planted with the same or a related crop. The phenomenon is not only limited to citrus but can be characterised by the appearance of small leaves, the formation of gummosis and trees showing low vigour with short internodes (Derrick and Timmer, 2000). This leads to a reduction in plant growth, a delay in the start of the production period and ultimately lower yield of the orchard (Bent *et al*., 2009).

The retarded and stunted growth of newly planted trees has been ascribed to biotic and abiotic factors. A build-up of phytotoxins in the soil, released by the decomposition of the remaining roots in the soil from the previous crop, has been reported as an abiotic factor that contribute to replant problems in citrus (Proebsting and Gilmore, 1941). Other abiotic factors may also be possibly involved such as nutrient imbalances and the deterioration of soil physical characteristics. Biological agents including several soilborne pathogens have also been proposed as causal factors (Mai and Abawi, 1981; Cronje *et al*., 2002; Bent *et al*., 2009). However, limited studies have been conducted that have attempted to identify pathogens other than those that are known major soilborne pathogens of citrus including *Phytophthora* and *Pythium* spp. along with the citrus nematode. Furthermore, studies on citrus replant, have often only investigated the involvement of a limited number of these major pathogens within a specific study. The pathogens can infect almost all parts of the tree, but usually infect their host through roots and wounds near the orchard soil surface and colonise the vascular tissue resulting in blockage of the vascular system (Ahmed *et al*., 2012; Das *et al*., 2016). The infection of mature trees is characterised by fibrous root rot, foot rot, leaf blight, the formation of gummosis on trunks and branches, girdling of the affected branches, leading to the death of the entire tree (Alvarez *et al*., 2008; Ahmed *et al*., 2012; Das *et al*., 2016). Young replanted trees can die rapidly due to trunk and branch canker infection with replant pathogens, especially when exposed to abiotic stress factors (Alvarez *et al*., 2008). Citrus growers can face major financial losses when the infections are severe enough for the orchards to become uneconomical, since replant problems limit the longevity and reduce the yield of trees. Affected orchards also need to be replanted prematurely.

Fumigation with methyl bromide has been shown to significantly improve the productivity of replanted citrus orchards in South Africa. Orchards planted on soils where

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methyl bromide soil fumigation was done prior to replanting, had a significantly higher net income and higher volume of exportable fruit, compared to replant sites where no pre-plant fumigation was applied (Le Roux *et al.*, 1998). However, the use of methyl bromide as a pre-plant fumigant is no longer an option. This is due to the phasing out of methyl bromide in January 2015 in South Africa (EPA, 2017). Finding alternatives to methyl bromide in citrus production is therefore important to control soilborne pathogens and nematodes involved in replant disease, in order to prevent a decrease in fruit production and quality. Many chemical fumigants have been evaluated as possible alternatives to methyl bromide, but most are effective against only fungal soilborne pathogens or only against nematodes (Duniway, 2002; Schneider *et al.*, 2003; Ruzo, 2006). Therefore, mixtures of fumigants are often used to target all the biotic agents involved in a specific crop.

For some other replant diseases, such as apple replant disease, effective management strategies that do not involve fumigation have been developed. These include for example the use of mixtures of brassica seed meals, which yield tree growth responses and yields similar to, or exceeding those of soil fumigation treatments (Mazzola *et al.*, 2015). The development of these alternative strategies was only possible through the development of a good understanding of the biological agents involved, and monitoring these agents during the evaluation and optimization of management strategies. The known biological agents involved in apple replant disease include nematodes (*Pratylenchus* spp.), oomycetes (*Phytophthora, Phytopythium* and *Pythium* spp.) and fungi (*Rhizoctonia* spp. and '*Cylindrocarpon*'-like fungi) (Mazzola and Manici, 2012). It is therefore important to know which biological agents are involved in replant disease of a specific crop.

This literature study will seek to examine replant pathogens associated with replant disease on citrus. The epidemiology and symptoms of these pathogens and possible management practices will be reviewed. However, limited information is available on the aforementioned subjects. Therefore, a brief overview of the causative agents of apple replant disease and managements strategies will also be provided. Apple replant disease is the tree fruit crop where replant disease has been best studied. Reviewing these aspects can contribute to a better understanding of replant disease pathogens involved in the death of young citrus trees in South Africa.

GLOBAL CITRUS PRODUCTION

Citrus cultivation dates back to China about 2200 BC, where the earliest reference of citrus was found in ancient Chinese manuscripts and documents (Florida Citrus Manual BMP, 2012). The Citrus industry is the third largest horticultural industry after deciduous fruits and vegetables (FAO, 2015). Citrus is considered one of the most important horticultural crops, due to its economic export value and local consumption (Ahmed *et al.*, 2012). Citrus is the

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fourth largest global fruit production industry after bananas, watermelons, and apples, with an estimated 81.1 million tons of citrus produced worldwide during 2015/2016 (South African Fruit Trade Flow, 2016; USDA, 2017). The global export of citrus amounted to a total of 8.9 million tons for the 2015/2016 season (South African Fruit Trade Flow, 2016). The major export destinations for South African citrus are Europe and the Middle East, representing 37% and 20% of total exports, respectively (CRW, 2017).

The top eleven major citrus growing regions in the world firstly include China followed by Brazil, India, the United States of America, Mexico, Spain, Egypt, Turkey, Italy, Iran and South Africa (CGA, 2016a). In the Northern hemisphere, China is the leading producer with 38 million tons per year followed by India, the United States of America and Mexico (FAO, 2015; CGA, 2016b). South Africa, although only being the eleventh largest citrus producer in the world, is the second largest producer in the Southern hemisphere with a production forecast of 3.2 million tons for the 2014/2015 marketing year (FAO, 2015; CGA, 2016b). Citrus in South Africa is the second largest fruit crop after grapes in terms of production volumes. South Africa is furthermore the second largest exporter of citrus worldwide, with 1.7 million tons of fresh citrus exported during the 2014/2015 season (CGA, 2016a; CRW, 2017). Citrus is mainly cultivated in Mediterranean climatic regions and is therefore confined to areas with mild and almost frost free winters, where temperature seldom drop below 2°C. Citrus show internal freeze damage when temperatures drop to between -3°C to -1.6°C for longer than two hours.

THE SOUTH AFRICAN CITRUS INDUSTRY

Production areas

South Africa is known for its diverse weather and climatic conditions and is globally known as a producer and exporter of citrus. In South Africa, more than 68 000 hectares (ha) are currently planted with citrus in different production regions. These are located in several provinces including the Limpopo, Mpumalanga, Western Cape, and Eastern Cape provinces; with Limpopo responsible for approximately 42% of the total citrus production and exports (CGA, 2016b). The main production areas in Limpopo include Tzaneen, Letsitele, Makhado (Louis Trichardt), Polokwane, Lephalale (Ellisras), Mokopane (Potgietersrus) and Modimolle (Nylstroom) (Capespan SA, 2015). The area under citrus production has steadily increased from ~53 224 ha in 2012 to 68 272 ha in 2016 (CGA, 2016b). The quantity of citrus produced and exported from these production regions has also increased over the past 5 years with an estimate of 1.4 million pallets exported in 2016 (CGA, 2016a; SA Fruit Journal, 2017).

The most frequently planted citrus cultivars include Eureka lemons (*Citrus sinensis*, 8 262 ha) and Nules clementines (*Citrus reticulate*, 15 930 ha). The most frequently used rootstocks that these cultivars are grafted onto are *Citrus sinensis* x *Citrus trifoliata* (Carizzo

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citrage and Troyer citrage), *Citrus jambhiri* (Rough lemon) and *Citrus paradise "Duncan" Macf* x *Citrus triofoliata* (Swingle citrumelo).

Orchard establishment cost

The South African Citrus tree census of 2016 showed that 16% of the trees in current orchards are younger than 5 years old and 57% of the trees are between 6 and 20 years old (CGA, 2016a; SA Fruit Journal, 2017). Citrus bud-wood sales have increased over the past 5 years, with an estimate of 5 million for 2016, with soft citrus bud-wood sales being the highest that currently represent the majority of new and replanting taking place in the South African industry (CGA, 2016b).

PATHOGENS CONTRIBUTING TO REPLANT DISEASE ON CITRUS TREES

The pathogens involved in citrus replant disease have not been studied extensively in South Africa although Le Roux *et al*., (1998) reported that oomycetes, nematodes and other soilborne fungi are probably involved. It is likely that the replant pathogens will include some of the known soilborne pests and pathogens of citrus. The major soilborne pathogens of citrus include the citrus nematode *Tylenchulus semipenetrans*, *Phytophthora nicotianae* and *Phytophthora citrophthora* (Le Roux *et al*., 1998). *Fusarium solani* is also a known soilborne pathogen of citrus, but its role as pathogen and symptoms caused have been somewhat unknown as it is also known as being involved with dry root rot of citrus (Labuschagne *et al*., 1987). Although *Pythium* spp. have been associated with citrus tree roots (Maseko and Coutinho, 2002; Benfradj *et al*., 2017), their pathogenicity towards citrus is unknown. *Pythium* is a genus that is well known for being involved in replant diseases such as apple replant (Mazzola and Manici, 2012), and this group of pathogens will thus also be discussed in this section. The severity of disease is influenced by the virulence of the organism or infectious agent involved, the susceptibility and growth stage of the host and environmental conditions such as moisture and temperature (Dreistadt, 2012).

Soilborne pathogens that infect the root system and trunk of citrus trees are few in number, but cause a variety of symptoms, resulting in yellowing of foliage, dieback of terminal shoots and branches, poor growth, leaf drop, gradual decline, girdling and death of the entire tree (Graham and Menge, 1999; Graham and Timmer, 2003; Alvarez *et al*., 2008). Fibrous roots, which take up nutrients and water from the soil are primarily affected, followed by infection of major roots and trunk tissue. The roots and trunk tissue deliver water, nutrients and photosynthetic products between root- and shoot systems. Soilborne pathogens play a direct or indirect role in reducing fruit size, total yield, postharvest quality and tree survival under favourable pathogen conditions (Graham and Menge, 1999).

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Phytophthora foot and root rot

The genus *Phytophthora* belongs to the class Perenosporomycetes, placed in the Straminipila kingdom, under the phylum Oomycota (Riisberg *et al*., 2009). *Phytophthora* species are the most damaging and widespread soilborne fungi that attack citrus because they can infect any part of the tree at all stages of development (Erwin and Ribeiro, 1996; Graham and Menge, 1999; Agrios, 2005). There are at least ten *Phytophthora* spp. capable of infecting citrus trees, but the greatest reduction in fruit yield can be attributed to *Phytophthora nicotianae* and *Phytophthora citrophthora* (Erwin and Ribeiro, 1996; Cacciola and di San Lio, 2008; Schutte and Botha, 2010). Abiotic stress, such as root asphyxiation, mineral disorders, and phytotoxicity can also affect the health of roots and enhance the appearance of *Phytophthora* disease symptoms (Dreistadt, 2012).

Phytophthora nicotianae

Phytophthora nicotianae was first isolated from tobacco at the end of the 19th century and is considered one of the most widespread oomycete plant pathogens, with a host range of more than 255 species (Cline *et al*., 2008; Panabières *et al*., 2016). *P. nicotianae* is usually found in subtropical areas, causes foot- and root rot, and seldom infect above ground tree parts. The pathogen can also sometimes cause brown rot on fruit in the canopy, and fallen fruit on the ground. Brown rot epidemics are associated with prolonged wet periods and a temperature range of 23°C to 32°C (Graham *et al*., 1998; Puglisi *et al*., 2017). Foot rot occurs when *Phytophthora* spp. infect the scion area near the ground level, which results in lesions that extend upward to the bud union on resistant rootstocks, or up the trunk into main branches (Graham and Menge, 1999; Graham *et al*., 1998, 2012; Graham and Feichtenberger, 2015). Foot- and root rot can be severe on susceptible rootstocks growing in infested orchard soil, causing tree decline and yield losses. In advanced stages, the production of new fibrous roots is slow and the tree is unable to maintain adequate water, mineral uptake, and nutrient reserves. This results in the reduction of fruit size and yield, loss of leaves, and twig dieback of the entire tree. Gorter (1977) ascribed root and collar rot symptoms associated with citrus in the Western Cape to *Phytophthora nicotianae*. Following the discovery of *P. nicotianae*, four studies were conducted to identify the distribution of *P. nicotianae* in South Africa. A study done by Wehner *et al*. (1987) reported that *P. nicotianae* was the only *Phytophthora* sp. isolated from citrus nursery plants and soil from the Eastern Cape, Limpopo, Mpumalanga and Gauteng provinces. Thompson *et al*. (1995) and Maseko and Coutinho (2002) both identified *P. nicotianae* as the most commonly isolated *Phytophthora* spp. from root and soil samples taken from citrus orchards and nurseries. The most recent study by Meitz-Hopkins *et al*. (2013) confirmed the finding that *P. nicotianae* is the most widely distributed *Phytophthora* species in the seven citrus producing provinces within South Africa.

Disease cycle

Phytophthora nicotianae is a hemibiotrophic pathogen that can live as a parasite or saprophyte on another organism (Panabières, 2016). The pathogen can survive and infect a wide range of plants and plant organs but can also survive without a host. The disease cycle of *P. nicotianae*, similar to most *Phytophthora* spp., begins with the production of multinucleated sporangia on specialised hyphal structures (sporangiophores), releasing large numbers of uninucleate motile zoospores (Graham and Timmer, 2003; Walker and van West, 2007). Under optimal conditions, zoospores encyst, germinate to develop a germ tube and penetrate plant tissue. The optimum temperature for mycelial growth is 30°C to 32°C. Sporangial production is favoured by small deficits in matrix water potential (Graham and Timmer, 2003). The availability of free water and oxygen, together with nutritional depletion and light, stimulate the production of sporangia from mycelium. The indirect germination of sporangia to produce zoospores requires free water and a decrease in temperature. Under moist and at temperatures below 15°C, sporangia have the ability to germinate directly by producing a germ tube (Sharma, 1989). Zoospores are attracted to roots through chemotaxis and electrotaxis from root exudates released by living, damaged or stressed roots. They can also swim short distances by flagellar movements (Walker and van West, 2007).

Phytophthora nicotianae is known to survive unfavourable conditions through the production of chlamydospores and oospores (Erwin and Ribeiro, 1996; Graham and Menge, 1999; Graham and Timmer, 2003). Chlamydospore production occurs under unfavourable conditions including low oxygen levels, temperatures between 15°C and 18°C and nutrient depletion. Under unfavourable conditions chlamydospores can survive for several months. The water requirements for germination of chlamydospores are similar to those for sporangia. Germination is also stimulated when temperatures are favourable for root growth together with nutrients in the form of root exudates. At temperatures below 15°C, chlamydospores become dormant, and when favourable conditions return, they germinate. Chlamydospores can germinate indirectly to produce sporangia and zoospores, or directly to produce mycelium (Graham *et al.*, 1998; Graham and Timmer, 2003). Oospores are thick-walled and resistant to desiccation and cold temperatures, and form when opposite mating types (A1 and A2) are present (Andres *et al.*, 2003; Nerkar *et al.*, 2012). The maturation of oospores are slower than chlamydospores. However, the requirements for oospore germination are almost identical to those of chlamydospores. Chlamydospores and oospores germinate and form sporangia that release zoospores, under well-aerated, moist conditions (Shivankar *et al.*, 2015).

Phytophthora citrophthora

Phytophthora citrophthora was the first *Phytophthora* sp. reported from citrus in South Africa (Doidge, 1925). It was mainly associated with citrus foot and root rot in the Limpopo Province, and was later also reported from Mpumalanga, the Western Cape and Eastern Cape (Doidge

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and Bottomley, 1931; Hector and Loest, 1937; Meitz-Hopkins *et al*., 2013). The reported symptoms include trunk and branch cankers, gum exudates on branches and similar root rot symptoms associated with *P. nicotianae* induced root rot (Alvarez *et al*., 2008, 2009b, 2011). *P. citrophthora* is also known to cause gummosis in other Mediterranean climates and is the most common cause of brown rot on fruit (Graham and Menge, 1999). Gummosis are observed where the inner bark of the scion is damaged. Small cracks form on the bark, exuding abundant gum. Citrus gum is water-soluble and wash away after heavy rains. Lesions can spread around the trunk, slowly girdling the tree, leading to the appearance of pale green leaves with yellow veins (Le Roux, 2003; Schutte and Botha, 2010). A study by Schutte and Botha (2010) showed that *P. citrophthora* is more active at moderate temperatures below 30°C, and *P. nicotianae* at higher temperature >30°C. This confirmed the high activity and isolation of *P. citrophthora* in Mediterranean climatic areas (Alvarez *et al*., 2008; Schutte and Botha, 2010). *Phytophthora citrophthora* also infects the root cortex leading to the decay of fibrous roots. This is primarily a problem in seedbeds and nurseries, where frequent irrigation and high planting densities create favourable conditions for infection (Timmer *et al*., 1991). The root cortex of fibrous roots becomes soft, discoloured and appears water-soaked. The fibrous roots slough their cortex, leaving only the white, threadlike stele protruding at their ends, and giving the root system a stringy appearance (Graham *et al*., 1998, 2012; Graham and Menge, 1999; Le Roux, 2003). There is distinct genetic variation among *P. citrophthora* isolates associated with citrus (Spies *et al*., 2014). A study using isozyme analyses of *P. citrophthora* indicated that the species can be divided into three distinct subgroups. CTR 1 included isolates of *P. citrophthora* from a wide host range including citrus and CTR 2 comprised of Brazilian isolates from cacao. The third group, CTR 3 included Indonesian isolates also isolated from cacao like CTR 2 (Mchau and Coffey, 1994). Cohen *et al.* (2003) found that *P. citrophthora* isolates from Corsica can be further divided into four clonal lineages, G1 to G2, based on sequence data of the internal transcribed spacer regions (ITS), random amplified micro-satellite (RAMS). Alvarez *et al.* (2011) subsequently reported that isolates representing lineages G1, G2 and G3 were also present in Spain. Spies *et al.* (2014) subsequently used random amplified polymorphic DNA (RAPD) marker data, and phylogenetic analyses of the ß-tubulin, *cytochrome c oxidase* subunit I and the ITS region to (i) confirm the presence of the G1 and G2 lineages in South Africa and (ii) show that the two lineages did not represent different phylogenetic species when isolates from South Africa and Spain were analysed (Spies *et al*., 2014). Mating-type analyses showed that most isolates within the G1 lineage were sterile, although some could be identified as belonging to A1 mating type when paired with a fertile *Phytophthora* spp. The G2 lineage isolates were all A2 mating types (Spies *et al*., 2014).

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Disease cycle

The development of mycelia, sporangia, and release of zoospores in *P. citrophthora* are very similar to that mentioned above for *P. nicotianae*. The optimum temperatures for *P. citrophthora* mycelial growth are 23°C to 28°C, whereas those for *P. nicotianae* are 30°C to 32°C (Timmer *et al*., 1989). *Phytophthora citrophthora* rarely produces chlamydospores and never produces oospores. Therefore, the mechanism of survival of the pathogen is uncertain. However, both species can persist as mycelium as well as chlamydospores in infected, living roots or as sporangia in the soil (Graham and Menge, 1999).

Inoculum sources of P. nicotianae *and* P. citrophthora

The way in which *Phytophthora* spp. are spread to new citrus orchards is mainly through infected nursery trees. The pathogen may be present in the soil or in the roots of nursery trees, without any disease symptoms being visible (Graham and Timmer, 2003). Other sources of inoculum of *Phytophthora* spp. include contaminated soil on equipment and vehicles that move from one infested orchard to non-infested areas, or within a nursery. Irrigation plays an important role in the distribution of the pathogens, especially where furrow and flood irrigation is used (Le Roux, 2003). Heavy rains can also result in pathogens being carried with surface water into canals or dams used for irrigation. Zoospores are usually carried over long distances by soil surface water, irrigation water, and hydroponic solutions (Fry and Grünwald, 2010). Infested soil under citrus trees serves as an inoculum source for fruit infections when fruit touch the ground, or when contaminated soil is splashed onto fruit. When conditions are favourable, sporangia are produced on the surface of the fruit, and splashing- or windblown rain spread the sporangia throughout the canopy to other fruit (Graham and Menge, 1999). Wind, without rain, is not a major factor in the dispersal of *Phytophthora* spp., but wind-blown soil may contaminate fumigated soils. Various animals, such as birds, termites or snails are also involved in the dispersal of *Phytophthora* spp. inoculum, since they serve as vectors. Chlamydospores can survive in the gastrointestinal tracts and faeces of these vectors (Weste, 1983; Alvarez *et al*., 2009a).

***Pythium* spp.**

Pythium spp. belong to the family Pythiaceae, order Peronosporales in the Oomycota class and are more closely related to chromophyte algae than eumycotan fungi (Lévesque and De Cock, 2004). *Pythium* spp. are widely distributed and are important plant pathogens, but some species are also non-pathogenic and can even cause plant growth enhancements (Plaats-Niterink, 1981). *Pythium* spp., can thus exist as parasites or saprophytes in soil, water, in other fungi, and on various plants and small marine animals (Martin and Loper, 2010). These oomycete species are widely distributed ranging from tropical to temperate and arctic to antarctic regions (Knox and Paterson, 1973; Plaats-Niterink, 1981; Hoshino *et al*., 1999). Plant

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pathogenic *Pythium* spp. have a wide host range including some woody hosts for example apple, citrus, Eucalyptus, rubber and other woody trees and shrubs. Pathogenic *Pythium* spp. are known to cause necrosis of feeder roots on various tree crops and aerial symptoms such as wilting, chlorosis, and sparse leaf production of the entire canopy (Zea-Bonilla *et al*., 2007). Various conditions such as, the type of soil, host and the management strategies have an important impact on the *Pythium* populations, diversity and severity of symptoms expressed (Al-Sadi, 2012).

The role that *Pythium* spp. play in citrus replant disease is still unknown, even though these species have frequently been isolated from the rhizosphere of diseased citrus trees (Maseko and Coutinho, 2002; Benfradj *et al*., 2017). The pathogenicity of the isolated species has not been tested in most of the studies reporting on the association of *Pythium* spp. with citrus. Thompson *et al*. (1995) reported that *P. irregulare, P. aphenidermatum* and *P. ultimum* are usually associated with citrus in South Africa. *Pythium ultimum* was the first reported *Pythium* spp. to cause root rot on citrus in Cambodia. But since its discovery, other studies have revealed that it is commonly associated with citrus in other countries like Australia, Japan, South Africa, Brazil, Canada, and China (Hendrix and Campbell, 1970; Ohazuruike and Obi, 2000; Kean *et al*., 2010). Benfradj *et al*. (2017) isolated *P. ultimum, P. dissotocum*, *P. aphanidermatum, Phytopythium vexans* and *Phytopythium mercuriale* from citrus trees in Tunisia and according to this study, two of these species were also in citrus nurseries in South Africa. Studies done by Citrus Research International (CRI) in South Africa showed that *Pythium* spp. occurs at significant levels in the irrigation water and potting media of citrus nurseries (personal communication, M. C. Pretorius, CRI, South Africa). However, to date the identity of these isolates along with the potential damage that they can cause, are unknown. The combination of *Phytophthora* and *Pythium* spp. present in citrus soils may lead to increased disease symptoms and rapid death of entire trees or orchards (Benfradj *et al*., 2017).

Taxonomy of the genus Pythium

The identification of *Pythium* spp. is important for diagnosing diseases and developing and implementing control strategies (Kageyama *et al*., 2005). Until the advent of the sequencing and phylogenetic eras, *Pythium* species were identified using morphological criteria. The main criteria for morphological identification include whether the species is homothallic or heterothallic, the number of antheridium that can be observed per oogonium and the type of sporangia. The oogonium itself has various morphological characteristics, which include the difference in size of the oogonium and oospores, the oogonium wall and the character of the oospores in the oogonium. The oogonium wall can be ornamented or smooth and the oospores within the oogonium can be plerotic or aplerotic (Schroeder *et al*., 2013). Identifying

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Pythium isolates to species level using only morphological structures are difficult due to heterothallism (two opposite mating types are required for sexual reproduction), absence in culture of reproductive structures that are important for identification, and similar and overlapping size ranges of the reproductive structures (Mathew *et al.*, 2003; Kageyama, 2014). For morphological identification, *Pythium* are usually plated out on corn meal agar (CMA), potato-carrot agar (PCA), potato dextrose agar (PDA) and 20% (V/V) V8 juice agar with a colony morphology of chrysanthemum, cottony, rosette and semi-cottony on the agar (Kageyama, 2014).

Molecular identification of *Pythium* spp. is more accurate than morphological identifications (Kageyama, 2014). Polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) of the internal transcribed spacer region (ITS) and the cytochrome c oxidase subunit 2 (cox2) fragments can be used as a first step in molecular species identification. PCR-RFLPs can be used to first group isolates with similar sequences into PCR-RFLP groups when large collections of isolates are investigated. Only a subset of the isolates representing the different PCR-RFLP groups are then selected for sequencing and phylogenetic analyses (Lévesque and De Cock, 2004; Choi *et al.*, 2015).

Phylogenetic analyses of the ITS region of the genus *Pythium* have shown that the genus consists of eleven clades, named phylogenic clades A to K (Lévesque and De Cock, 2004). Clade K has been re-described as a new genus *Phytopythium,* since the genus shares morphological characteristics with *Pythium* and *Phytophthora* and is phylogenetically distinct from the genus *Pythium* (De Cock *et al.*, 2014). Lévesque and De Cock (2004) showed that the ITS sequence length of oomycetes is between 750 to 1050 base pairs (bp) and that of Eumycota are shorter, 300 to 700 bp. Robideau *et al.* (2011) first proposed the ITS region and the cytochrome c oxidase subunit 1 (cox1) region as barcoding regions for the oomycetes. A study by Choi *et al.* (2015) indicated that the cox2 gene region should be used as universal barcode for oomycetes, since it results in better amplification and PCR results compared to cox1.

Some *Pythium* isolates remain difficult to identify to the species level, even when using phylogenetic analyses of barcoding genes and additional gene regions. Such isolates are referred to as belonging to species complexes, such as the *P. irregulare* species complex. Robideau *et al.* (2011) stated that *Pythium* isolates in the *P. irregulare* complex are unresolved using only cox1 and ITS sequences. Spies *et al.* (2011) also investigated the *P. irregulare* complex using the cox2 and β-tubulin regions in addition to the ITS and cox1 regions used by Robideau *et al.* (2011). Using these four gene regions, Spies *et al.* (2011) found that *P. irregulare* is genetically diverse and represents only one phylogenetic species. Furthermore, some isolates within the *P. irregulare* complex that were re-described as new species, for

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example *P. cryptoirregulare,* are not phylogenetically distinct within the *P. irregulare* complex (Spies *et al.*, 2011).

Disease cycle

Pythium species are present in abundance in cultivated soil near the root zone. Many species of *Pythium* are known to form sporangia and zoospores as asexual reproductive structures. Zoospores are formed in a vesicle and not in the sporangium itself. This is a characteristic trait that differentiate *Pythium* spp. from *Phytophthora* spp., (Plaats-Niterink, 1981). Hyphal swelling and the formation of sporangia are stimulated by Mg^{2+}, K^{+} and Ca^{2+} ions while the germination of sporangia and mycelial growth is stimulated by root exudates and germinating roots (Yang and Mitchell, 1965; Stanghellini and Hancock, 1971). *Pythium* spp. can be homothallic or heterotallic and sexual reproductive structures are produced as survival structures (Pérez-Jiménez, 2008). Sexual reproduction occurs when an oogonia (female organ) and antheridia (male organ) are present. When the antheridial cell meets the oogonium, a fertilisation tube is formed which penetrates the oogonium and form a zygote also known as the oospore (Plaats-Niterink, 1981). Oospores go through a dormant phase before germination is initiated. Dormancy is lifted by external carbohydrate and calcium sources, high water potential, optimum pH levels and temperatures. Oospores serves as important survival structures and can survive for up to 12 years in the soil (Stanghellini and Hancock, 1971). Nzungize *et al.* (2012) stated that each *Pythium* species has an optimum growth temperature, which may vary between species. A characteristic shared amongst most *Pythium* species is the optimum temperature that is usually around 30°C and the maximum temperature are between 35 and 40°C (Souli *et al.*, 2014). *Pythium ultimum* prefers temperatures that range between 10 and 15°C while the growth of *P. irregulare* is inhibited in warmer soil temperatures between 25 and 36°C. Greater yield losses will also be observed during higher temperatures than in cooler seasons (Petkowski *et al.*, 2013). Soil pH not only influences the dormancy of oospores, but also disease expression in the host and the overall lifecycle of the pathogen. The soil pH play an important role in the availability and uptake of minerals in the soil by the host, and can therefore be altered to supress disease (Martin and Loper, 1999). *Pythium* spp. are more abundantly found in soil with a pH ranging between 6.8 to 7.2, but *Pythium* has been found in soils with a pH above 8.0 (Martin and Loper, 1999). Conditions such as low light intensity, high moisture, low pH, high salt concentrations and temperatures above 28°C should be avoided due to the fact that *Pythium* is more dominant in these environments. *Pythium* spp. are distributed in the same way as *Phytophthora* spp., which was discussed previously.

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Fusarium diseases on citrus

Fusarium spp. are commonly found in soils of citrus orchards (Le Roux, 1985; Nemec *et al.*, 1989) and nurseries (Wehner *et al.*, 1987). The most predominant *Fusarium* species associated with citrus is *Fusarium solani* (Menge and Nemec, 1997), however, *Fusarium oxysporum* has also been isolated from citrus roots and rhizosphere soil but at lower frequencies (Labuschagne *et al.*, 1987). Yaseen and D'Onghia (2012) mentioned that Baker *et al.* (1981) and Nemec *et al.* (1980) implicated *Fusarium solani* as a possible causal agent of citrus blight. Citrus blight is an important decline disease affecting citrus in many countries (Yaseen and D'Onghia, 2012). The symptoms associated with citrus blight include, wilt, defoliation and dieback of the canopy, reduced water uptake and zinc accumulation (Baker *et al.*, 1981; Yaseen and D'Onghia, 2012) that causes dieback and wilt of the entire canopy and the development of poor growth flushes (Derrick and Timmer, 2000; Futch *et al.*, 2005). Blight infected trees seldom die, but trees stay in an unproductive state for many years after infection (Anderson and Calvert, 1970; Polizzi *et al.*, 1992). Baker *et a*l. (1981) and Nemec *et al.* (1980) further investigated and stated that *F. solani* is the possible causal agent of citrus blight. *Fusarium solani* is associated with fibrous and scaffold root rot, and seem to be the only disease symptoms related to blight. Blight symptoms are identical to the symptoms of dry root rot on citrus trees. (Menge and Nemec, 1997). Nemec *et al.* (1980) showed that pathogenic strains of *F. solani* isolated from fibrous roots induced the same blight-like symptoms when it was re-inoculated on healthy roots. Blight- and dry root rot disease have been associated with climatic and soil factors, and that all the citrus rootstocks are susceptible to both diseases (Bender, 1985; Nemec and Myhre, 1992).

Dry root rot is a sporadic, destructive and localized disease complex that may take years before severe symptoms develop. The disease severity usually increase in combination with other root pathogens, nematodes and abiotic factors (Dreistadt, 2012). Dry root rot is often observed on the crown and scaffold roots. Trees develop a moist, dark decay of the bark that overlays the wood infected by the pathogen. Over time the infected bark and wood dries and have a dry and brown stained appearance where pathogen colonization occurred (Graham and Menge, 1999; Adesemoye *et al.*, 2011). Other more visible symptoms associated with dry root rot include wilting, defoliation, dull green leaf colour, die-back of small twigs and the production of small fruit. Under extensive root damage, the leaves suddenly wilt and dry on the tree (Dandurand and Menge, 1992; Yaseen and D'Onghia, 2012; Dreistadt, 2012; Kunta *et al.*, 2015; Marais, 2015). Overall, dry root rot symptoms observed in the canopy are like those caused by *Phytophthora* spp. and other agents that damage the roots or girdle the trunk. Nemec *et al.* (1978) found that abovementioned symptoms and physiological disruptions are due to fibrous root rot and necrosis of major roots infected by *F. solani*.

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Fusarium taxonomy in the F. solani and Fusarium oxysporum species complex

Fusarium solani was classified into the section Martiella by Booth (1971) and can be divided into 50 subspecific lineages. It is a well-known plant pathogen associated with a wide range of plants, causing a variety of diseases and symptoms (Kolattukudy and Gamble, 1995). The identification of *Fusarium* species at the morphological level is based on distinctive characters such as the shape and size of the macro- and microconidia (Leslie and Summerell, 2006). The presence and absence of chlamydospores, colony appearance, pigmentation and growth rate are also indications of *F. solani*. The macroconidia are relatively wide, straight and robust with 5- to 7-septa and present in abundance in the sporodochia. Microconidia is more oval, ellipsoid, reniform and fusiform with 0- to 2- septa. The monophialides is often long and the microconidia are present in abundance in the aerial mycelia. Chlamydospores are globose to oval with smooth or rough cell walls. The chlamydospores can be found within the hyphae (intercalary) or formed terminally on short branches and may be present in short chains (Leslie and Summerell, 2006).

Fusarium solani can be divided into two different morphotypes, I and II (Chehri *et al.*, 2015). Morphotype I, produces short macroconidia (27.0 to 37.3 µm) with 3 to 5 septations, whereas morphotype II has longer macroconidia (36.6 to 46.2 µm) with 3 to 7 septa (Hafizi *et al.*, 2013; Chehri *et al.*, 2011, 2015). The macroconidia of morphotype I are narrower with sparse to abundant cottony forming mycelium, which are pale brown to brown, brown-greenish with a pale brown to yellowish brown pigmentation. Morphotype II has wider macroconidia, with white-creamy to white-greyish cottony mycelium colour. Pigmentation for Morphotype II may be absent or white-creamy with dark brown zonation (Hafizi *et al.*, 2013). Two different coloured sporodochia (cream and blue) can be observed within Morphotype I, whereas morphotype II only produce cream sporodochia and chlamydospores can be observed in both morphotypes (Hafizi *et al.*, 2013).

Phylogenetic analyses revealed that there are variabilities among the members of the *Fusarium solani* Species Complex (FSSC), which led to the identification of three clades (O'Donnell *et al.*, 1998, 2008; O'Donnell, 2000; Nalim *et al.*, 2011). Clade 1 included two known species*, Nectria illudens* and *Nectria plagianthi*, which were used as outgroups in the phylogenetic analyses of *F. solani* isolates in this study (Chapter 3). Members of Clade 2 consisted of pathogens that cause sudden death syndrome on soy-bean (Aoki *et al.*, 2003, 2005, 2012). A study done by Nalim *et al.* (2011) further indicated that members of Clade 2 are paraphyletic. Clade 3 are known to contain the most common *Fusarium* spp. associated with plant diseases and include *Fusarium falciformis* and *Fusarium keratoplasticum* (O'Donnell, 2000; Zhang *et al.*, 2006; O'Donnell *et al.*, 2008; Short *et al.*, 2013, 2014). The most haplotype-diverse species were also placed within Clade 3 (O'Donnell *et al.*, 2008; Nalim *et al.*, 2011; Short *et al.*, 2014).

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The *Fusarium oxysporum* Species Complex (FOSC) is known as an anamorphic species and widespread fungus found in soil (Kistler, 1997; Leslie and Summerell, 2006). *F. oxysporum* contains both pathogenic and non-pathogenic isolates (Gordon and Martyn, 1997). Pathogenic and non-pathogenic strains of *F. oxysporum* can be found in many native plant groups, in soils that have never been cultivated as well as in agricultural soils throughout the world (Gordon and Martyn, 1997; Gordon *et al*., 1992). The pathogenic *F. oxysporum* strains are referred to as *formae speciales* based on the host plant attacked. For example, isolates that are pathogenic towards banana are named *F. oxysporum* f.sp. *cubense.* A *formae speciales* usually has a narrow host range, usually consisting of only one host plant species (Gordon and Martyn, 1997). There are more than 70 described *formae speciales* (f. sp) causing diseases in over 100 plant species (Gordon and Martyn, 1997). Sequence analysis showed that many *formae speciales* are polyphyletic or paraphyletic meaning it is derived from more than one common evolutionary ancestor or ancestral group (O'Donnell *et al*., 1998; Skovgaard *et al*., 2001).

The taxonomy of *F. oxysporum* was initially based on the morphology of the asexual reproductive structures, but as for all fungi this been complemented and to some extent replaced with phylogenetic sequence analyses. This is due to the limited number of morphological characteristics available for identifying in the *F. oxysporum* species complex (Hafizi *et al*., 2013; Lazarotto *et al*., 2014). A multi locus phylogenetic study indicated that *F. oxysporum* consists of three clades, designated as Clades 1, 2, and 3 (O'Donnell *et al*., 1998; 2004). Subsequently, a fourth Clade was identified within the FOSC, with the addition of clinical isolates found by O'Donnel *et al*. (2004). The four clades were further divided into two phylogenetic species, PS I (Clades 1) and PS II (Clades 2, 3 and 4) (Laurence *et al*., 2014).

Disease cycle

Although *F. solani* has been implicated as a causal agent of citrus blight and dry root rot, controversy exists regarding its status as a pathogen and is usually regarded as a saprophyte and opportunistic pathogen within the roots, stems, and bark of citrus trees (Yaseen and D'Onghia, 2012). Dry root rot and citrus blight usually starts when the causal agent infects larger roots that have been injured by *Phytophthora* spp., water-saturated soil, mechanical damage or root burn caused by an overdose of fertilizers or pesticides. This infection of living tissue occurs from surrounding dead tissue where the fungus remains in an inactive state. Under frequent stress conditions the starch levels of the citrus tree drop, causing the natural resistance of the tree to break down, predisposing the citrus trees to infection (Dandurand and Menge, 1992). Infection of wood and cambial tissue is high during cool and wet seasons when the citrus tissue appears dormant and slow to heal (Graham and Menge, 1999).

After infection, the disease spreads into the crown, where the underground portion of the crown appear moist and dark, the moisture dries over time and the lesion stay on the wood. The fungus produces abundant macroconidia and microconidia on dead roots, leaves, bark and organic matter in the soil (Dreistadt, 2012). Macroconidia are relatively wide, straight, slightly curved with 3- to 7-septa and rounded ends. Microconidia are formed in round false heads on long mono-phialides. The microconidia can be oval, ellipsoidal or reniform with 0- to 1-septum. Chlamydospores are abundantly produced in pairs on hyphae and in agar, with a globose to oval shape and can be smooth or rough walled (Snyder and Hansen, 2006). The development of dry root rot and citrus blight disease is enhanced by the presence of other root rot pathogens such as *Phytophthora* spp. and *Pythium* spp., (Polizzi *et al*., 1992).

Inoculum sources

Fusarium solani has a cosmopolitan distribution and can be found in numerous native soils. Perithecia are commonly observed on plant debris in wet tropical areas and serve as survival structures (Snyder and Hansen, 2006).

Citrus nematode

The citrus nematode (*Tylenchulus semipenetrans*) is a soilborne pest, and one of the most important parasitic root nematodes of trees causing severe damage on citrus (Ayazpour *et al*., 2010; Le Roux, 2003). The damage citrus nematodes inflict on citrus is often called citrus slow decline (Dreistadt, 2012). According to El-Borai *et al*. (2002), Thomas (1913) first discovered that the citrus nematode infects citrus trees in California, after which Cobb (1914) described the nematode as a new species and named it, *T. semipenetrans*. Since its discovery, *T. semipenetrans* has been recorded in every citrus growing region of the world (Duncan, 2005). The slow decline caused by *T. semipenetrans* results in a significant reduction in fruit yield and size (Duncan and Cohn, 1990). Leaves of severely infested trees are dull green and smaller than normal. Small twigs die back, giving the canopy a sparse appearance (Le Roux, 2003).

Life cycle

Tylenchulus semipenetrans is a dimorphic species that exhibit sexual dimorphism at both the juvenile and adult stage. The life cycle is typical of a plant-parasitic nematode beginning as an egg, which contains the first juvenile stage (J1). The J1 then moults into the second stage juvenile (J2) within the egg, which will hatch from the egg and begin to search for new host roots (Dreistadt, 2012). The motile and vermiform J2 female moults into J3 and J4, and finally into a sedentary adult female. Female nematodes have the ability to reproduce without mating (parthenogenesis), although mating and sexual reproduction in the presence of a male may

occur. The development of a female juvenile into a sedentary adult requires feeding on the epidermis and superficial layers of the cortical parenchyma of the rootlets (Thorne, 1961).

The nematode is a semi-endoparasite of the cortical cells, where the immature female penetrates the outer surface of the roots into the deep cortical layers, without reaching the endodermis. The female induces several nurse cells in the root cortex and establishes a permanent feeding site, while the posterior part of the nematode remains exposed in the soil (Van Gundy, 1958; Cohn, 1965; El-Borai *et al*., 2002). With maturation, the posterior part of the body swells and protrudes from the root surface while its elongated neck and head remain embedded in the cortex. The female becomes immobile and feeds from the nurse cells for its entire reproductive life. The mature female lays about 75 to 100 eggs, which are deposited through the excretory pore in a gelatinous matrix onto the fibrous root surface (Cobb, 1914). The length of the female life cycle from egg to egg can range from 6 to 8 weeks under optimal temperatures of 25°C to 30°C (Van Gundy, 1958; El-Borai *et al*., 2002; Agrios, 2005). Eggs hatch under wet and moist conditions, following irrigation or rain and when the soil temperature is above 20°C. The eggs can survive for up to nine years in the soil (Le Roux, 2003). The development of the J2 male into an adult is completed in seven days and does not require feeding. This is due to the fact that the feeding apparatus, the stylet, and oesophagus, of the adult males, are poorly developed and are difficult to observe.

Infection sources

Tylenchulus semipenetrans evolved in the Far East with citrus and was dispersed to many citrus growing areas of the world with nematode infested nursery stock. High nematode populations are commonly found in citrus orchards established in fine-textured soils or in sandy soils with high organic matter content. Fluctuations in soil salinity from high to low favours reproduction, while sandy soils poor in organic matter suppresses population increase to an extent (Timmer *et al*., 2003). The citrus nematode moves slowly through the soil, and under flood irrigation and irrigation with nematode infested water, nematodes are spread more rapidly from one tree to another (Le Roux, 2003). Low populations of the citrus nematode can survive in leftover roots and soil for up to 5 years after host removal (Dreistadt, 2012).

MANAGEMENT STRATEGIES TO CONTROL CITRUS REPLANT PATHOGENS

Disease management is highly reliant on the prevention of infection mainly through the production and use of clean planting material, disease-free soil, nursery hygiene, and the use of resistant rootstocks (Das *et al*., 2016). The improvement of drainage and aeration of the soil should also be maintained for adequate growth of root systems (Menge and Nemec, 1997). In a study done by Le Roux *et al*. (1998) they noted that there can be differences in replant soils in terms of soilborne pathogens and plant parasitic nematode populations. It is

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therefore important to identify the soilborne pathogens and nematodes in the soil prior to replanting. This will ensure the development of optimal management strategies that are effective against all the identified pests and diseases. Management strategies should also be effective under all the diverse soil conditions where citrus production occurs. It is also important that management strategies should be economically feasible and environmentally acceptable, making it suitable for use in replant disease management.

Cultural practices

Cultural control practices, such as sanitation and monitoring of soilborne pests and pathogens through fruit- and leaf baiting, should be used to detect, reduce and prevent infections. Plant material from nurseries should be free from citrus-specific pathogens. Inspection of fibrous roots in the nursery and orchard before planting is therefore recommended (Graham *et al.*, 2012).

It is important to know the history of an orchard when planning to establish a new citrus block. Orchards not previously planted with citrus or similar species are probably free from citrus specific *Phytophthora* spp. and other soilborne pathogens damaging citrus roots. When planting the trees, the bud union should be planted well above the soil line, on raised soil beds, to avoid contact between the susceptible scion bark and potentially infected soil (Graham and Menge, 1999; Graham *et al.*, 2012). If soils will be fumigated, it is important to remove dead, fungal contaminated and diseased parts of the tree, as well as dead trees and fruit from the orchard soil, otherwise fumigation will not be effective (Grout, 2003).

Steam sterilisation

The traditional approach is to heat the soil with steam at 100°C for about 30 to 40 minutes. Sheet steaming is the most frequently used steam soil sterilisation method and requires a temperature of 95°C at a depth of 30 cm (Bungay, 1994; Nerderpel, 1979). Steaming the soil at high temperature alters the chemical composition of the soil to some extent and it is, therefore, important to lime the soil to decrease the accumulation of high concentrations of water-soluble and exchangeable manganese (Dawson *et al.*, 1965; Rabie, 2001). If aerated steam is used, effective disease control can be reached at soil temperatures as low as 65°C (Gullino and Mezzalama, 1993). This method offers several advantages including the possibility of eliminating pathogens, without affecting certain components of the resident saprophytic microflora (Baker, 1970). Steaming the soil with temperatures of up to 50°C do not improve growth of trees in replant orchard soil (Otto, 1972). However, steam sterilising the soil with temperatures ranging from 65°C to 95°C can contribute to the increase in tree growth (Sharma and Bhardwaj, 1999).

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Resistant rootstocks

There are no rootstocks that are tolerant toward all the putative biological agents involved in citrus replant. Most commercial rootstocks are to some extent resistant toward bark and root infections. There are some rootstocks known for having some resistance to *Phytophthora* spp. Swingle citrumelo *(Citrus paradise* Macf. x *Poncirus trifoliate* (L.) Raf.) is considered to be resistant due to the fact that it tolerates high populations of *Phytophthora* spp. in the soil (Graham, 1990, 1995; Graham and Menge, 1999; Le Roux, 2003). These rootstocks have the ability to regenerate fibrous roots after infection, but resistance is less effective in poorly drained soil (Adair *et al*., 2000; Castle *et al*., 2004). Volkamer lemon (*C. volkameriana* Tan. And Pasq.) is seen as tolerant to *Phytophthora* infection because new fibrous roots develop in the presence of *Phytophthora* spp. and when infection is observed on the roots (Graham, 1995). Other rootstocks are susceptible to root rot and may suffer root loss when heavily infected (Graham, 1990, 1995). These susceptible rootstocks include Carrizo citrange (*C. sinensis* x *P. trifoliata*), sour orange (*C. aurantium* L.), Cleopatra mandarin (*C. reticulate* Blanco), sweet orange (*C. sinensis* L.), and Palestine sweet lime (*C. limettioides* Tanaka (Agostini *et al*., 1991; Timmer *et al*., 1991). Some of the *Phytophthora* resistant rootstocks may be susceptible to other diseases, show incompatibility towards commercial scion cultivars, are horticulturally unacceptable or fail to adapt to soil conditions (Bright *et al*., 2004; Feichtenberger *et al*., 1992).

Commercial scion cultivars are susceptible to *Phytophthora* bark infection; however, some scion-rootstock combinations show moderate resistance toward infection (Feichtenberger *et al*., 1992; Graham and Feichtengerger, 2015).

Cover crops and crop rotation

The principle of crop rotation is to plant other species of crops in an orchard before replanting the soil with the desired crop (Biggs *et al*., 1997). This method can promote growth of newly planted trees through enhanced nutrient availability and by suppressing plant pathogenic nematodes, and it can also prevent the build-up of pathogenic microorganisms in the soil. In most cases, crop rotation can effectively control soilborne diseases (Mazzola and Gu, 2000). However, the biological components that cause citrus replant disease are known to be stable once established in the soil and are difficult to control with cover crops and crop rotation since citrus trees are perennial and cannot be rotated frequently (Timmer *et al*., 2000). Rotation with annual crops for 1 to 3 years before replanting is advisable, but this is not feasible for the control of soilborne diseases in orchard systems due to the perennial nature of the crops (Mazzola and Gu, 2000).

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Chemical control

Fungicide treatments

The use of fungicides to control soilborne diseases has resulted in a reduction of pathogen populations, increased fibrous root densities and in some cases, increased yields (Sandler *et al*., 1989; Timmer *et al*., 1989). The application of fungicides registered against *Phytophthora*, *Pythium,* and *Fusarium* alone, or in combination with a nematicide can be used to reduce root and foot rot on citrus trees. This may also result in improved tree vigour and health (Mckenzie, 1985; Le Roux *et al*., 1991). There are a few fungicides available for the management of oomycete species. The standard treatment for this species, with systemic properties, are products with etridiazole and mefenoxam as active ingredient (Porter *et al*., 2009). Ridomil Gold® 480 SL, is a group code 4 fungicide and contains mefenoxam as active which is a phenylamide. Mefenoxam is the most effective systemic fungicide with preventative and curative activity against *Phytophthora* and *Pythium* spp. in soil and plant tissue. Mefenoxam inhibit the ribosomal RNA polymerase with it's single-site mode of action, thus inhibiting sporulation and growth of mycelium (Hu and Li, 2014). It is advisable to apply mefenoxam every alternative season and when planting new trees as well as applying a nematicide at transplant. A nematicide with active ingredient cadusafos, under the trade name of Rugby® 10 ME, can be applied to control nematodes in the soil. It is advisable to apply cadusafos at an interval of two months as soon as the rain season start. Cadusafos is a contact action nematicide and only controls the mobile stages of the nematodes in the soil. The product must be drenched into the top layer of the soil by irrigation up to 20 to 35 mm.

Fumigation

Methyl bromide is a widely used fumigant for both pre-plant and post-harvest pest and pathogen control (Schneider *et al*., 2003). Fumigation with methyl bromide as a pre-plant fumigant has been used for more than 50 years to manage soilborne pathogens and pests (Duniway, 2002). Methyl bromide is volatile and can penetrate the soil for some distance from the point of application. It is a highly effective and easy to use fumigant, but was categorised as a Class 1 ozone-depleting substance and therefore the use and production of methyl bromide was phased out internationally in 2005 by the Montreal Protocol and US Clean Air Act (Duniway, 2002; Schneider *et al*., 2003; Desaeger *et al*., 2017). For developing countries, the reduction and use of methyl bromide was more gradual and the phased-out period was delayed until 2015. Since the phasing-out of methyl bromide as a pre-plant and post-harvest fumigant there is a high demand for alternative management strategies with the same desired effect as methyl bromide. There are a few fumigation alternatives that are currently being used with broad-spectrum activities in the soil, such as 1,3-dichloropropene (1,3-D), chloropicrin (CP), methyl isothiocyanate (MITC) and metam sodium (Duniway, 2002; Gilreath *et al*., 2005;

Desaeger *et al.*, 2017). These chemicals can be used alone, or can be mixed with one or more of the above-mentioned alternatives. The main disadvantage that all the fumigants have compared to methyl bromide, is that they are less volatile and mobile, and are not uniformly distributed in the soil (Ruzo, 2006).1,3-Dichloropropene was initially developed as a soil nematicide and chloropicrin has strong fungicidal activity and is less effective against nematodes (Duniway, 2002; Ruzo, 2006).

In South Africa 1,3-dichloropropene (1,3-D) and chloropicrin (CP) are used in combination as a soil fumigation for effective nematode, plant pathogenic fungi and oomycete control. Trials done by Schneider *et al.* (2003) and Jhala *et al.* (2011) for the control of citrus nematode (*Tylenchulus semipenetrans*) and soilborne fungi found that pre-plant soil fumigation with 1,3-D and CP had a positive effect on eliminating nematodes, fungi and oomycetes in the soil. However, the use of these chemicals provided little weed management, and is less active against dormant seeds in the soil (Schneider *et al.*, 2003; Jhala *et al.*, 2011). Soil fumigation in citrus replant situations can, however significantly contribute to long-term profitability of newly replanted orchard.

APPLE REPLANT DISEASE AS A MODEL SYSTEM FOR REPLANT DISEASE

Replant disease of apples have been extensively studied over the past decades. Although several studies initially ascribed several abiotic factors as being the cause of replant problems, it is now mostly accepted that the disease is caused by biological agents (Mazzola and Manici, 2012). This has been shown using several approaches. In glasshouse studies the biological nature of the disease was shown convincingly by (i) the fact that apple seedlings show a significant increase in growth when grown in pasteurized soil relative to seedlings grown in untreated soil, (ii) adding 10 or 15% of untreated replant soil to pasteurized soil results in a reduction in seedling growth relative to seedlings grown in pasteurised soil and (iii) some biocide treatments result in a significant increase in apple seedling growth (Mazzola, 1998; Tewoldemedhin *et al.*, 2011a). Under orchard conditions, trees on fumigated soil show an increased growth response in comparison to trees grown in untreated soil, further providing evidence for the biological nature of apple replant disease (Mazzola and Manici, 2012).

The biological agents involved in apple replant disease have been elucidated using a combination of approaches. Isolations studies have been conducted quite extensively in South Africa, Italy and the United states. The isolation studies were mainly done using seedling bioassays, since isolations from orchard tree roots result in an overwhelming number of saprophytic fungi, which mask the presence of pathogens (Mazzola, 1998; Tewoldemedhin *et al.*, 2011a). Isolation studies have shown the association of oomycetes (*Pythium* spp., *Phytopythium* and *Phytophthora* spp.), fungi (*Cylindrocarpon*-like fungi, *Rhizoctonia solani,* bi-nucleate *Rhizoctonia*, *Fusarium* spp.) and parasitic nematodes (*Pratylenchus* spp.). The

pathogens can be site specific, for example *R. solani* occurs in Italy and the USA in replant soils, but not in South Africa. *Pratylenchus* spp., which are known to be parasitic on apple, occur in replant orchards in all investigated countries, but only in some orchards. Isolations studies alone cannot prove the involvement of fungi and oomycetes with replant diseases. Therefore, pathogenicity assays have also been conducted to determine which of the associated species are involved. Pathogenicity studies have shown that only selective *Pythium* spp. (mainly *P. ultimum, P. irregulare* and *P. sylvaticum*), *Phytophthora* spp., a few bi-nucleate *Rhizoctonia* species, *Rhizoctonia solani* AG-5 and AG-6 and *Cylindrocarpon*-like fungi are involved (Mazzola, 1998; Manici *et al.,* 2003*;* Tewoldemedhin *et al.,* 2011a, 2011b, 2011c).

The involvement and pathogenicity of '*Cylindrocarpon'*-like fungi are not clear due to major taxonomic changes in this group of fungi. Furthermore, most '*Cylindrocarpon'* species that were identified as containing pathogenic isolates, also contained isolates non-pathogenic to apple (Tewoldemedhin *et al.*, 2011a; Lombard *et al.*, 2014). In South Africa, Italy and the USA *Fusarium oxysporum,* although extensively isolated from replant seedling roots, were not pathogenic (Mazzola 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011a). The *Fusarium* spp., *F. solani, F. sambucinum* and *F. avenaceum* in general have low virulence towards apple seedlings and not all isolates within a specific species are pathogenic. *Fusarium* spp. are thus not considered as major apple replant disease pathogens (Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011b). Some studies have furthermore shown using pathogenicity studies that some of the known replant pathogens interact synergistically for example *'Cylindrocarpon'*-like spp. and some *Pythium* spp. (Braun 1995; Mazzola 1998; Tewoldemedhin *et al*., 2011a).

Another approach that has been used to determine which groups of biological agents are involved, is the application of biocides to apple seedlings under glasshouse conditions (Mazzola, 1998; Tewoldemedhin *et al*., 2011a). For example, the application of metalaxyl that target oomycetes, significantly improved the growth of seedlings in most replant soils in South Africa. This indicated an important role of this group of pathogens in South Africa (Tewoldemedhin *et al*., 2011a). The involvement of nematodes in some replant orchards has been shown through applications of fenamiphos (Mazzola, 1998; Tewoldemedhin *et al*., 2011a).

It is interesting that of the known apple replant pathogens, several are only able to incite disease on young trees. Once trees reach the age of 3-years, these pathogens are no longer damaging to trees. Therefore, the protection of trees at planting and in the early years of orchard establishment is very important (Mazzola and Manici, 2012).

Elucidation of the biological agents involved in apple replant disease, as well as the development of molecular markers to rapidly quantify the pathogens from roots, has led to the

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development of sustainable management strategies. The best example is the use of brassica seed meals. A range of orchard trials were conducted by first evaluating single brassica seed meal species (Mazzola *et al.*, 2015). These were shown to be ineffective when used independently (Cohen and Mazzola, 2006; Mazzola *et al.*, 2009). However, knowledge on which brassica species suppressed specific pathogens were ultimately used to develop a mixture of seed meals that can be used effectively to manage apple replant disease. The mixture of seed meals applied and incorporated into soil, followed by plastic tarping of the soil, yielded tree growth improvement and yields that exceeded those attained with fumigation treatments (Mazzola *et al.*, 2015).

CONCLUSION

Citrus are one of the most important deciduous fruit crops being produced in South Africa, contributing to the revenue produced from exporting fruit, mainly to Europe and the Middle East (CRW, 2017). Losses due to soilborne diseases are of economical concern for citrus growers in South Africa. Based on the literature study there are still many gaps in literature based on the etiology and management of citrus replant disease in South Africa. The pathogens involved have not yet been characterized using modern molecular studies combined with phylogeny and fundamental research into the causal organism complex. It is therefore important to accurately characterize the biological agents involved and to develop an effective, sustainable management strategy. This will provide the citrus industry with knowledge regarding the best approach for establishing replant orchards, leading to productive orchards with high quality fruit for the export market.

The aim of this thesis study was to better understand citrus replant disease in South Africa with the ultimate aim of developing a methyl bromide free management strategy. The objectives were to:

1. determine if the basis of citrus replant disease is biotic or abiotic.
2. determine the main oomycete, fungal and nematode species associated with citrus replant disease.
3. conduct molecular characterization and/or phylogenetic studies on a selected group of fungi (*Fusarium* spp.) and oomycetes (*P. citrophthora, P. nicotianae and P. irregulare*) found to be associated with replant soils.

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REFERENCES

Adair, R.C., Mehta, N.K. and Graham, J.H. 2000. A pilot study of spatial distribution of *Diaprepes abbreviates* (L.) (Cleoptera: Curculionidae) and *Phytophthora* spp. in citrus. Proceedings of the Florida State Horticultural Society, 113: 82-88.

Adesemoye, A., Eskalen, A., Faber, B., O'Connell, N., Kallsen, C. and Shea, T. 2011. Current knowledge on *Fusarium* dry rot of citrus. Citrograph Nov/Dec: 29-33.

Agostini, J.P., Timmer, L.W., Castle, W.S. and Mitchell, D.J. 1991. Effect of citrus rootstocks on soil populations of *Phytophthora parasitic*a. Plant Disease, 75: 296-300.

Agrios, G.N. 2005. Plant pathology. 5th Ed. Elsevier Academic Press, San Diego, USA.

Ahmed, Y., D'Onghia, A.M., Ippolito, A., E.l Shimy, H., Cirvilleri, G. and Yaseen, T. 2012. *Phytophthora nicotianae* is the predominant *Phytophthora* species in citrus nurseries in Egypt. Phytopathologia Mediteranea, 51(3): 519-527.

Al-Sadi, A.M. 2012. Efficacy of mefenoxam is affected by a lag period between application and inactivation of *Pythium* species. Phytopathologia Mediteranea, 51: 292−297.

Alvarez, L.A., Vincent, A., Soler, J.M., Dela Roca, E., Bascon, J. and Garcia-Jimenez, J. 2008. Comparison of application methods of systemic fungicides to suppress branch cankers in clementine trees caused by *Phytophthora citrophthora*. Plant Disease, 92(9): 1357-1363.

Alvarez, L.A., Gramaje, D., Abad-Campos, P. and Garcia-Jimenez, J. 2009a. Role of the *Helix aspersa* snail as a vector of *Phytophthora citrophthora* causing branch cankers on clementine trees in Spain. Plant Pathology, 58: 956-963.

Alvarez, L.A., Gramaje, D., Abad-Campos, P. and Garcia-Jimenez, J. 2009b. Seasonal susceptibility of citrus scion to *Phytophthora citrophthora* and *P. nicotianae* and the influence of environmental and host-linked factors on infection development. Plant Pathology, 124: 621-635.

Alvarez, L.A., Leom, M., Abad-Campos, P., Garcia-Jimenez, J. and Vincent, A. 2011. Genetic variation and host specificity of *Phytophthora citrophthora* isolates causing branch cankers in Clementine trees in Spain. Plant Pathology, 129: 103-117.

Anderson. C.A. and Calvert, D.V. 1970. Mineral composition of leaves from citrus trees affected with declines of unknown etiology. Proceedings of the Florida State Horticultural Society, 83: 41-45.

Andrés, J.L., Rivera, A. and Fernández, J.A. 2003. *Phytophthora nicotianae* pathogenic to pepper in Northwest Spain. Plant Pathology, 58: 91-98.

Aoki, T., O'Donnell, K. and Geiser, D.M. 2003. Sudden-death syndrome of soybean is caused by two morphologically and phylogenetically distinct species within the *Fusarium solani* Species Complex – *F. virguliforme* in North America and *F. tucumaniae* in South America. Mycologia, 95: 660-684.

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

Aoki, T., O'Donnell, K. and Scandiani, M.M. 2005. Sudden-death syndrome of soybean in South America is caused by four species of *Fusarium*: *Fusarium brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F. tucumaniae* and *F. virguliforme*. Mycologia, 46: 162-183.

Aoki, T., Scandiani, M.M. and O'Donnell, K. 2012. Phenotypic, molecular phylogenetic and pathogenetic characterisation of *Fusarium crassistipitatum* sp. nov., a novel soybean sudden death syndrome pathogen from Argentina and Brazil. Mycoscience, 53(3): 167-186.

Ayazpour, K., Hasanzadeh, H. and Arabzadegan, M.S. 2010. Evaluation of the control of citrus nematode (*Tylenchulus semipenetrans*) by leaf extracts of many plants and their effects on plant growth. African Journal of Agricultural Research, 5(14): 1876-1880.

Baker, K.F. 1970. Selevtive killing of soil microorganisms by aerated steam. In: Tousson T.A, Bega, R.V and Nelson, P.E (eds) Root Diseases and Soilborne Pathogens, 234-239. University of California Press, Berkeley. CA, USA.

Baker, R.A., Tatum, J.H. and Nemec, S. 1981. Toxin production by *Fusarium solani* from fibrous roots of blight-diseased citrus. Phytopathology, 71: 951-954.

Bender, G.S. 1985. Dry root rot of citrus – Factors which increase the susceptibility of trees to infection by *Fusarium solani*. Ph.D. dissertation, University of California Riverside, CA.

Benfradj, N., Migliorini, D., Luchi, N., Santini, A. and Boughalleb-M'Hamdi, N. 2017. Occurrence of *Pythium* and *Phytophthora* species isolated from citrus trees infected with gummosis disease in Tunisia. Archives of Phytopathology and Plant Protection, 50: 286-302.

Bent, E., Loffredo, A., Yang, J.I., McKenry, M.V., Becker, J.P. And Borneman, J. 2009. Investigation into peach seedling stunting caused by replant soil. FEMS Microbiology Ecology, 68(2): 192-200.

Biggs, A.R., Baugher, T.A., Collins, A.R., Hogmire, H.W., Kotcon, J.B., Glenn, M., Sexstone, A.J. and Byers, R.E. 1997. Growth of apple trees, nitrate mobility and pest populations following a corn versus fescue crop rotation. American Journal of Alternative Agriculture, 12: 162-172.

Booth, C. 1971. The genus Fusarium. Kew, United Kingdom, Commonwealth Mycological Institute, 1-237.

Braun, P.G. 1995. Effects of *Cylindrocarpon* and *Pythium* species on apple seedlings and potential role in apple replant disease. Canadian Journal of Plant Pathology, 17: 336-341.

Bright, D.B., Irey. MS., Baucum. L.E. and Graham, J.H. 2004. Soil, rootstock and climatic factors affecting populations of *Phytophthora nicotianae* in South Florida citrus plantings. Proceedings of the Florida State Horticultural Society, 117: 167-173.

Bungay, D.P. 1994. Principles of Steam Pasteurization. South African Avocado Growers' Association Yearbook, 17: 53-55.

Cacciola, S.O. and di San Lio, G.M. 2008. Management of citrus diseases caused by *Phytophthora* spp. In A. Ciancio and K.G. Mukerji (Eds.), Integrated management of diseases caused by fungi, Phytoplasma, and bacteria. Netherlands: Springer, 61-84.

Capespan, 2015. Capespan South Africa, Citrus production areas in South Africa. Online: http://www.capespan.com/index.php?c=global_operations/page&title=sa/production-areas-sa

Castle, W.S., Bauer, M., Bowman, B., Obreza, T. and Stover, E. 2004. Matching soils with rootstocks, especially Swingle citrumelo. Citrus Industry, 85(7): 15-17.

CGA, 2016a. Citrus Growers' Association of South Africa – Annual Report 2016. Online: http://3b5dca501ee1e6d8cd7b905f4e1bf723.cdn.ilink247.com/ClientFiles/cga/CitrusGowersAssociation/Company/Documents/CGA%20AR%202016e(1).pdf

CGA, 2016b. Citrus Growers' Association of South Africa – Key Industry Statistics for Citrus Growers. Online: https://www.citrusresourcewarehouse.org.za/home/document-home/information/cga-key-industry-statistics/3610-cga-key-industry-statistics-2016/file

Chehri, K., Salleh, B., Yli-Mattila, T., Reddy, K.R.N. and Abbasi, S. 2011. Molecular characterisation of pathogenic *Fusarium* species in cucurbit plants from Kermanshah province, Iran. Biological Sciences, 18(4): 341-351.

Chehri, K., Salleh, B. and Zakaria, L. 2015. Morphological and phylogenetic analysis of *Fusarium solani* species complex in Malaysia. Microbiology Ecology, 69: 457-471.

Choi, Y., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., Ploch, S., Shin, H., Shivas, R. G., Telle, S., Voglmayr, H. and Thines, M. 2015. Towards a universal barcode of oomycetes – a comparison of the cox1 and cox2 loci. Molecular Ecology Resources, 15: 1275–1288.

Cline, E.T., Farr, D.F. And Rossman, A.Y. 2008. Synopsis of Phytophthora with accurate scientific names, host range, and geographic distribution. Plant Health Progress, 1-12.

Cobb, N.A. 1914. Citrus-root nematode. Journal of Agriculture Research, 3: 217-230.

Cohen, M.F. and Mazzola, M. 2006. Resident bacteria, nitric oxide emission and particle size modulate the effect of in *Brassica napus* seed meal on disease incited by *Rhizoctonia solai* and *Pythium* spp. Plant Soil, 286: 75-86.

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

Cohen, S., Allasia, V., Venard, P., Notter, S., Verniere, C. and Panabieres, F. 2003. Intraspecific variation in *Phytophthora citrophthora* from citrus trees in Eastern Corsica. European Journal of Plant Pathology, 109: 791-805.

Cohn, N.A. 1965. On the feeding and histopathology of the citrus nematode. Nematologica, 11: 594-600.

Cronje, C., Le Roux, H. F., Truter, M., van Heerden, I. and Phillips, H. 2002. Long-term effect of preplant soil solarization on growth of replant citrus trees in South Africa. Plant Protection, 8:41–49.

CRW, 2017. Citrus Resource Warehouse – Province makes sizeable contribution to SA citrus crop. Online: https://citrusresourcewarehouse.org.za/home/document-home/news-articles/bizcommunity/4418-bizcommunity-province-makes-sizeable-contribution-to-sa-citrus-crop-14-03-17/file

Dandurand, L.M. and Menge, J.A. 1992. Influence of *Fusarium solani* on citrus root rot caused by *Phytophthora parasitica* and *Phytophthora citrophthora*. Plant and Soil 144: 13-21.

Das, A.K., Nerkar, S., Kumar, A. and Bawage, S. 2016. Detection, identification, and characterization of *Phytophthora* spp. infecting citrus in India. Plant Pathology, 98: 55-69.

Dawson, J.R., Johnson, R.A.H., Adams, P. and Last, F.T. 1965. Influence of steam/air mixtures, when used for heating soil, on biological and chemical properties that effect seedling growth. Annual Applied Biology, 56: 243-251.

De Cock, W.A.M., Lodhi, A.M., Rintoul, T.L., Bala, K., Abad, A.G., Coffey, M.D., Shahzad, S. and Lévesque, C.A. 2014. *Phytopythium*: molecular phylogeny and systematics. Persoonia, 34: 25-39.

Derrick, K.S. and Timmer, L.W. 2000. Citrus blight and other diseases of recalcitrant etiology. Phytopathology, 38: 181-205.

Desaeger, J., Dickson, D.W. and Locascio, S.J. 2017. Methyl bromide alternatives for control of root-knot Nematode (*Meloidogyne* spp.) in tomato production in Florida. Journal of Nematology, 49(2): 140-149.

Doidge, E.M. 1925. Brown rot in citrus fruits (*Phythiacystis citrophthora* (R. and E. Sm.). Journal of the Department of Agriculture, Union of South Africa 1920, 10: 499-503.

Doidge, E.M. and Bottomley, A.M. 1931. A revised list of plant diseases occurring in South Africa. Memoir Botanical Survey of South Africa, 11: 1-78.

Dreistadt, S.H. 2012. Integrated Pest Management for Citrus. University of California State-wide Integrated Pest Management Program. Oakland: UC ANR Publication 3303.

Duncan, L.W. 2005. Nematode Parasites of Citrus. In: Plant parasitic nematodes in subtropical and tropical agriculture, 2nd Edition, 11: 437 – 492.

Duncan, L.W. and Cohn, E. 1990. Nematodes parasites on citrus. 321-346 in: Plant-parasitic nematodes in subtropical and tropical agriculture (M. Luc, R.A. Sikora, and J. Bridge, eds.). Wallingford, England: CAB International.

Duniway, J.M. 2002. Status of Chemical Alternatives to Methyl Bromide for pre-plant fumigation of soil. Phytopathology, 92(12): 1337-1343.

El-Borai, F.E., Duncan, L.W. and Graham J.H. 2002. Infection of citrus roots by *Tylenchulus semipenetrans* reduces root infection by *Phytophthora nicotianae*. Nematology, 34: 384-389.

EPA, 2017. Phasing-out Methyl Bromide in developing countries, United Nations Environment Programme, UNEP 2014; United States Environmental Protection Agency, EPA 2017. Online:
https://www.epa.gov/ods-phaseout/methyl-bromide

Erwin, D.C. and Ribeiro, O.K. 1996. Phytophthora diseases worldwide. American Phytopathological Society (APS) Press, St Paul, MN, USA.

FAO, 2015. Food and Agriculture Organization of the United Nations – Statistical Pocketbook World food and agriculture.
Online:
http://www.fao.org/3/a-i4691e.pdf

Feichtenberger, E., Rossetti, V., Pompeu, J Jr., Teófilo Sobrinho, J. and Figueiredo, JO. 1992. Evaluation of tolerance to *Phytophthora* species in scion rootstock combinations of citrus in Brazil. Proceedings of the International Society of Citriculture, 7: 854-858.

Florida Citrus Manual BMP, 2012. Water quality/quantity best management practices for Florida Citrus.
Online:
http://freshfromflorida.s3.amazonaws.com/Bmp_FloridaCitrus2012.pdf

Fry, W.E. and Grünwald, N.J. 2010. Introduction to Oomycetes. The Plant Health Instructor, Cornell University and USDA-ARS.

Futch, S.H., Derrick, K.S. and Brelansky, R.H. 2005. Field identification of Citrus Blight. IFAS Extension University of Florida.
Online:
http://edis.ifas.ufl.edu

Gilreath. J.P., Santos, B.M., Gilreath, P.R. and Noling, J.W. 2005. Validation of 1,3-dichloropropene plus chloropicrin broadcast application in tomato grower fields. Journal of Vegetable Science, 11: 133-139.

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

Gordon, T.R. Okamoto, D. and Milgroom, M.G. 1992. The structure and interrelationship of fungal population in native and cultivated soils. Molecular Ecology, 1: 241-249.

Gordon, T.R. and Martyn, R.D. 1997. The evolutionary biology of *Fusarium oxysporum*. Annual Review of Phytopathology, 35: 111-128.

Gorter, G.J.M.A. 1977. Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Report of the South African Department of Agriculture Technical Services Plant Protection Research Institute Science Bulletin, 392: 1-177.

Graham, J.H. 1990. Evaluation of tolerance of citrus rootstocks to Phytophthora root rot in chlamydospores-infested soil. Plant Disease, 74: 743-746.

Graham, J.H. 1995. Root regeneration and tolerance of citrus rootstocks to root rot caused by *Phytophthora nicotianae*. Phytopathology, 85: 111-117.

Graham, J.H., Timmer, L.W., Drouillard, D.L. and Peever, T.L. 1998. Characterization of *Phytophthora* spp. causing outbreaks of citrus brown rot in Florida. Phytopathology, 88: 724-729.

Graham, J.H. and Menge, J.A. 1999. Root Diseases. 4(12): 126-13 in: Citrus health management (L.W. Timmer, and L.W. Duncan, eds.). APS Press, St. Paul, Minnesota, USA.

Graham, J.H. and Timmer, L.W. 2003. *Phytophthora* diseases of citrus. University of Florida IFAS Extension ,127: 1-11.

Graham, J.H., Timmer, L.W. and Dewdney, M.M. 2012. Florida Citrus Pest Management Guide: *Phytophthora* foot rot and root rot. University of Florida IFAS Extension, 156: 1-6.

Graham, J.H. and Feichtenberger, E. 2015. Citrus *phytophthora* diseases: Management challenges and successes. Citrus Pathology, 2(1): 1-11.

Grout, T.G. 2003. Cultural Practices. 3(2)13-17 in: Integrated production guidelines for export Citrus. Citrus Research International (Pty) Ltd, Nelspruit, SA.

Gullino, M.L. and Mezzalama, M. 1993. Influence of cultural practices and chemical treatments on soilborne diseases of fruit crops. Acta Horticulture, 477: 107-113.

Hafizi, R., Salleh, B. and Latiffah, Z. 2013. Morphological and molecular characterisation of *Fusarium solani* and *Fusarium oxysporum* associated with crown disease of oil palm.

Hector, J. and Loest, F. 1937. Preliminary note on brown-rot gummosis of citrus in the Eastern Cape. South African Journal of Science, 33: 643-644.

Hendrix, F.J.R. and Campbell, W.A. 1970. Distribution of *Phytophthora* and *Pythium* species in soils in the Continental United States. Canadian Journal of Botany, 48: 377-384.

Hoshino, T., Tojo, M., Okada, G., Kanda, H., Ohgiya, S. and Ishizaki, K. 1999. A filamentous fungus, *Pythium ultimum* Trow var. *ultimum*, isolated from moribund moss colonies from Scalbard, Northern Islands of Norway. Polar Bioscience, 12: 68-75.

Hu, J. and Li, Y. 2014. Inheritance of mefenoxam resistance in *Phytophthora nicotianae* populations from a plant nursery. European Journal of Plant Pathology, 139: 545–555.

Jhala, A.J., Gao, S., Gerik, J.S., Qin, R. and Hanson, B.D. 2011. Effects of surface treatments and application shanks on nematode, pathogen and weed control with 1,3-dichloropropene. Pest Management Science, 68: 225-230.

Kageyama, K. 2014. Molecular taxonomy and its application to ecological studies of *Pythium* species. Journal of General Plant Pathology, 80: 314-326.

Kageyama, K., Nakashima, A., Kajihara, Y., Suga, H. and Nelson, E.B. 2005. Phylogenetic and morphological analyses of *Pythium graminicola* and related species. The Journal of General Plant Pathology, 71: 174-182.

Kean, S., Soytong, K. and To-anun, C. 2010. Application of biological fungicides to control citrus root rot under field conditions in Cambodia. Agricultural Technology, 6(2): 219-230.

Kistler, H.C. 1997. Genetic Diversity in the Plant-Pathogenic Fungus *Fusarium oxysporum*. Phytopathology, 87(4): 474-479.

Knox, J.S. and Patterson, R.A. 1973. The occurrence and distribution of some aquatic Phycomycetes on Ross Island and the Dry Valleys of Victoria Land, Antartica. Mycologia, 65: 373-387.

Kolattukudy, P.E. and Gamble, D.L. 1995. Nectria haematococca: pathogenesis and host specificity in plant diseases. In: Kohmoto, K., Singh US, Singh RP, editors. Pathogenesis and Host Specificity in Plant Pathogenic Fungi and Nematodes. Vol. 2. Eukaryotes Pergamon, Oxford, 83-102.

Kunta, M., Salas, B., Gonzales, M. and Da Graça, J.V. 2015. First report of citrus dry root rot caused by *Fusarium solani* on sour orange rootstock in Texas. Citrus Pathology, 2(1): 1-5.

Labuschagne, N., Kotzé, J.M. and Putterill, J.F. 1987. Incidence of *Fusarium solani* and *F. oxysporum* in citrus roots and infection by *F. solani*. Phytophylactica, 19: 315-318.

Laurence, M.H., Summerell, B.A., Burgess, L.W. and Liew, E.C.Y. 2014. Genealogical concordance phylogenetic species recognition in the *Fusarium oxysporum* species complex. Fungal Biology, 118: 812-819.

Lazarotto, M., Milanesi, P.M., Muniz, M.F.B., Reiniger, L.R.S., Beltrame, R., Harakva, R. and Blume, E. 2014. Morphological and molecular characterization of *Fusarium* spp. pathogenetic to pecan trees in Brazil. Genetics and Molecular Research, 13(4): 9390-9402.

Le Roux, H.F. 1985. Effect of Soil Solarisation and Chemical Treatments on Root Disorders of Citrus. University of Pretoria, M.Sc. (Agric.) Thesis. Pretoria, South Africa.

Le Roux, H.F. 2003. Soilborne Diseases, 3(7): 201-217 in: Integrated production guidelines for export citrus. Citrus Research International (Pty) Ltd, Nelspruit, SA.

Le Roux, H.F., Wehner, F.C., Kotzè, J.M. and Grech, N.M. 1991. Combining fosetyl-al trunk injection or metalaxyl soil drenching with soil application of aldicarb for control of citrus decline. Plant Disease, 75: 1233-1236.

Le Roux, H.F., Ware, A.B., Petorius, M.C. and Wehner, F.C. 1998. Comparative efficacy of preplant fumigation and postplant chemical treatment of replant citrus trees in an orchard infested with *Tylenchulus semipenetrans*. Plant Disease, 82(12): 1323-1327.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual, 1st Edition. Blackwell Publishing, Iowa.

Lévesque, C.A., and De Cock, A.W.A.M. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Research, 108: 1363-1383.

Lombard, L., van der Merwe, N.A., Groenewald, J.Z. and Crous, P.W. 2014 Lineages in *Nectriaceae*: re-evaluating the generic status of Ilyonectria and allied genera. Phytopathologia Mediterranea, 53(3): 515-532.

Mai, W.F. and Abawi, G.S. 1981. Controlling replant diseases of pome and stone fruits in North-eastern United States by pre-plant fumigation. Plant Disease, 65(11): 859-864.

Manici, L.M., Ciavatta, C., Kelderer, M. and Erschbaumer, G. 2003. Replant problems in South Tyrol: role of fungal pathogens and microbial population in conventional and organic apple orchards. Plant and Soil, 256: 315-324.

Marais, L.J. 2015. Efficacy of water soluble silicon in managing *Fusarium* Dry Root Rot of Citrus. Acta Horticulture, 1065: 993-999.

Martin, F.N. and Loper, J.E. 1999. Soilborne Plant Diseases Caused by *Pythium* spp.: Ecology, Epidemiology, and Prospects for Biological Control. Critical Reviews in Plant Sciences, 18: 111-181.

Martin, F.N. and Loper, J.E. 2010. Soilborne Plant Disease caused by Pythium spp.: Ecology, Epidemiology, and Prospects for Biological Control. Critical Reviews in Plant Sciences, 18(2): 111-181.

Maseko, B.O.Z. and Coutinho, T.A. 2002. Pathogenicity of *Phytophthora* and *Pythium* species associated with citrus root rot in South Africa. South African Journal of Botany, 68: 327-332.

Mathew, R., Singh, K.K. and Paul, B. 2003. *Pythium campanulatum* sp. nov., isolated from the rhizosphere of maize, its taxonomy, ITS region of rDNA, and comparison with related species. FEMS Microbiology Letters, 226: 9-14.

Mazzola, M. 1998. Elucidation of the Microbial Complex Having a Causal Role in the Development of Apple Replant Disease in Washington. Phytopathology, 88(9): 930-938.

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

Mazzola, M., Brown, J., Zhao, X., Izzo, A., and Fazio, G. 2009. Interaction of brassicaceous seed meal and apple rootstock on recovery of *Pythium* spp. and *Pratylenchus penetrans* from roots grown in replant soils. Plant Disease, 93:51-57.

Mazzola, M. and Gu, Y. 2000. Impact of wheat cultivation on microbial communities from replant soils and apple growth in greenhouse trials. Phytopathology, 90: 114-119.

Mazzola, M., Hewavitharana, S.S. and Strauss, S.L. 2015. *Brassica* Seed Meal Soil Amendments Transform the Rhizosphere Microbiome and Improve Apple Production Through Resistance to Pathogen Reinfestation. Phytopathology, 105(4): 460-469.

Mazzola, M. and Manici, M. 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. Annual Review of Phytopathology, 50: 45-65.

Mchau, G.R.A. and Coffey, M.D. 1994. An integrated study of morphological and isozyme patterns found within a worldwide collection of *Phytophthora citrophthora* and a redescription of species. Mycological Research, 98(11): 1291-1299.

Mckenzie, D. 1985. The influence of metalaxyl (Ridomil) on *Phytophthora* root and collar rot of citrus in South Africa. Citrus Subtropical Fruit Journal, 618: 16-20.

Meitz-Hopkins, J.C., Pretorius, M.C., Spies, C.F.J., Huisman, L., Botha, W.J., Langenhoven, S.D. and McLeod, A. 2013. Phytophthora species distribution in South African citrus production regions. European Journal of Plant Pathology, 138(4): 733-749.

Menge, J.A. and Nemec, S. 1997. Citrus. Pages 185-227 in: Soilborne diseases of tropical crops (R.J. Hillocks and J.M. Waller, eds.). CAB International.

Nalim, F.A., Samuels, G.J. and Wijesundera, R.L. 2011. New species from *the Fusarium solani* Species Complex derived from perithecia and soil in the old-World tropics. Mycologia, 103: 1302-1330.

Nederpel, L. 1979. Soil solarisation and pasteurisation. In: D. Mulder (ed.). Soil disinfestation. Elsevier Scientific Publishing Company, Amsterdam, 29-37.

Nemec, S., Burnette, H.C. and Patterson, M. 1978. Observations on a citrus fibrous root rot involving *Fusarium solani* in blight-diseased groves. Soil and Science Society of Florida Proceedings, 37: 43-47.

Nemec, S., Baker, R. and Burnett, H. 1980. Pathogenicity of *Fusarium solani* to citrus roots and its possible role in blight aetiology. Proceedings of the Florida State Horticultural Society, 93: 36-41.

Nemec., S. and Myhre, D.L. 1992. Citrus Blight. In: J. Kumar, H.S. Chaube, U.S. Singh and A.N. Mukhopadhyay (ed.). Plant diseases of international importance. Diseases of fruit crops, Patience Hall, England Cliffs N.J., 3: 209-225.

Nemec, S., Zablotowicz, R.M. and Chandler, J.L. 1989. Distribution of *Fusarium* spp. and selected micro-flora in citrus soils and rhizospheres associates with healthy and blight-diseased citrus in Florida. Phytophylactica, 21: 141-146.

Nerkar, S.C., Bawage, S.S., Kumar, A. and Das, A.K. 2012. Uni-gene-derived microsatellite marker based variation study of *Phytophthora nicotianae* isolates infecting Citrus. Microbiology, 52: 489-491.

Nzungize, J.R., Lyumugabe, F., Busogoro, J. and Baudoin, J. 2012. *Pythium* root rot of common bean: biology and control methods. A review. Biotechnology, Agronomy, Society and Environment, 16: 405-413.

O'Donnell, K., Kistler, C.K., Cigelnik, E. and Ploetz, R. 1998. Multiple evolutionary origins of fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences USA, 95: 2044-2049.

O'Donnell, K. 2000. Molecular Phylogeny of the *Nectria haematococca-Fusarium solani* Species Complex. Mycologia, 92(5): 919-938.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Magnon, K.C., Cox, P.A., Revankar, S.G., Sanche, S., Geiser, D.M., Juba, J.H., Van Burik, J.A.H. and Padhye, A. 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multi-locus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. Journal of Clinical Microbiology, 42(11): 5109-5120.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E. and Geiser, D.M. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. Journal of Clinical Microbiology, 46(8): 2477-2490.

Ohazuruike, N.C. and Obi, V.I. 2000. Basic Principles of Plant Pathology. Totan publishers Ltd, Nigeria, 127.

Otto, G. 1972. Studies on the cause of specific replant disease of fruit trees. Experiments on the transfer of specific replant disease by the roots. Bacteriologica, Parasitica and Hygiene, 127(3): 279-289.

Panabières, F., Ali, G.S., Allagui, M.B., Dalio, R.J.D., Gudmestad, N.C., Kuhn, M., Guharoy, S., Shena, L. and Zampounis, A. 2016. *Phytophthora nicotianae* diseases worldwide: new knowledge of a long-recognised pathogen. Phytopathologia Mediterranea, 55(1): 20-40.

Pérez-Jiménez, R. 2008. Significant Avocado Diseases Caused by Fungi and Oomycetes. Plant Science and Biotechnology, 2(1): 1-24.

Petkowski, J.E., De Boer, R.F., Norng, S., Thomson, F. and Minchinton, E.J. 2013. *Pythium* species associated with root rot complex in winter-grown parsnip and parsley crops in south eastern Australia. Australasian Plant Pathology, 42: 403-411.

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

Plaats-Niterink, A.J.VD. 1981. Monograph of the genus *Pythium*. Studies in Mycology, 21: 1-242.

Polizzi, G., Mangnano di San Lio, G. and Catara, A. 1992. Dry root rot of citranges in Italy. Proc. Int. Soc. Citriculture, 2: 890-893.

Porter, L.D., Hamm, P.B., David, N.L., Gieck, S.L., Miller, J.S., Gundersen, B. and Inglis, D.A. 2009. Metalaxyl-M-Resistant *Pythium* Species in Potato Production Areas of the Pacific Northwest of the U.S.A. American Journal of Potato Research, 86: 315–326.

Proebsting, E. and Gilmore, A. 1941. The relation of peach root toxicity to the re-establishing of peach orchards. Horticultural Science, 38: 21-26.

Puglisi, I., De Patrizio, A., Schena, L., Jung, T., Evoli, M., Pane, A., Van Hoa, N., Van Tri, M., Wright, S., Ramstedt, M., Olsson, C., Faedda, R., Di San Lio, G.M. and Cacciola, S.O. 2017. Two previously unknown *Phytophthora* species associated with brown rot of Pomelo (*Citrus grandis*) fruits in Vietnam. PLOS One, 12(2): 1-19.

Rabie, L. 2001. An evaluation of the specific apple replant problem in Western Cape orchard soils. MScAgric thesis University of Stellenbosch, Stellenbosch, South Africa, 22-23.

Riisberg, I., Orr, R.J.S., Kluge, R., Shalchina-Tabrizi, K., Bowers, H.A., Patil, V., Edvardsen, B. and Jakobsen, K.S. 2009. Seven gene phylogeny of heterokonts. Protista, 160: 191-204.

Robideau, G.P., De Dock, A.W.A.M., Coffey, M.D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D.W., Désaulniers, N., Eggertson, Q.A., Gachon, C.M.M., Hu, C., Küpper, F.C., Rintoul, T.L., Sarhan, E., Verstappen, E.C.P., Zhang, Y., Bonants, P.J.M., Ristaino, J.B. and Lévesque, C.A. 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Molecular Ecology Resources, 11: 1002–1011.

Ruzo, L.O. 2006. Physical, chemical and environmental properties of selected chemical alternatives for the pre-plant use of methyl bromide as soil fumigant. Pest Management Science, 62: 99-113.

Sandler, H.A., Timmer, L.W., Graham, J.H. and Zitko, S.E. 1989. Effect of fungicide applications on populations of *Phytophthora parasitica* and on feeder root densities and fruit yield of citrus trees. Plant Disease, 73(11): 902-906.

Schneider, S.M., Rosskopf, E.N., Leesch, J.G., Chellemi, D.O., Carolee, T.B. And Mazzola, M. 2003. United States Deprtment of Agriculture – Agricultural Research Service research on alternatives to methyl bromide: pre-plant and post-harvest. Pest Management Science, 59: 814-826.

Schroeder, K.L., Martin, F.N., De Cock A.W.A.M., Lévesque, C.A., Spies, C.F.J., Okubara, P.A. and Paulitz, T.C. 2013. Molecular Detection and Quantification of *Pythium* Species: Evolving Taxonomy, New Tools, and Challenges. Plant Disease, 97: 4-20.

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

Schutte, G.C. and Botha, W.J. 2010. *Phytophthora citrophthora* trunk and branch canker on Clementine mandarins in the Western Cape province of South Africa. Plant and Soil, 27(3): 215-220.

Sharma, O.P. 1989. Textbook of Fungi. Tata McGraw-Hill Publishing Company Ltd.

Sharma, S.K. and Bhardwaj, L.N. 1999. Apple replant problem. In Disease of Horticultural Crops. Ed, Verma, L.R. and Sharma, R.K. 1999. Indus Publishing Co., New Delhi.

Shivankar, V.J., Ghosh, D.K., Das, A.K. and Rao, C.N. 2015. Tropical and Subtropical Citrus Health Management. Satish Serial Publishing, India.

Short, D.P.G., O'Donnell, K., Thrane, U., Nielsen, K.F., Zhang, N., Juba, J.H. and Geiser, D.M. 2013. Phylogenetic relationships among members of the *Fusarium solani* Species Complex in human infections and the descriptions of *F. keratoplasticum* sp. nov. and *F. petroliphilum* stat. nov. Fungal Genetics and Biology, 53: 59-70.

Short, D.P.G., O'Donnell, K. and Geiser, D.M. 2014. Clonality, recombination, and hybridization in the plumbing-inhabiting human pathogen *Fusarium keratoplasticum* inferred from multilocus sequence typing. Evolutionary Biology, 14: 1-14.

Skovgaard, K., Nirenberg, H.I., O'Donnell, K. and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology, 91: 1231-1237.

Souli, M., Abad-Campus, P., Pérez-Sierra, A.M., Fattouch, S., Armengol, Forti, J. and Boughalleb-M'Hamdi, N. 2014. Etiology of apple tree dieback in Tunisia and abiotic factors associated with the disease. African Journal of Microbiology Research, 8: 2272-2281.

South African fruit trade flow, 2016. National Agricultural Marketing Council – Markets and Economic Research Centre.
Online:
http://www.kzndard.gov.za/images/Documents/MARKET_INFORMATION/South-African-Fruit-flow-report_June-2016_Issue-22.pdf

South African Fruit Journal. 2017. April and May edition, p. 28 – 32.

Spies, C.F.J., Mazzola, M. and McLeod, A. 2011. Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa. European Journal of Plant Pathology, 131: 103-119.

Spies, C.F.J., Meitz-Hopkins, J.C., Langenhoven, S.D., Pretorius, M.C. and McLeod, A. 2014. Two clonal lineages of *Phytophthora citrophthora* from citrus in South Africa represent a single phylogenetic species. Mycologia, 106(6): 1106-1118.

Snyder, W.C. and Hansen, H.N. 2006. *Fusarium solani* (Matius) Appel and Wollenweber emend. 1(13): 250-255 in: The *Fusarium* Laboratory Manual (JF. Leslie, and BA. Summerell). Blackwell Publishing Ltd, Iowa, USA.

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

Stanghellini, M.E. and Hancock, J.G. 1971. The sporangium of *Pythium ultimum* as a survival structure in soil. Phytopathology, 61: 157.

Tewoldemedhin, Y.T., Mazzola, M., Mostert, L. and McLeod, A. 2011 a. *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. European Journal Plant Pathology, 129: 637-651.

Tewoldemedhin, Y.T., Mazzola, M., Botha, W.J., Spies, C.F.J. and McLeod, A. 2011 b. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple replant orchards in South Africa. European Journal of Plant Pathology, 130, 215-229.

Tewoldemedhin, Y.T., Mazzola, M., Labuschagne, I. and McLeod, A. 2011 c. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agaents, with some agents acting synergistically. Soil Biology and Biochemistry, 43: 1917-1927.

Thomas, E.E. 1913. A preliminary report of a nematode observed on citrus roots and its possible relation with the mottled appearance of Citrus trees. California Agricultural Experiment Station Publications in Citriculture, 85: 14-18.

Thompson, A.H., Phillips, A.J.L. and Nel, E. 1995. *Phytophthora* and *Pythium* associated with feeder root rot of citrus in the Transvaal province of South Africa. Phytopathology, 14: 37-41.

Thorne, G. 1961. Principles of Nematology. McGraw-Hill Book Company, Inc. New York, Toronto, London.

Timmer, L.W., Agostini, J.P., Graham, J.H. and Castle, W.S. 1991. Relationship of citrus rootstock of *Phytophthora* root rot and populations of *Phytophthora parasitica*. Proceedings of the Florida State Horticultural Society, 104: 173-178.

Timmer, L.W., Garnsey, S.M. and Broadbent, P. 2003. Diseases of citrus. 136-196 in: Diseases of Tropical Fruit Crops. CAB International, UK.

Timmer, L.W., Garnsey, S.M. and Graham, J.H. 2000. Compendium of Citrus Diseases. APS Press, Minnesota, USA, 2: 9-19.

Timmer, L.W., Sandler, H.A., Graham, J.H. and Zitko, S.E. 1989. *Phytophthora* feeder root rot of bearing citrus: fungicide effects on soil populations of *Phytophthora parasitica* and citrus tree productivity. Proceedings of the Florida State Horticultural Society, 102: 5-9.

USDA, 2017. United States Department of Agriculture – Citrus: World Market and Trades. Online: https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf

Van Gundy, S.D. 1958. The life history of the citrus nematode *Tylenchulus semipenetrans* Cobb. Nematologica, 3: 283-294.

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Walker, C.A. and van West, P. 2007. Zoospore development in oomycetes. Fungal Biology Reviews, 21: 10-18.

Wehner, F.C., Combrink, H. and Kotzé, J.M. 1987. Root pathogens in South African citrus nurseries. Phytophylactica, 19: 107-108.

Weste, G. 1983. *Phytophthora cinnamomi*: The Dynamics of Chlamydospore Formation and Survival. Journal of Phytopathology, 106(2): 163-176.

Yang, C.Y.D. and Mitchell, J.F. 1965. Cation effect on reproduction in *Pythium* spp. Phytopathology, 55: 1127-1131.

Yaseen, T. and D'Onghia, A.M. 2012. *Fusarium* spp. Associated to Citrus dry root rot: An emerging issue of Mediteranean Citriculture. Acta Horticulture, 940: 647-656.

Zea-Bonilla, T., Martín-Sánchez, P.M., González-Sánchez, M.A. and Pérez-Jiménez, R.M. 2007. *Neofusicoccum parvum* and *Pythium vexans*: new avocado pathogens detected in Andalusia. Book of Abstracts of the VI World Avocado Congress, 112.

Zhang, N., O'Donnell, K., Sutton, D.A., Nalim, F.A., Summerbell, R.C., Padhye, A.A., Geiser, D.M. 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. Journal of Clinical Microbiology, 44(6): 2186-2190.

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CHAPTER 2

Identification and characterization of soilborne pest, diseases and abiotic factors potentially associated with citrus replant disease

ABSTRACT

Citrus replant disease is a soilborne disease that occurs when new orchards are established on sites where citrus has been cultivated for many years. Tree stunting and yield reductions are associated with replant disease. The causative agents (fungi, oomycetes and nematodes) involved in citrus replant disease in South Africa, have only been studied to a limited extent. The aim of this study was to investigate the etiology of citrus replant disease in South Africa in four orchard soils. Four citrus replant orchard soils were sampled in the Addo, Patensie, Hoedspruit and Letsitele production areas. Analyses of orchard soil samples showed that *Pythium irregulare*, *Phytophthora citrophthora* and *Phytophthora nicotianae* were present in the soil from all four production areas. Infestation levels of juveniles of the citrus nematode *T. semipenetrans* was significantly higher in the soil samples of the Patensie and Hoedspruit orchards, than in the other two orchard soils. The involvement of biological replant agents were further investigated by growing Carrizo citrange rootstock seedlings in a soil bioassay trial in the glasshouse, where six treatments were evaluated. Biological agents were involved in three of the orchard soils (Addo, Hoedspruit and Letsitele), since seedling growth (height or weight) was significantly higher for seedlings grown in pasteurized soil versus seedlings grown in the untreated control soil. Furthermore, a dilution of 20% of these replant soils into pasteurized soil resulted in a significant reduction in seedling growth relative to the control and the pasteurized treatments. In the Patensie orchard soil, the involvement of biological agents was unclear since soil pasteurized did not significantly increase seedling growth relative to the control seedlings. However, dilution of the Patensie soil did result in significantly lower seedling growth than the control. The remaining seedling bioassay treatments consisted of the independent application of three biocides including mefenoxam, difenoconazole, and cadusafos that target oomycetes, fungi, and nematodes respectively. None of the biocide treatments indicated the involvement of specific biological agents targeted by each biocide. The exception was the Letsitele orchard where oomycetes were likely involved due to a significant increase in the length of mefenoxam treated seedlings relative to the control. Isolations from the roots of the control bioassay seedlings in all orchards showed that isolates belonging to the '*Fusarium solani'* species complex dominated in all orchards except the Letisitele orchard. *Fusarium oxysporum* species complex isolates and oomycetes (*Phytophthora nicotianae, Phytophthora citrophthora* and *Pythium irregulare*) were also associated with the citrus roots at more or less similar frequencies within each orchard.

INTRODUCTION

Replant disease is a phenomenon that is observed in cases where young, healthy nursery trees are planted on old orchard sites, previously planted with the same crop or closely related species. Replant disease symptoms is characterised by newly planted trees that are stunted, with small leaves, exhibiting poor growth (Derrick and Timmer, 2000). The retarded and stunted growth of newly planted trees has been ascribed to the accumulation of phytotoxins in the soil, development of nutrient imbalances, deterioration of soil physical characteristics and the establishment of soil pathogens that damage the root systems of young trees (Cronje *et al.*, 2002). The casual agents associated with citrus replant disease in South Africa according to a previous study done by Le Roux *et al.* (1998) include the citrus nematode, *Tylenchulus semipenetrans* and the oomycete pathogens, *Phytophthora nicotianae* and *Phytophthora citrophthora*. Previous citrus replant studies only focused on the involvement of *Phytophthora* spp. and the citrus nematode. It is therefore important to further investigate any possible pathogens involved in the disease complex instead of focusing only on *Phytophthora* spp. and the citrus nematode.

The biological agents involved in apple replant disease are much more complex than what has been reported for citrus replant disease. Isolation and pathogenicity studies in Italy, South Africa, Australia and the United states have shown that various fungal (*Rhizoctonia* spp.), oomycete (*Pythium, Phytopythium* and *Phytophthora*) and nematode (*Pratylenchus* spp.) species are involved. These organisms have been shown to be cite specific, and it is therefore important to investigate several replant sites in order to determine the different replant pathogens involved. *Phytophthora cactorum* occurs worldwide in apple replant soils, whereas *P. cambivorum* has been reported less frequently. Many *Pythium* species have been associated with apple replant soils, but only a few are pathogenic. Some of the most virulent and wide-spread species include *P. ultimum, P. irregulare* and *P. sylvaticum.* Only a few of bi-nucleate *Rhizoctonia* species are pathogenic, but some isolates within the bi-nucleate anastomosis groups are non-pathogenic (Dullahide *et al.*, 1994; Mazzola, 1998; Manici *et al.*, 2003; Tewoldemedhin *et al.*, 2011a, b, c; Mazzola and Manici, 2012). *Rhizoctonia solani* AG-5 and AG-6 have only been identified in Italy and Washington State in the USA, where they are known as highly pathogenic species (Mazzola, 1998; Manici *et al.*, 2003). '*Cylindrocarpon'*-like fungi are known to be involved in apple replant disease although isolates in general have low virulence (Dullahide *et al.*, 1994; Mazzola, 1998; Tewoldemedhin *et al.*, 2011a). Several *Fusarium* spp. were also found to be involved with apple replant disease (Verma and Sharma, 1999). However, the species involved are not clear due to recent major taxonomic changes in this group of fungi (Lombard *et al.*, 2014). The *Fusarium* spp., *F. solani, F. sambucinum. F. tricinctum* and *F. avenaceum* have been identified as apple replant pathogens, but these are considered as unimportant in apple replant disease. This is due to most isolates within a

species not being pathogenic, and those isolates that are pathogenic having low virulence (Dullahide *et al*., 1994; Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011 b, c). Some studies have furthermore shown through pathogenicity studies that some of the known apple replant pathogens interact synergistically for example *'Cylindrocarpon'*-like spp. and some *Pythium* spp. (Braun, 1995; Mazzola, 1998; Tewoldemedhin *et al*., 2011a).

Peach replant is also caused by biological agents, although the agents involved has been studied to a lesser extent than for apple replant. Replant in peach, have been shown to be associated with '*Cylindrocarpon' destructans*, '*Cylindrocarpon' lucidum*, *Pythium* spp., *Phytophthora cactorum* and *Rhizoctonia solani* (Jaffee *et al*., 1982; Browne *et al*., 2006; Bent *et al*., 2009).

In citrus replant, the fumigation of soil with methyl bromide pre-plant significantly increased the net income and the total volume of exportable fruit compared to the untreated replant soils (Le Roux *et al*., 1998; Duniway, 2002; Schneider *et al*., 2003). However, this management option is no longer available, since methyl bromide was phased out for developing countries such as South Africa in 2015 (Duniway, 2002; Schneider *et al*., 2003; Desaeger *et al*., 2017). Finding alternatives to methyl bromide for citrus production on old orchard sites are important, since if soilborne pathogens and nematodes are not controlled, this can lead to significant losses in production and fruit quality.

The increase in citrus production in South Africa has necessitated the establishment of new orchards on sites where citrus has previously been cultivated for many years (Burger and Small, 1983). Since our understanding of the biological agents involved in citrus replant disease is limited, it is important to elucidate the biological agents involved. This can ultimately lead to the development of sustainable management strategies that target the complex group of biological agents that might be involved in citrus replant disease. This study was therefore aimed at providing the citrus industry with knowledge regarding whether biological agents were involved and the specific agents involved.

MATERIALS AND METHODS

Soil sampling

Root and soil samples were collected from four citrus orchards, aged between 37 and 47 years, prior to removal of the old trees. The orchards were situated in two of the main citrus growing regions in South Africa, i.e. Eastern Cape (Addo and Patensie areas) and Limpopo provinces (Hoedspruit and Letsitele areas). In each orchard, 90 soil samples (250 kg) with some roots were randomly collected at a depth of 10 to 30 cm underneath the tree canopy, leading up to 360 soil samples in total. Sampling was conducted between April and May 2016. Between orchards, the shovel used to take samples were cleaned and sterilised by spraying with 70% ethanol. The 90 soil samples taken from each orchard, was pooled and mixed

together and subdivided for use in a glasshouse seedling bioassay trial. A small portion of the pooled soil per orchard were used to determine the physical characteristics of the soil (NviroTek Labs, Hartbeespoortdam) and another portion were used to quantify and identify oomycetes and parasitic nematodes present within the soil (Grimm and Alexander, 1973).

Physical and chemical characterization of soil

The portion of soil per orchard sent away were subjected to standard chemical and physical soil analyses. Soil pH was determined in a KCl solution, while phosphorous content was determined using the Bray I method. The results from the different orchards were compared using analysis of variance (ANOVA) while the means were compared using the Fisher's LSD test at a 95% confidence level (SAS software version 9.4).

Quantification of oomycetes and nematodes from orchard soils

Semi-quantitative analyses of oomycetes

The presence of oomycetes (*Pythium* and *Phytophthora*) in the soils were determined using a leaf disk baiting method to analyse the portion of soil from each orchard subjected for oomycete quantification (Grimm and Alexander, 1973; Linde *et al.*, 1994; Timmer *et al.*, 1988, 1990). For each of the 10 soil samples, one ice tray, containing 14 cubicles were used. One teaspoon (5 mL) of soil was placed into each cubicle of an ice tray with 14 cubicles and filled with sterile distilled water to cover the soil. Unsprayed lemon leaves were surface sterilised with 70% ethanol for 10 seconds and left to dry in the laminar flow. Two circular citrus leaf disks with a diameter of 5 mm were placed on the soil mixture in each cubicle and covered with cardboard. The ice trays were incubated for 48 hours at room temperature before removing the leaf disks. After removing the leaf pieces from the soil-water slurry, it was blotted dry on a paper towel to remove excess water before plating out the 28 leaf disks for each orchard soil. A total of four plates with seven leaf pieces (28 in total) each was plated out for each ice tray and sub-sample on corn meal agar (CMA, Sigma-Aldrich) amended with pimaricin-ampicillin-rifampicin-PCNB-hymexazol (PARPH) and corn meal agar (CMA, Sigma-Aldrich) pimaricin-ampicillin-rifampicin-PCNB (PARP) selective medium as modified by Timmer *et al.* (1988). The Petri dishes were incubated in the dark for 48 hours at 29°C. After the incubation period, the number of leaf disks yielding hyphal growth into the medium were sub-cultured onto CMA medium for further morphological identification. Five inoculum plugs (5 mm^2) of each oomycete isolate were plated in a sterile petri dish (65 mm) containing non-sterile soil water extract (20 g soil suspension in 1L distilled water and filtered). The plates were incubated at 25°C under cool white fluorescent light for 24h until sporulation was observed (Jeffers, 2006). The plates were examined under a compound light microscope at different magnifications. Identification of *Phytophthora* spp. was identified up to genus level

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based on sporangia characteristics (Gallegly and Hong, 2008). *Pythium* spp. was identified based on the presence of non-papillated round sporangia (Plaats-Niterink, 1981). From here on representative isolates based on the preliminary identification was used for further molecular identification.

Nematode extraction and quantification

Nematodes were extracted from each of 10 soil samples per orchard. For the extraction of juvenile nematodes, soil samples were subjected to the Baermann pan extraction method (Whitehead and Hemming, 1965). The plastic pan, plastic gauze and tissue paper were prepared and placed onto one another. Soil samples were mixed thoroughly before pouring 250 cm^3 soil from each sample onto the tissue paper and filling the plastic pan with sterile distilled water until the soil was saturated. The plastic pan was left to stand at room temperature for 48 hours allowing the juvenile nematodes to migrate through the tissue layer to the water in the plastic pan. After 48 hours, the plastic gauze with the tissue paper and soil was removed from the plastic pan and the remaining water, possibly containing nematodes, was washed through a 38 µm sieve using slow running water. The nematodes were then rinsed from the 38 µm sieve into a 250 mL beaker to a suspension volume of 100 mL. The suspension was mixed thoroughly by a stream of air bubbles and 1 ml of the suspension was drawn up using a 1 mL Eppendorf pipette and pipetted into a Hawksley nematode counting chamber. The citrus nematode juveniles were counted two times and the average for each sample was calculated.

For the extraction of female citrus nematodes, five grams (5g) from the pooled soil and roots collected from each orchard were weighed off and washed with water to remove excess soil. The roots were cut into 1 cm pieces, placed in a blender with 250 ml water and blended for 20 seconds at low speed (Greco and D'Addabbo, 1990; Galeano *et al.*, 2003). The blended roots were then collected in a three-layer Labotec Test sieve and washed through the sieves from the largest to smallest diameter (600µm, 150µm, 38µm) to collect the female nematodes. The nematodes were then washed with a 2% acidic acid solution into a small container with a 38 µm mesh and stained with a Phenol solution (10 mL 1% acid fuchsin, 10 mL 1% orcein, 200 mL distilled water, 188 mL phenol, 165 mL lactic acid and 318 mL glycerol) for 60 min (Greco and D'Addabbo, 1990; Fourie *et al.*, 2017). After staining, the root tissue was rinsed with tap water and washed into a beaker. A 1 mL subsample was taken from the 100 mL nematode suspension and pipetted into a Peters' counting slide, and observed under a microscope (Luc *et al.*, 2005; Fourie *et al.*, 2017).

Glasshouse seedling bioassay trail

Plant material

Carrizo citrange seeds were collected from the Citrus Foundation block near Uitenhage in the Eastern Cape province. The seeds were sown in March 2016 into seedling pots containing a steam pasteurized perlite and peat moss (50:50) mixture. After 3 months, the seedlings were selected for uniformity before being transplanted in treated or untreated soil.

Trial treatments

The soil from each of the four orchards were divided into six equal portions that each received one of six different treatments. Treatments included (i) steam pasteurization, (ii) 20% dilution of each orchard soil into the corresponding steam pasteurized soil, and the application of (iii) mefenoxam, (iv) difenoconazole, (v) cadusafos and (vi) untreated control. After the soils received the different treatments, the treated soil of each treatment was dispensed into six 500 mL plastic pots. Prior to planting two Carrizo citrange rootstock seedlings per pot, the weight of each seedling was recorded. The height of each seedling was also recorded after planting. The potted Carrizo citrange seedlings were placed in a randomize block design in a glasshouse, located in Nelspruit at Citrus Research International (CRI). The temperature of the glasshouse was at ambient temperature and if temperature raised above 30°C, fans automatically switch on and decrease the temperature. The seedlings were watered as needed. The trial was repeated two weeks apart and left to grow for a period of 7 months.

Steam pasteurization

The required amount of soil from each orchard was placed in a cement mixer and mixed for 3 min. After mixing the soil, the steamers were placed into black dustbins, together with the soil and was left to double pasteurize for 90 min (180 min total) at temperatures ranging from 82 to 95 °C. Using running water, the steamers and the cement mixer were washed thoroughly between the treatment of soils from different orchards.

Soil Dilution

A 20% soil dilution mixture was prepared for each orchard by adding 20% (v/v?) of untreated control soil to steam pasteurized soil from the same orchard.

Fungicide and nematicide treatments

Mefenoxam (480 a.i. g/L) and difenoconazole (250 a.i. g/L) were used to target oomycetes and fungi respectively, while cadusafos (100 a.i. g/L) were used to suppress any potential nematodes in the soil. In different watering cans, mefenoxam at 0.2 ml/L, difenoconazole at 1ml/L and cadusafos at 15 ml/L solutions was prepared using tap water. Of these solutions

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500 mL was applied per pot. This resulted in 0.048g a.i. of mefenoxam, 0.125g a.i. difeneconazole and 0.75g a.i. cadusafos being applied per pot receiving these different biocide treatments. The soil was left to dry for two days before planting the seedlings, two per pot.

Trial evaluation

Seedlings growth measurements

*A*fter seven months of growth, the glasshouse seedling trial was terminated. The number of dead seedlings per treatment w*as* noted. The remaining seedlings were removed from the soil. The root systems were washed with sterile distilled water, removing excess soil*,* and seedlings were then left to air-dry on sterile tissue paper. The length of the above ground growth was measured using an electronic calliper and data logger. The total seedling mass (combined root and above ground mass) of each seedling were measured and compared to data taken at planting. Subsequently, the increase in seedling weight and height was calculated.

Isolation for fungi and oomycetes from roots

The roots of the untreated control seedling were used for isolations to determine which fungi and oomycetes colonized the roots of seedlings. After seedling weight measurements at trial termination, root systems were removed using a pruning knife (blades spray-sterilised with 70% ethanol) and washed with sterile distilled water and left to air-dry on sterile tissue paper in the laminar flow cabinet. Thirty-six root pieces per pot, each approximately 10 mm in length, were removed from the root systems using a flame sterilised scalpel and plated out onto four different mediums in 90 mm Petri dishes, (i) 2% potato dextrose agar (Difco, Becton, Dickinson and Company) amended with 1 mL streptomycin sulphate solution (40 mg/L, Calbiochem, Merck) (PDA+s), (ii) 1.5% bacteriological agar (Difco, Becton, Dickson and Company) amended with streptomycin sulphate (40 mg/L, Calbiochem, Merck) and metalaxyl (250 a.i. g/L) (WA+s+m), as well as (iii) PARPH amended with benomyl (500 a.i. g/kg) (PARPH+B) and (iv) PARP amended with benomyl (0.2g/100mL) (PARP+B). Nine root pieces were plated onto each medium, thus eight 90mm Petri dishes per pot. Plates were incubated at 29°C for 2 to 3 days. When fungal growth emerging from the roots was observed, subcultures representing each fungal colony was transferred to PDA+s Petri dishes (65 mm). The subcultured plates were incubated under the same conditions as mentioned previously. Growth that was observed on PARPH+B and PARP+B were purified by hyphal-tipping to WA and sub-cultured onto corn meal agar (CMA) Petri dishes (65 mm) and incubated at 29°C for further identification.

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Five CMA inoculum plugs (5 mm^2) of each oomycete isolate were plated in a sterile petri dish (65 mm) containing non-sterile soil water extract (20 g soil suspension in 1L distilled water and filtered). The plates were incubated at 25°C under cool white fluorescent light for 24h until sporulation was observed (Jeffers, 2006). The plates were examined under a compound light microscope at different magnifications. *Phytophthora nicotianae* was identified by the sporangia being papillated and more ovoid in shape. The sporangia of *Phytophthora citrophthora* is also papillated but more asymmetrically in shape, and often with more than one apex. The preliminary identification of *P. citrophthora* at this stage was based on the presence of papillated sporangia with two apices (Gallegly and Hong, 2008). *Pythium* spp. was identified based on the presence of non-papillated round sporangia (Plaats-Niterink, 1981).

The sub-cultured fungi and oomycetes were identified to the species level as described in the section below under "Molecular identification of isolated fungal species".

Isolation of nematodes

No isolations of the female citrus nematode were made after seven months due to the lack of root development. There were not enough roots to extract female nematodes, and therefore not meaningful to compare the data before and after seven months. The isolation of juveniles from the soil would also not have given any meaningful information due to the females influencing the growth of the roots.

Statistical analysis

Two experimental trials were conducted. For each trial, the experimental design was a randomised block with the 24 treatment combinations (six treatments applied to soil from four production areas) replicated at random in four blocks. An experimental unit consisted of 10 seedlings in total (five pots each containing two seedlings). The average increase in weight and length per seedling were calculated for each experimental unit.

For each trial, the increase in weight and length per seedling were subjected to analyses of variance (ANOVA) using GLM (General Linear Models) Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Trial results were also combined in one analysis of variance (John and Quenouille, 1977) after confirmation of trial homogeneity of variance using Levene's test (Levene, 1960). Shapiro-Wilk test was performed on to test for deviation from normality (Shapiro and Wilk, 1965). Square root transformation was applied to improve weight increase deviation from normality (Snedecor and Cochran, 1980). Fisher's least significant difference was calculated at the 95% level to compare means for significant effects (Ott, 1998). A probability level of 95% was considered significant for all significance tests.

Molecular species identification of isolated fungal and oomycete isolates

The sub-cultured oomycete (soil and root) and fungal (roots) isolates were classified into different groups based on their cultural and morphological characteristics on PDA+s for fungal and CMA media for oomycetes. Cultural characteristics used included colony size, texture, shape and colour. Oomycetes were further identified by observing the morphology of sporangia in non-sterile soil water extract under a compound light microscope as previously described in the section "Quantification of oomycetes and nematodes from orchard soils". The *Fusarium* isolates were grouped according to the colour of the mycelia and growth pattern on PDA+s. Microscopic slides of five representative isolates from each group were prepared to observe the conidial shape, size and colour using a bright-field microscopy (Leslie and Summerell, 2006). The oomycete and fungal groups were identified as belonging to the families Peronosporaceae, Pythiaceae and Nectriaceae. All genera that have not been previously identified as being involved in replant disease associated with apples, peach and citrus were discarded. These genera included *Penicillium*, *Alternaria* and *Trichoderma*. The isolates of importance were stored for further identification. Oomycete isolates were stored in sterile distilled H_2O with lemon leaf pieces, and the fungi was stored on ½ strength PDA slants and in sterile distilled H_2O.

DNA extraction

DNA was extracted from 3-week-old fungal cultures growing on PDA+s and oomycete cultures growing on CMA+s. The DNA extraction protocol of Osmundson *et al*. (2013) was used with minor adjustments. Harvested mycelium was placed into 2-mL Eppendorf tubes with 0.5 mg glass beads and 500 µl 0.5M NaOH. The Eppendorf tubes were shaken for 5 min at 30 Hz using a Retsch Mixer Mill (Retsch MM 400, Germany) after which the tubes were centrifuged for 2 min at 13 500 rpm. Five microliters of the supernatant were extracted and added to a new 1.5 mL Eppendorf tube containing 495 µl Tris-HCL (100 Mm; pH 8.0) and vortexed.

Polymerase chain reaction (PCR) and electrophoresis

The ITS regions and 5.8S gene for the oomycete isolates were amplified using universal primers ITS-6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke and Duncan, 1997; Cooke *et al*., 2000) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). In a total reaction volume of 25 µl, the PCR reaction contained 2 µl of DNA, 2X Promega G2 GoTaq Master Mix (Promega Madison, WI USA), 0.3µM of each primer and 9 µl PCR H2O. The PCR reaction conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 32 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, and a final extension step at 72°C for 5 min.

The putative *Fusarium* spp. isolates were identified by amplifying the translation elongation factor 1-alpha (EF 1-α) gene using primers EF1 (5'-ATGGGTAAGGARGACAAGAC-3') and EF2 (5'-GGARGTACCAGTSATCATGTT-3') (O'Donnell *et al.*, 1998). A fragment length of 700 bp were amplified for the TEF-1α gene region. The PCR reactions contained 2 µl of DNA, 2X Promega G2 GoTaq Master Mix (Promega Madison, WI USA), 0.32µM of each primer per PCR reaction as described above and 9 µl PCR H2O. Reaction conditions consisted of an initial denaturation step at 95°C for 3 min followed by 30 cycles of 1 min at 95°C and 45 s at 55°C, 1 min at 72°C with a final extension of 3 min at 72°C. All PCR reactions were performed in an Applied Biosystems 2700 PCR machine (Carlsbad, California, USA). A non-template water control was also included in each PCR run.

PCR products were separated by electrophoresis on a 1.5% (w/v) SeaKem® LE agarose gel (Lorenza Rockland, ME USA) in TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5) after ethidium bromide staining. The GeneGenius Gel Documentation and Analysis System (Syngene, UK) were used to visualize the gel under ultraviolet (UV) light alongside a 100-bp DNA ladder (GeneRuler, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Restriction fragment length polymorphism (RFLP) of oomycete species

The ITS-PCR products of the oomycete isolates were used to obtain restriction fragment length polymorphism banding profile patterns, for the classification and identification of the organisms by using a single and double-restriction enzyme RFLP. In a total reaction volume of 20 µl the ITS-RFLP double-enzyme reaction contained 8 µl ITS-PCR product, 2 µl CutSmart buffer (BioLabs NEB, New England), 1 µl *HhaI* and 1 µl *HinfI* restriction enzymes (BioLabs NEB, New Enland) and 8 µl nuclease free water (Promega Madison, WI USA). For the single reaction, 1µl *HhaI* enzyme was used and 9 µl nuclease free water instead of 8 µl mentioned in the double restriction enzyme reaction. The restriction enzyme reactions were all incubated at 37 °C for 15 min. PCR-RFLP products were separated by electrophoresis on a 3% (w/v) SeaKem® LE agarose gel (containing ethidium bromide) in TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5) for 90 min at 75 V/cm. The GeneGenius Gel Documentation and Analysis System (Syngene, UK) were used to visualize the gel under ultraviolet (UV) light alongside a 100-bp DNA ladder (Promega, Madison, WI USA). Isolate that contained the same banding patterns were grouped into the same PCR-RFLP group.

Sequencing of PCR products

The PCR products were purified using the MSB Spin PCRapase Kit (Invitek, Berlin, Germany) according to manufacturer's instructions. In the final step, DNA was eluted from the column

using 15 µl water. The cleaned PCR products were then sent for sequencing to the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University. Sequencing was conducted using the forward and reverse primers used in the initial PCRs. Sequencing was conducted using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, United States) and an ABI 3130xl DNA sequencer (Applied Biosystems, Foster City, California, United States)

RESULTS

Chemical and physical soil analyses

Results of the physical and chemical analyses of the sampled soils in the different orchards showed that the different soils varied greatly with regards to their physical and chemical characteristics. This variation is illustrated in Table 1, where it is clear that in many cases there were significant differences ($P \leq 0.05$; ANOVA not shown) in the levels of pH, different minerals and the clay, silt and sand percentages contained in the different soils from the different areas. There was no significant difference in soil pH between the Addo (6.36), Hoedspruit (6.60) and the Letsitele (6.50) soils. The soil pH of Patensie differed significantly from the other three areas, with a lower soil pH of 5.82 (Table 1). The pH of the soil should be adjusted according to the citrus needs during the period of planting and establishing new orchards. In this study, the pH of the soil was not adjusted prior to planting Carrizo citrange seedlings into the pots.

There was a significant difference ($P < 0.0001$; Table 1) in P levels (mg/kg) between Patensie (138.62 mg/kg), Addo (30.21 mg/kg) and Hoedspruit (4.57 mg/kg) soil, and no significance was observed between Hoedspruit (4.57 mg/kg) and Letsitele (7.52 mg/kg; Table 1). There was a significant difference in soil Calcium (Ca) levels per area ($P < 0.0001$; Table 1). Patensie soil had the highest Ca level (2260.21 mg/kg), statistically higher than any of the other areas. Hoedspruit (1532.42 mg/kg) and Addo (1181.83 mg/kg) had statistically similar Ca levels, but significantly differed from the Ca level in Letsitele (685.50 mg/kg; Table 1). In terms of magnesium (Mg) content significant differences were also observed between the different areas. Patensie (248.51 mg/kg) and Addo (299.12 mg/kg) soils had statistically similar Mg content. Hoedspruit at 420.57 mg/kg had significantly the highest Mg content of all the areas. Letsitele was the area with the lowest Mg content in the soil of 180.59 mg/kg (Table 1).

The clay percentage of the different soils showed significant variation ($P <0.0001$) where the percentage of clay in Patensie (21.70%) and Addo (21.20%) was statistically similar (Table 1). The clay percentage of Hoedspruit (16.50%) and Letsitele (13.10%) was significantly different from one another and that of Patensie and Addo (Table 1). Patensie soil had a significantly higher ($P < 0.0001$) silt percentage of 26.17% than Addo (17.59%). The silt content for both Patensie and Addo soils were also significantly higher than Hoedspruit

(9.84%) and Letsitele (7.67%; Table 1). There was a significant difference (*P* = <0.0001) in the percentage sand of the soil from all four areas. Letsitele had significantly the highest sand percentage of 79.23% followed by Hoedspruit (73.66%), Addo (61.21%) and Patensie (52.13%; Table 1).

Quantification of oomycetes and nematodes from orchard soils

Semi-quantitative analyses of oomycetes

Oomycete isolates were obtained from all four orchard soils and in total consisted of 91, 132, 110 and 140 isolates from the Addo, Patensie, Hoedspruit and Letsitele orchards respectively. *Pythium* spp. were the most frequently isolated oomycete in all four orchards (Figure 1). Orchard soils from Letsitele and Patensie had the highest mean percentage (>90%) leaf discs colonized by *Pythium* spp. *Phytophthora* spp. were also isolated from the soil through soilbaiting. Letsitele had the highest mean percentage (≥90%) leaf discs colonized by *Phytophthora* spp., whereas Patensie, Hoedspruit and Addo had a lower mean percentage (≤40%) *Phytophthora* spp. colonized leaf discs (Figure 1).

RFLP analyses of oomycetes

Representative isolates obtained from soilbaiting were identified through Restriction fragment length polymorphism (RFLP) of ITS-PCR (Figure 2) amplicons and sequencing. Ten *Phytophthora* and ten *Pythium* isolates from each of the four orchards was selected for further identification based on morphology observed in non-sterile soil water extract. A total of three different RFLP groups were identified and from each RFLP group, three isolates were sequenced (Figure 2).

Nucleotide sequence analyses of isolates representing the three-different oomycete PCR-RFLP groups resulted in RFLP group 1 being identified as *P. nicotianae,* RFLP group 2 as *P. citrophthora* and RFLP group 3 as *P. irregulare*. Representative oomycete isolates, obtained from soilbaiting was identified through RFLP of ITS-PCR (Figure 2) amplicons and sequencing. *Phytophthora nicotianae* (lanes 6 and 12), *Pythium irregulare* (lanes 2, 3 and 8) and *Phytophthora citrophthora* (lanes 1, 4, 5, 7, 9 to 11) was identified as the oomycetes colonising citrus leaf disks from soilbaiting (Figure 2).

Nematode extraction and quantification

Citrus nematodes *Tylenchulus semipenetrans*, were isolated from all four orchard soils. Analyses of variance (ANOVA) of mean juvenile and female counts obtained from soil and root samples from the different orchards, indicated a significant difference in mean juvenile counts between areas (*P* <0.001; Table 2). With regards to the female counts, the ANOVA indicated no significant difference in female counts between areas (*P* = 0.788; Table 2). In

Addo no analysis for females could be done due to the trees in the orchard being very small without sufficient root mass (Table 3). Between the other three areas, Patensie, Hoedspruit and Letsitele, there were no significant difference between the female counts. The counts ranged from 1720 females per 10 g roots to 2140 females per 10 g roots (Table 3). With regards to juvenile counts, the mean juvenile counts in soils from Patensie (4290) and Hoedspruit (4940) were statistically similar but significantly higher than the mean counts recorded for the Addo (75) and Letsitele (1040) soils. The last two means were statistically similar but numerically there were a marked difference between the mean juvenile counts in the Addo and Letsitele soils (Table 3).

Glasshouse seedling bioassay trail

Seedling growth measurements

A total of 18 seedlings died during the seven-month period in the glasshouse. These seedlings were excluded from all analyses. The analyses of variance of the seedling weight and length increase indicated no significant differences between trials ($P = 0.5524$). As a result, the data from the two trial repetitions could be combined in all the analyses. Further analysis showed that there was a significant production area x treatment interaction for mean weight increase ($P < 0.0001$) and mean seedling length increase ($P = <0.0001$; Table 4). Therefore, the data of each area was considered separately.

In Patensie, the mean seedling weight increase (1.76 g) for seedlings grown in steam pasteurized soil was statistically similar to the weight increase observed in the untreated soil (1.69 g; Table 5). However, the mean weight increase of seedlings in the soil dilution was significantly lower (0.68g) compared to the weight increases observed in both the steam pasteurized and untreated soils (Table 5). The biocide treatments performed, in terms of mean weight increase, statistically similar (mefenoxam and difenoconazole) or poorer (cadusafos) compared to the steam pasteurized treatment and the untreated soil (Table 5). The mean seedling length increase for Patensie seedlings grown in steam pasteurized soil, was 8.74 mm. This value was statistically lower than that observed for the seedlings in the untreated control (10.38 mm) but significantly better compared to the soil dilution treatment (5.51 mm; Table 5). For this measurement, mefenoxam was the only biocide that caused a mean seedling length increase that was statistically similar to the untreated control (Table 5).

The mean weight increase of seedlings grown in steamed soil from Addo was 1.94 g, and was statistically different from the untreated control (1.52 g) and soil dilution (1.20 g) treatments (Table 5). Both the cadusafos (1.44.g) and difenoconazole (1.41 g) treatments performed for this measurement statistically similar to the untreated control. The mefenoxam treatment caused a significantly lower mean weight increase compared to the untreated control (Table 5). In terms of the mean length increase of seedlings grown in the Addo soil,

the mean length increase for the untreated soil was 8.81 mm, statistically similar to that observed in the steam pasteurized (8.65 mm) soil and soil dilution (7.89 mm) treatments (Table 5). All three the biocides performed in terms of the mean length increase significantly poorer than the untreated control treatment (Table 5).

In the steam pasteurized soil of Hoedspruit, the mean increase in seedling weight and length (2.02g and 10.02 mm) was statistically better compared to the untreated control (1.34 g and 7.46 mm) and soil dilution (0.78 g and 6.30 mm) treatments (Table 5), respectively. In terms of mean weight increase all three biocides caused significantly lower increases compared to the untreated control (Table 5). The mean length increase caused by the mefenoxam (6.51 mm) and difenoconazole (6.21 mm) treatments were statistically similar to the untreated control while the increase seen for the cadusafos treatment (4.30 mm) was significantly lower than the untreated control (Table 5).

In the steam pasteurized treated soil of Letsitele the mean seedling weight and length increases (2.12 g and 10.96 mm) was statistically higher than the untreated soil (0.86 g and 5.03 mm) and soil dilution (0.53 g and 6.52 mm) treatment (Table 5). The mean weight increase seen in seedlings from the soil dilution treatment (0.53 g) were statistically similar to that of the untreated control (0.86 g). However, in terms of mean length increase of seedlings, the soil dilution treatment had a mean (6.52 mm) length significantly better than the untreated control (5.03 mm; Table 5). In terms of mean weight increase the different biocides had statistically the same effect as the untreated control treatment (Table 5). However, the mean length increase results showed that mefenoxam (7.23 mm) had a significantly better mean length increase compared to the untreated control. The cadusafos and difenoconazole (5.32 mm) treatment had a mean length increase (4.64 mm) that was statistically the same as the untreated control (Table 5).

Isolation of fungi and oomycetes from roots

Root isolations from untreated control seedlings showed that *Fusarium solani* was the most predominant genus isolated in all four areas followed by oomycetes (*P. citrophthora*, *P. nicotianae* and *P. irregulare*) and *Fusarium oxysporum* (Figure 3). In three of the orchards (Patensie, Addo and Hoedspruit) isolates belonging to the *Fusarium solani* species complex was the most abundant species isolated (Figure 3). BLAST analyses of these isolates, showed that the isolates had 99.8% sequence identity to several Genbank accession belonging to the FSSC complex (Sandoval-Denis *et al.*, 2017) including *F. solani* (LT746338), *Neocosmospora* spp. (LT746330) (Sandoval-Denis *et al.*, 2017), *Fusarium falciformis* (KF255514) and *Fusarium* spp. (EF469980; O'Donnell *et al.*, 2007). The *F. oxysporum* isolates was confirmed with a BLAST analyses (LT746314; Sandoval-Denis *et al.*, 2017 and LT841210; Brankovics *et al.*, 2016) of 99.8% identical sites.

Nucleotide sequence analyses of isolates representing the three-different oomycete PCR-RFLP groups resulted in RFLP group 1 being identified as *P. nicotianae,* RFLP group 2 as *P. citrophthora* and RFLP group 3 as *P. irregulare*. Representative oomycete isolates, obtained from root isolations was identified through RFLP of ITS-PCR (Figure 4) amplicons and sequencing. *Phytophthora nicotianae* (lanes 1 to 16 and 20 to 36), *Pythium irregulare* (lanes 17 and 19) and *Phytophthora citrophthora* (lane 18) was identified as the oomycetes colonising citrus roots (Figure 4). The RFLP group 1 *P. nicotianae* sequences (769 to 877 bp) had 99.3% sequence similarity to published *P. nicotianae* sequences in Genbank (KT455619, KT337714, Yang and Hong, 2015; Sanahuja *et al*., 2016). The PCR-RFLP group 2 *P. citrophthora* sequences (785 to 825 bp) had 99.7% identity to published Genbank sequences in BLAST analyses (KU877816, Das *et al*., 2016). The PCR-RFLP group 3 *P. irregulare* isolate sequences (830 to 931 bp) from citrus roots had 99.7 % identity to *P. irregulare* (KC855076, Bahramisharif *et al*., 2014).

DISCUSSION

In this study, the etiology of citrus replant disease was investigated in four citrus replant orchard soils, one from each of four production areas in South Africa. The disease was shown to be caused by biological agents in three of the orchards, but not in the fourth orchard. The application of biocides (mefenoxam, cadusafos, difenoconazole) to the orchard soils was unable to conclusively show the involvement of specific groups of biological agents (oomycetes, nematodes or fungi). The exception was in the Letsitele orchard where oomycetes were likely involved. Nonetheless, several known and putative replant pathogens were found associated with citrus roots in a seedling bioassay for all four orchards. These agents included the oomycetes *P. nicotianae, P. irregulare* and *P. citrophthora* and fungi belonging to the FSSC and FOSC. Soil analyses of the orchards supported the presence of the three oomycetes. Juveniles of the citrus nematode *T. semipenetrans* were furthemore also identified in soil analysed from all four orchards, and thus likely also play a role in citrus replant disease in South Africa.

In the current study, the growth response (weight and length increases) of the seedlings in the bioassay (glasshouse trial) indicated that, as with apple replant (ARD) disease (Tewoldemedhin *et al*., 2011a; Mazzola and Manici, 2012), the cause of citrus replant disease is biological in nature. This was true for three (Addo, Hoedspruit and Letsitele) orchards, but not the Patensie orchard. In the Hoedspruit and Letsitele orchards, the pasteurized control seedlings had a significantly higher increase in weight and length than the untreated control, whereas as for the Addo orchard this was only true for the weight increase. Hoestra (1968) and Sewell *et al*. (1992) indicated that weight rather than length increase is a better indication of apple replant disease severity, but that there is always a variation in severity between sites

as also seen in the current study. Further support for the biological nature of citrus replant disease in South Africa is provided by the fact that for all four orchards addling 20% of the untreated soil to steam sterilised soil resulted in significantly lower increase in weight compared to the steam and untreated control treatments. This can be attributed to the re-inoculation of the pathogens and nematode containing orchard soils into the biological vacuum created by the 80% steam sterilised soil. This probably resulted in rapid growth and proliferation of the biological agents and consequently severe infection of seedling roots. For apple replant disease, a significant reduction in growth (length and weight) of the soil dilution treatment relative to the pasteurized treatment has also been reported for most replant soils. However, a significant reduction in growth of the soil dilution treatment relative to the untreated control has not been reported (Tewoldemedhin *et al.*, 2011c). This might be due to a higher percentage of untreated soil (20%) used in the current study, than the 15% used by Tewoldemedhin *et al.* (2011c).

In the current study on citrus in South Africa, the effect on seedling growth of the biocide treatments consisting of mefenoxam, cadusafos and difenoconazole provided no support for the involvement of biological agents of citrus replant disease, with the exception of the Letsitele orchard. The Letsitele orchard was the only orchard where seedlings grown in one of the biocide treatments had a significant higher growth (length or weight) than the untreated soil. In the Letsitele soil, oomycetes were likely involved since the mefenoxam treatment seedlings had a significantly higher increase in seedling length than the untreated control. The fact that none of the biocide treatments resulted in an increase in seedling growth relative to the untreated control was unexpected, considering the association of several known citrus pathogens and parasitic nematodes with the roots and soil of all four orchards, as discussed below. A few reasons for the lack of response in improved seedling growth to the two biocide applications could firstly be that the biocide dosages were too high resulting in damage of the seedlings. For example, the cadusafos and difenoconazole treatments resulted in significantly lower height or weight increases of seedlings compared to the untreated in three of the orchards. Alternatively, it could be that the application of only one biocide resulted in the specific targeted group of pathogens being suppressed, but then resulted in the excessive proliferation of another group of pathogens that caused severe seedling damage. This has sometimes been reported in apple replant disease when management practices are applied that do not suppresses all of the biological agents involved (Mazzola and Manici, 2012). In citrus, the negative effect of some biological agents on others have been reported, and could thus result in an increase in some groups when one specific group is suppressed. For example, the suppression of nematodes by cadusafos could have resulted in an increase and more aggressive infections by *Fusarium solani.* Chandel and Sharma (1989) and El-Borai *et al.* (2002b) found that *Tylenchulus semipenetrans* supressed the growth of *F. solani* while

it also reduced host infection by this pathogen in dual inoculations. It has furthermore also been found that citrus root infections by *Phytophthora nicotianae* were reduced by root infection by *Tylenchulus semipenetrans* (Chandel and Sharma, 1989; El-Borai *et al.*, 2002a, b).

The current study did not only focus on the pathogens previously identified to be associated with citrus replant disease, but investigated the involvement of other possible pathogens. In previous studies on this topic in South Africa (Le Roux *et al.*, 1991, 1998; Cronje *et al.*, 2002) soil from fewer orchards in one production area were evaluated, and only the presence of nematodes and *Phytophthora* spp. were investigated. In the current study, *P. irregulare*, *P. nicotianae* and *P. citrophthora* together with *F. solani*, *F. oxysporum* and the citrus nematode *T. semipenetrans* was the predominant pathogen species found to be associated with citrus replant disease. In previous studies that focused on citrus replant disease, it was shown that the citrus nematode, *T. semipenetrans*, two *Phytophthora* spp. (*P. nicotianae* and *P. citrophthora*) and *F. solani* were associated with the disease complex (Baines *et al.*, 1978; Nemec *et al.*, 1978, 1980; Labuschagne *et al.*, 1987; Le Roux *et al.*, 1998). In the current study, it is clear that *Pythium* spp. also might play a role in citrus replant disease which was not previously indicated. However, pathogenicity studies will have to be conducted. In apple replant disease, *Pythium* species are known to play an important role (Mazzola, 1998). Other apple replant pathogens such as '*Cylindrocarpon*'–like fungi and *Rhizoctonia* spp. were not found to be associated with citrus replant disease in South Africa. It is possible that some of the FSSC and *Phytophthora* spp. identified in the current study, could interact in causing replant disease. Dandurand and Menge (1992) reported that the severity of citrus root rot caused by *Phytophthora nicotianae* and *P. citrophthora* were increased when co-inoculation with *Fusarium solani* was done.

This indicates that the citrus- and apple replant disease complex differs from one another but that there are similarities. Methyl bromide was previously used to fumigate soils in replant situations as it had a broad-spectrum effect on oomycetes, soilborne fungi and nematodes (Le Roux *et al.*, 1998). However, as this is no longer available, abovementioned soil survey prior to replanting gains major importance as it will determine which soil fumigants or mixture of fumigants to employ as chloropicrin and 1,3-dichloropropene does not have the same broad-spectrum efficacy (Jhala *et al.*, 2011). Ultimately the effective prevention of citrus replant disease using non-methyl bromide fumigation is dependent on knowing what is present in the soil and making the correct decisions based on this knowledge.

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REFERENCES

Bahramisharif, A., Lamprecht, S.C., Spies, C.F.J., Botha, W.J. and McLeod, A. 2014. *Pythium* spp. associated with rooibos seedlings, and their pathogenicity towards rooibos, lupin and oat. Plant Disease, 98(2): 223-232.

Baines, R.C., Small, R.H., De Wolfe, T.A., Martin, J.P. and Stolzy, L.H. 1978. Control of citrus nematode and *Phytophthora* spp. by vapours. Plant Disease, 41: 405-414.

Bent, E., Loffredo, A., Yang, J., McKenry, M.V., Becker, J.O. and Borneman, J. 2009. Investigations into peach seedling stunting caused by a replant soil. Microbiology Ecology, 68(2): 192-200.

Brankovics, B., Van Dam, P., Rep, M., De Hoog, G.S., Van der Lee, TA.J., Waalwijk, C. and Van Diepeningen, A.D. 2016. Recombination within an asexual species: what the mitochondria genomes reveal in the *Fusarium oxysporum* species complex. Medical Mycology, CBS, Netherlands.

Braun, P.G. 1995. Effects of *Cylindrocarpon* and *Pythium* species on apple seedlings and potential role in apple replant disease. Canadian Journal of Plant Pathology, 17: 336-341.

Browne, G.T., Connell, J.H. and Schneider, S.M. 2006. Almond replant disease and its management with alternative pre-plant soil fumigation treatments and rootstocks. Plant Disease, 90(7): 869-876.

Burger, W.P. and Small, J.G.C. 1983. Allelopathy in citrus orchards. Scientia Horticulture, 20: 361-375.

Chandel, Y.S. and Sharma, N.K. 1989. Interction of *Tylenchulus semipenetrans* with *Fusarium solani* on citrus. Indian Journal of Nematology, 19(1): 21-24.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G. and Brasier, C.M. 2000. A Molecular Phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology, 30: 17-32.

Cooke, D.E.L. and Duncan, J.M. 1997. Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of ribosomal DNA. Mycological Research, 101: 667-677.

Cronje, C., Le Roux, H.F., Truter, M., van Heerden, I. and Phillips, H. 2002. Long-term effect of pre-plant soil solarisation on growth of replant citrus trees in South Africa. African Plant Protection, 8: 41-49.

Dandurand, L.M. and Menge, J.A. 1992. Influence of *Fusarium solani* on citrus root rot caused by *Phytophthora parasitica* and *Phytophthora citrophthora*. Plant and Soil, 144: 13-21.

Das, A.K., Kumar, A., Nerkar, S.G. and Thakre, N. 2016. Characterization of *Phytophthora* spp. isolated from citrus orchards in India. Plant Pathology, Central Citrus Research Institute, 98(1): 55-69.

Derrick, K.S. and Timmer, L.W. 2000. Citrus blight and other diseases of recalcitrant etiology. Phytopathology, 38: 181-205.

Desaeger, J., Dickson, D.W. and Locascio, S.J. 2017. Methyl bromide alternatives for control of root-knot Nematode (*Meloidogyne* spp.) in tomato production in Florida. Journal of Nematology, 49(2): 140-149.

Dullahide, S.R., Stirling, G.R., Nikulin, A. and Stirling, A.M. 1994. The role of nematodes, fungi, bacteria, and abiotic factors in the etiology of apple replant problems in the Granite Belt of Queensland. Australian Journal of Experimental Agriculture, 34: 1177-1182.

Duniway, J.M. 2002. Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. Phytopathology, 92(12): 1337-1343.

El-Borai, F.E., Duncan, L.W. and Graham, J.H. 2002 a. Infection of citrus roots by *Tylenchulus semipenetrans* reduces root infection by *Phytophthora nicotianae*. Journal of Nematology, 34(4): 384-389.

El-Borai, F.E., Duncan, L.W. and Graham, J.H. 2002 b. Eggs of *Tylenchulus semipenetrans* inhibit growth of *Phytophthora nicotianae* and *Fusarium solani* in vitro. Journal of Nematology, 34(3): 267-272.

Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. and De Waele, D. 2017. Nematology in South Africa: A view from the 21st Century. Springer International Publishing, Switzerland, 92-94.

Galeano, M., Verdejo-Lucas, S., Sorribas, F.J., Ornat, C., Forner, J.B. and Alcaide, A. 2003. New citrus selections from Cleopatra mandarin x *Poncirus trifoliata* with resistance to *Tylenchulus semipenetrans* Cobb. Nematology, 5(2): 227-234.

Gallegly. M.E. and Hong, C. 2008. *Phytophthora*: Identifying species by Morphology and DNA fingerprints. APS Press, St. Paul, Minnesota, USA.

Greco, N. and D'Addabbo, T. 1990. Efficient procedure for extracting *Tylenchulus semipenetrans* from Citrus Roots. Nematology, 22(4): 590-593.

Grimm, G.R. and Alexander, A.F. 1973. Citrus leaf pieces as traps for *Phytophthora parasitica* from Soil Slurries. Phytopathology, 63: 540-541.

Hoestra, H. 1968. Replant disease of apple in the Netherlands. Wageningen Publications, pp, 68.

Jaffee, B.A., Abawi, G.S. and Mai, W.F. 1982. Fungi associated with roots of apple seedlings grown in soil from an apple replant site. Plant Disease, 66: 942-944.

Jeffers, S.N. 2006. Identifying species of *Phytophthora*. Department of Entomology, Soil and Plant Sciences. Clemson University, SC, 1-8.

Jhala, A.J., Gao, S., Gerik, J.S., Qin, R. and Hanson, B.D. 2011. Effects of surface treatments and application shanks on nematode, pathogen and weed control with 1,3-dichloropropene. Pest Management Science, 68: 225-230.

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

Labuschagne, N., Kotzé, J.M. and Putterill, J.F. 1987. Incidence of *Fusarium solani* and *F. oxysporum* in citrus roots and infection by *F. solani*. Phytophylactica, 19: 315-318.

Le Roux, H.F., Wehner, F.C., Kotzé, J.M. and Grech, N.M. 1991. Combining fosetyl-Al trunk injection or metalaxyl soil drenching with soil application of aldicarb for control of citrus decline. Plant Disease, 75: 1233-1236.

Le Roux, H.F., Ware, A.B. and Pretorius, M.C. 1998. Comparitive efficacy of preplant fumigation and postplant chemical treatment of citrus trees in an orchard infested with *Tylenchulus semipenetrans.* Plant Disease, 82: 1323-1327.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual, 1st Edition. Blackwell Publishing, Iowa.

Levene, H. 1960. Robust testes for equality of variances. In Contibutions to Probability and Statistics (I. Olkin, ed.) Standford University Press, Palo Alto, 278-292.

Linde, C., Kemp, G.H.J. and Wingfield, M.J. 1994. *Pythium* and *Phytophthora* species associated with eucalyptus and pines in South Africa. European Journal of Plant Pathology, 24: 345-356.

Lombard, L., van der Merwe, N.A., Groenewald, J.Z. and Crous, P.W. 2014 Lineages in *Nectriaceae*: re-evaluating the generic status of *Ilyonectria* and allied genera. Phytopathologia Mediterranea, 53(3): 515-532.

Luc, M., Sikora, R.A. and Bridge, J. 2005. Plant parasitic nematodes in subtropical and tropical agriculture. CABO Publishing, Cambridge, USA, 2: 66-68.

Manici, L.M., Ciavatta, C., Kelderer, M. and Erschbaumer, G. 2003. Replant problems in South Tyrol: role of fungal pathogens and microbial population in conventional and organic apple orchards. Plant and Soil, 256: 315-324.

Mazzola, M. 1998. Elucidation of the microbial complex having a Causal role in the development of apple replant disease in Washington. Phytopathology, 88(9): 930-938.

Mazzola, M. and Manici, M. 2012. Apple Replant Disease: Role of microbial ecology in cause and control. Annual Review of Phytopathology, 50: 45-65.

Nemec, S., Burnette, H.C. and Patterson, M. 1978. Observations on a citrus fibrous root rot involving *Fusarium solani* in blight-diseased groves. Soil and Science Society of Florida Proceedings, 37: 43-47.

Nemec, S., Baker, R. and Burnett, H. 1980. Pathogenicity of *Fusarium solani* to citrus roots and its possible role in blight aetiology. Proceedings of the Florida State Horticultural Society, 93: 36-41.

O'Donnell, K., Kistler, C.K., Cigelnik, E. and Ploetz, R. 1998. Multiple evolutionary origins of fungus causing Panama disease of banana: Concordant evidence from nuclear and

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mitochondrial gene genealogies. Proceedings of the National Academy of Science. USA, 95: 2044-2049.

O'Donnell, K., Sarver, B.A., Brandt, M., Chang, D.C., Noble-Wang, J., Park, B.J., Sutton, D.A., Benjamin, L., Lindsley, M., Padhye, A., Geiser, D.M. and Ward, T.J. 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic Fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. Journal of Clinical Microbiology, 45(7): 2235-2248.

Osmundson, T.W., Eyre, C.A., Hayden, K.M., Dhillon, J. and Garbelotto, M.M. 2013. Back to basics: an evaluation of NaOH and alternative rapid DNA extraction protocols for DNA barcoding, genotyping, and disease diagnostics from fungal and oomycete samples. Molecular Ecology Resources 13: 66-74.

Ott, R.L. 1998. An Introduction to Statistical methods and data analysis. Belmont, California: Duxbury Press: pp 807-837.

Plaats-Niterink, A.J.VD. 1981. Monograph of the genus *Pythium*. Study in Mycology 21: 1-242.

Sanahuja, G., Lopez, P. and Palmateer, A.J. 2016. First report of *Phytophthora nicotianae* causing foliar blight on Zamioculas zamiifolia in Florida. Plant Disease, 100(4): 864.

Sandoval-Denis, M., Guarnaccia, V., Polizzi, G. and Crous, P.W. 2017. Symptomatic Citrus trees reveal a new pathogenic lineage in *Fusarium* and two new *Neocosmospora* species. Persoonia, 40: 1-25.

Schneider, S.M., Rosskopf, E.N., Leesch, J.G., Chellemi, D.O., Carolee, T.B. and Mazzola, M. 2003. United States Deprtment of Agriculture – Agricultural Research Service research on alternatives to methyl bromide: pre-plant and post-harvest. Pest Management Science, 59: 814-826.

Sewell, G.W.F., Roberts, A.L. and Elsey, R.F. 1992. Apple replant disease: the assessment and results of seedling bio-assays and growth responses to soil fumigation with chloropicrin. Annals of Applied Biology, 121(1): 199-209.

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

Snedecor, G.W. and Cochran, W.G. 1980. Statistical Methods, 7th Edition, The Iowa State University Press.

Tewoldemedhin, Y.T., Mazzola, M., Mostert, L. and McLeod, A. 2011 a. *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. European Journal Plant Pathology, 129: 637-651.

Tewoldemedhin, Y.T., Mazzola, M., Botha, W.J., Spies, C.F.J. and McLeod, A. 2011 b. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora*

and *Pythium*) associated with apple replant orchards in South Africa. European Journal of Plant Pathology, 130: 215-229.

Tewoldemedhin, Y.T., Mazzola, M., Labuschagne, I. and McLeod, A. 2011 c. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agaents, with some agents acting synergistically. Soil Biology and Biochemistry, 43: 1917-1927.

Timmer, L.W., Sandler, H.A., Graham, J.H. and Zitko, S.E. 1988. Sampling citrus orchards in Florida to estimate populations of *Phytophthora parasitica*. Phytopathology, 78: 940-944.

Timmer, L.W., Sandler, H.A., Graham, J.H. and Zitko, S.E. 1990. *Phytophthora* feeder root rot of bearing citrus: fungicide effects on soil populations of *Phytophthora parasitica* and citrus tree productivity. Proceedings of the Florida State Horticultural Society, 102: 5-9.

Verma, L.R. and Sharma, R.C. 1999. Diseases of Horticultural Crops: Fruits. Indus Publishing Company, New Delhi, p. 106.

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

Whitehead, A.G. and Hemming, J.R. 1965. A comparison of some quantitative methods of extracting small vermiform nematodes from soil. Annals of Applied Biology, 55(1): 25-38.

Yang, X. and Hong, C. 2015. Diversity and populations of *Phytophthora*, *Phytopythium* and *Pythium* species recovered from sediments in an agricultural runoff sedimentation reservoir. Plant Pathology, 65(7): 1118-1125.

TABLES AND FIGURES

Table 1. Mean pH, phosphorous (P), calcium (Ca), magnesium (Mg), clay, silt and sand levels of sampled top soil (0-30 cm) from the four citrus producing areas of Patensie, Addo, Hoedspruit and Letsitele.

Area	pH	P (mg/kg) Bray 1	Ca (mg/kg)	Mg (mg/kg)	Clay (%)	Silt (%)	Sand (%)
Patensie	5.82 b	138.62 a	2260.21 a	248.51 b	21.70 a	26.17 a	52.13 d
Addo	6.36 a	30.21 b	1181.83 b	299.12 b	21.20 a	17.59 b	61.21 c
Hoedspruit	6.60 a	4.57 c	1532.42 b	420.57 a	16.50 b	9.84 c	73.66 b
Letsitele	6.50 a	7.52 c	685.50 c	180.59 c	13.10 c	7.67 c	79.23 a
LSD	0.467	24.356	89.723	55.64	1.858	2.556	3.718
P-value	0.010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Means in a column followed by the same letter are not significantly different ($P < 0.05$) according to Fisher's least significant difference test.

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Table 2. Analysis of variance of mean juvenile and female *Tylenchulus semipenetrans* counts in sampled soil (juveniles) and roots (adult females) from the four citrus producing areas of Patensie, Addo, Hoedspruit and Letsitele.

	Juveniles				Females			
Source	**DF**	**SS**	**MS**	**SL**	**DF**	**SS**	**MS**	**SL**
Area	3	171401687,500	57133895,833	<0.001	2	924666,667	462333,333	0.788
Error	36	275353250,000	7648701,389			51845000,000	1920185,185	
Corrected Total	39	446754937,500				52769666,667		

DF Degrees of freedom
SS Sum of Squares
MS Mean Square
SL Significance level

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Table 3. Mean juvenile and female citrus nematode counts from ten soil and root samples collected from four old orchards in the citrus producing areas of Patensie, Addo, Hoedspruit and Letsitele.

Citrus producing areas	*Tylenchulus semipenetrans*	
	Juvenile[a]	Females[b]
Patensie	4290.0 a[c]	2140.0
Addo	75.0 b	*
Hoedspruit	4940.0 a	1850.0
Letsitele	1040.0 b	1720.0
LSD	2508.0	1272.0

[a] Mean count per 250 cm^3 sampled soil

[b] Mean count per 5 grams of roots

[c] Means in a column followed by the same letter are not significantly different ($P < 0.05$) according to Fisher's least significant difference test.

* No females were extracted from the roots

Table 4. Analysis of variance of mean weight- and length increase of citrus seedlings grown in four putative citrus replant orchard soils subjected to six different treatments and grown for seven months under glasshouse conditions.

		Weight increase			Length increase		
Source	**DF**	**SS**	**MS**	**SL**	**SS**	**MS**	**SL**
Trial	1	0.1119	0.1119	0.0717	0.2751	0.2751	0.5524
Trial (Block)	2	0.1862	0.0931	0.0696	10.1700	5.0850	0.0030
Area	3	3.3769	1.1256	<0.0001	54.1685	18.0561	<0.0001
Treatment (TRT)	5	12.0491	2.4098	<0.0001	122.2909	24.4581	<0.0001
Area x TRT	15	3.9281	0.2618	<0.0001	127.7945	8.5196	<0.0001
Trial x Area	3	0.2295	0.0765	0.0876	1.5441	0.5147	0.5745
Trial x TRT	5	0.06528	0.0130	0.8486	3.0483	0.6096	0.5594
Trial x Area x TRT	15	0.3977	0.0265	0.6658	14.1776	0.9451	0.2849
Error	45	1.4815	0.0329		34.5352	0.7674	
Corrected Total	94	21.8266			368.0045		

DF Degrees of freedom
SS Sum of Squares
MS Mean Square
SL Significance level

Table 5. Mean seedling weight (g) - and length (mm) increases of citrus seedlings in response to six treatments applied to four different citrus replant orchard soils.

	Patensie		**Addo**		**Hoedspruit**		**Letsitele**	
Treatment	**Weight (g)**	**Length (mm)**	**Weight (g)**	**Length (mm)**	**Weight (g)**	**Length (mm)**	**Weight (g)**	**Length (mm)**
Pasteurized	1.76 bc	8.74 st	1.94 ab	8.65 stu	2.02 a	10.02 qr	2.12 a	10.96 q
Untreated control	1.69 bcd	10.38 q	1.52 cdef	8.81 rst	1.34 fgh	7.46 uvw	0.86 jk	5.03 yz
Mefenoxam	1.62 cde	9.76 qrs	1.16 hi	7.25 vw	1.03 ij	6.51 wx	1.03 ij	7.23 vw
Cadusafos	1.26 ghi	8.66 stu	1.44 defg	7.41 uvw	1.03 ij	4.30 z	0.69 kl	4.64 z
Difenoconazole	1.55 cdef	7.35 vw	1.41 efgh	7.99 tuv	0.76 kl	6.21 wxy	0.61 kl	5.32 xyz
Soil dilution[a]	0.68 kl	5.51 xyz	1.20 ghi	7.89 tuv	0.78 jkl	6.30 wx	0.53 l	6.52 wx
LSD[b]	0.2602	1.2563						
P-value	<0.0001	<0.0001						

[a] The soil dilution treatment consisted of diluting 20% of the untreated control soil into pastuerized soil.

[b] t-LSD (least significant difference) was calculated at a 95% significance level.

Mean seedling weight (g) and length (mm) increase of Carrozo citrange rootstock seedlings were determined 7 months after trial establishment in the four citrus replant soils receiving the different treatments. The data is the average of two experiment, with each treatment containing twenty replicates. Means in columns and rows followed by the same letter are not significantly different (P > 0.05) according to Fisher's least significant difference test.

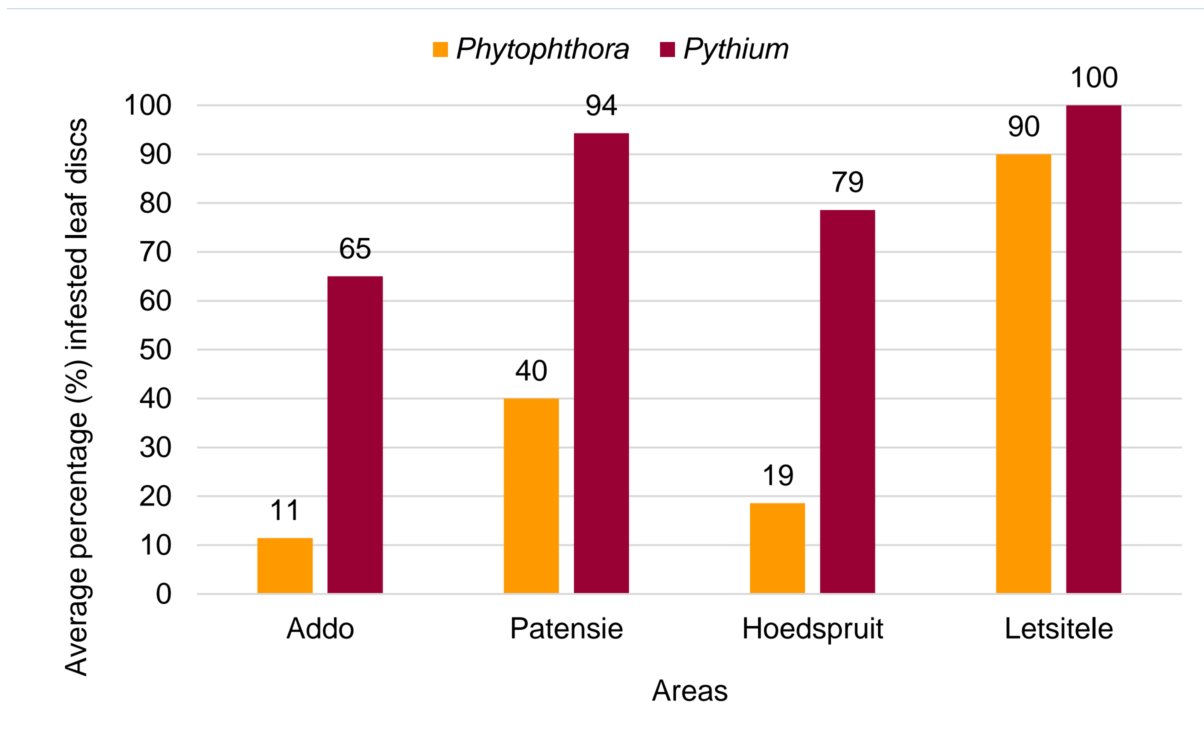

Figure 1. Average percentage (%) infested leaf discs following soilbaiting of citrus replant orchard soils collected from four major citrus producing areas.

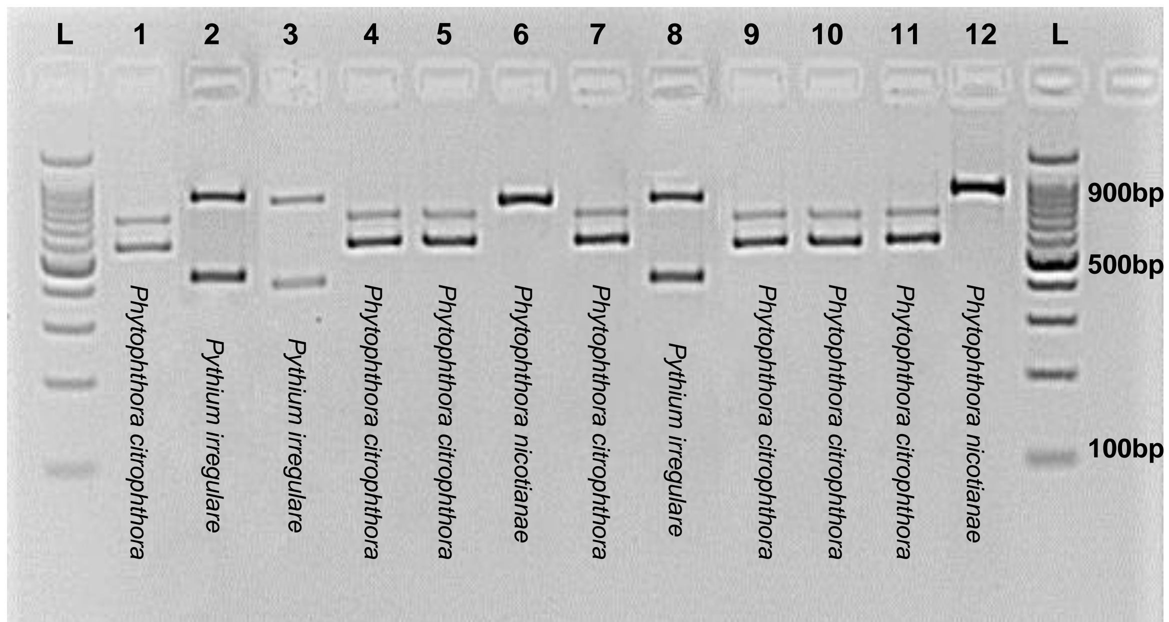

Figure 2. Restriction fragment length polymorphism (RFLP) analysis of the ITS PCR amplicons obtained from oomycete isolates from soilbaiting. Lanes 1, 4, 5, 7, 9 to 11 are *Phytophthora citrophthora* (two DNA fragments, 812-bp and 608-bp). Lanes 2, 3 and 8 represent *Pythium irregulare* with two DNA fragments one 920-bp and one 480-bp. *Phytophthora nicotianae* are represented by lane 6 and 12 with only one DNA fragment of 980-bp in size. The size fragments of a 100-bp DNA ladder are shown on the right and left side of the figure (lanes marked L).

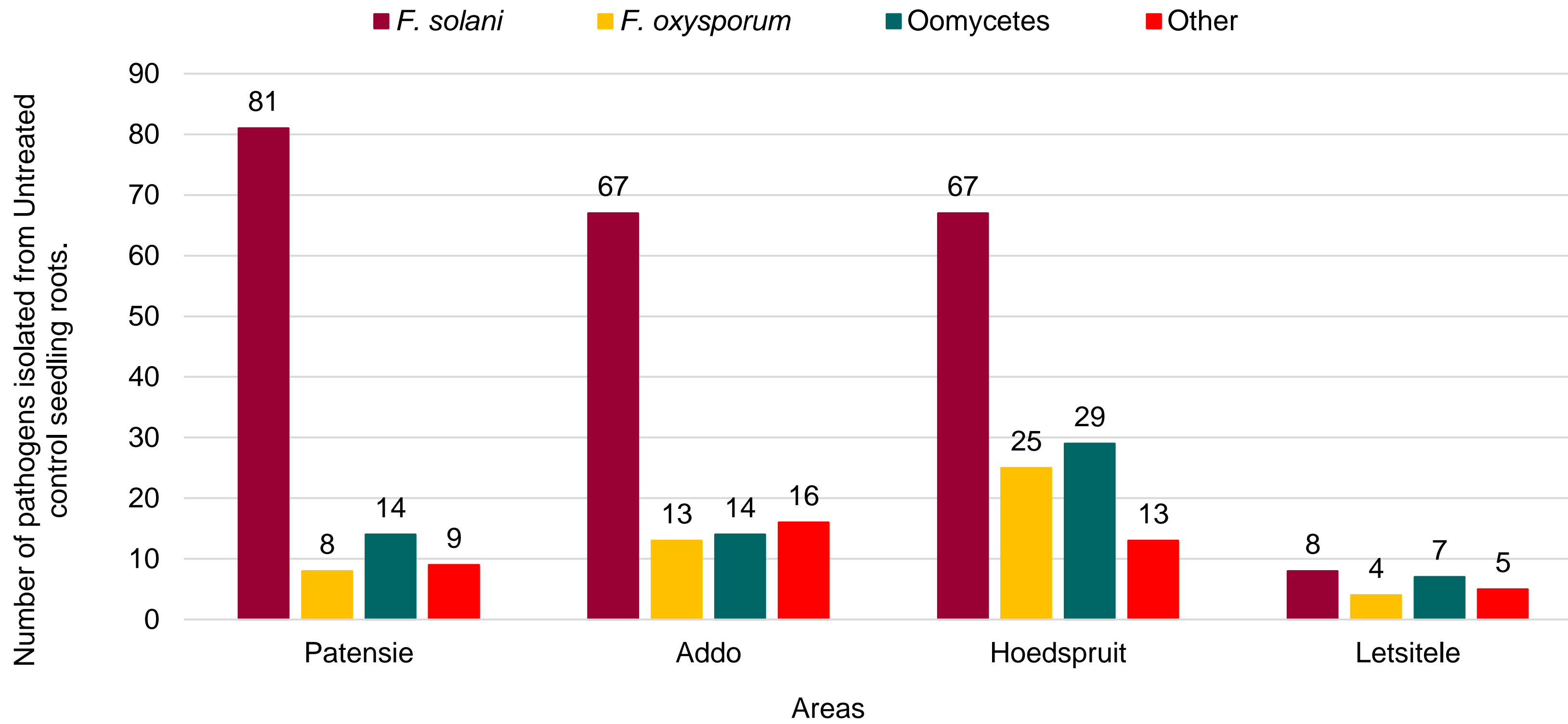

Figure 3. The number of isolates of oomycete and fungal pathogens isolated from citrus seedling roots grown in untreated control replant soil for seven months.

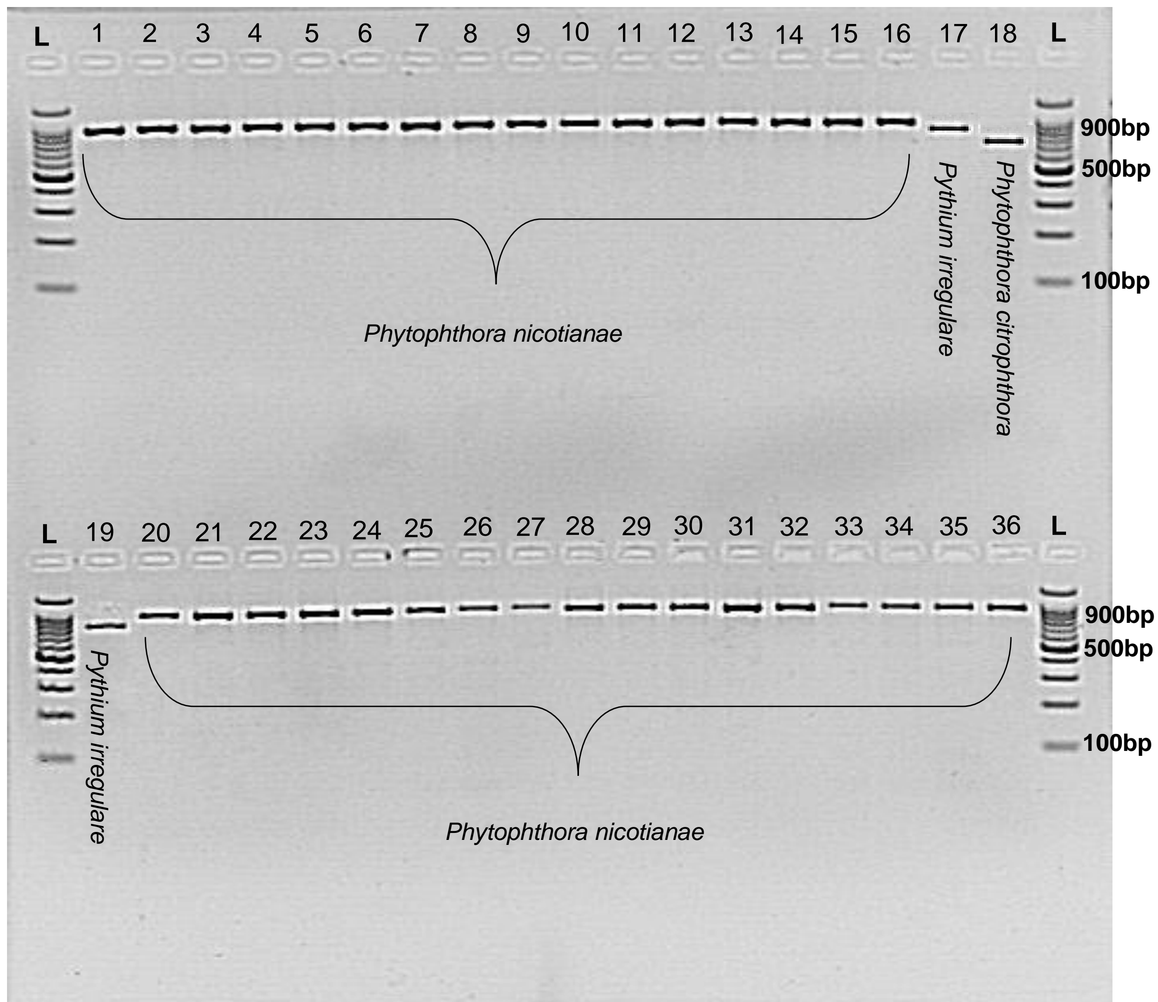

Figure 4. Restriction fragment length polymorphism (RFLP) analysis of the ITS PCR amplicons obtained from oomycete isolates from root isolations. Lanes 1 to 16 and 20 to 36 are *Phytophthora nicotianae* (one DNA fragment, 1000-bp). Lanes 17 and 19 represent *Pythium irregulare* with one DNA fragments 910-bp. *Phytophthora citrophthora* are represented by lane 18 with only one DNA fragment of 815-bp in size. The size fragments of a 100-bp DNA ladder are shown on the right and left side of the figure (lanes marked L).

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CHAPTER 3

Phylogenetic review of *Fusarium* and *Neocosmospora* species associated with Citrus in South Africa

ABSTRACT

Phylogenetic analyses were used in this study to determine the phylogenetic species identity and genetic diversity of '*Fusarium solani'* Species Complex (FSSC) and the *Fusarium oxysporum* Species Complex (FOSC) isolates from four different citrus production areas (Addo, Patensie, Hoedspruit and Letsitele) in South Africa. The isolates (13 *F. oxysporum* and 39 *'F. solani'*) were obtained from a previous citrus replant study (Chapter 2), which preliminarily identified the isolates as belonging to these two species complexes. The phylogenetic species identity of the isolates were determined separately for each species complex, using a concatenated multi-gene phylogeny of the translation elongation factor 1-alpha (TEF) and RNA polymerase II second largest subunit (RPB2) gene regions. Phylogenetic analyses of only the TEF region, which has traditionally been used for identification of these fungal groups, were also conducted for each species complex. The FSSC multi-gene phylogeny yielded a better resolution of clades than the TEF phylogeny, although several of the main clades in the multi-gene phylogeny had low or no bootstrap support. The multi-gene phylogeny of the FSSC isolates showed that the citrus isolates grouped into four clades including a *Neocosmospora solani* clade (25 isolates), *Neocosmospora croci* clade (one isolate), an unnamed *Fusarium* spp. clade (13 Isolates) with *F. falciformis* as the most related known *Fusarium* spp., and another clade (one isolate) containing an unnamed *Fusarium* species. The latter citrus isolate was obtained from Patensie, and may represent a putative new species. The citrus *Fusarium* spp. isolates that were related to *F. falciformis* also represent a putative new species and were obtained from Limpopo (Hoedspruit and Letsitele) production regions. The citrus *N. croci* isolate was only obtained from Addo production area. The most widely distributed FSSC species from citrus was *N. solani,* which occurred in all four production areas. The TEF phylogeny of the FOSC isolates resulted in a better resolution and support of clades than the multi-gene phylogeny. According to the TEF phylogeny, all the citrus FOSC isolates grouped within the *F. oxysporum* phylogenetic species II. The FOSC citrus isolates were furthermore distributed among two subclades, previously designated as Clade 3 (11 isolates) and Clade 4 (two Isolates) by O'Donnell et al. (1998, 2004). Both clades contained isolates from Patensie, Addo, Hoedspruit and Letsitele citrus production areas.

INTRODUCTION

The Ascomycota is a large and important group of fungi, characterised and distinguished from other fungi by a saclike ascus carrying haploid ascospores (Alexopoulos *et al.*, 1996). These fungi consist of over 32 000 species and form symbiotic, parasitic and saprobic relationships with both animals and plants (Hawksworth *et al.*, 1995; Alexopoulos *et al.*, 1996). The genus names of some members within the Ascomycota have recently changed. One of these include a change of some members of the genus *Fusarium* to the genus *Neocosmospora.* The genus name change was due to the fact that many genera within the *Nectriaceae* family were previously poorly characterised due to a lack of DNA sequence data and were thus solely identified based on phenotypic characters. These characters included, uniloculate ascomata that are yellow, orange-red to purple, with phialidic asexual morphs (Rossman *et al.*, 1999). The identification of *Fusarium* species at the morphological level is based on distinctive characters such as the shape and size of the macro- and microconidia (Leslie and Summerell, 2006). The genus name change of some members of *Fusarium* to *Neocosmospora* were based on a multi-gene phylogenetic analyses [translation elongation factor (TEF), internal transcribed spacer gene (ITS), RNA polymerase II second largest subunit (RPB1 and RPB2), the large subunit of the ATP citrate lyase (*acl1*), α-actin (*act*), β-tubulin (*tub2*), calmodulin (*cmdA*), histobe H3 (*his3*) and the nuclear large subunit 28S rDNA (NLSU) gene region] conducted for all available type and authentic strains of the known genera in *Nectriaceae*, as well as for genera of which no sequence data were previously available. These studies showed that the genus *Neocosmospora* contained members of some *Fusarium* spp., including some '*F. solani'* isolates*,* which were distinct from the genus *Fusarium* (Geiser *et al.*, 2013; O'Donnell *et al.*, 2013; Lombard *et al.*, 2014, 2015)

Members of the '*Fusarium solani'* Species Complex (FSSC) are known as plant, human and animal pathogens (O'Donnell *et al.*, 2008) and are frequently isolated from soil and mainly acts as decomposers. Some species act as parasites on plants, insects, humans and animals (Booth, 1971). '*Fusarium solani'* is known to be associated with the roots of symptomless as well as declining citrus trees and are commonly found in citrus soils (Labuschagne *et al.*, 1987; Smith *et al.*, 1988). Characteristic symptoms of infection on citrus includes colonisation and discoloration of the cortical tissue of feeder roots (Adesemoye *et al.*, 2011). Aboveground symptoms are evident as leaves that turn yellow, dieback and wilting of branches and the overall weakening of the tree with reduced fruit quality (Adesemoye *et al.*, 2011). '*Fusarium solani'* was classified into the section Martiella by Booth, (1971) and can be divided into 50 sub-specific lineages based on the molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex (O'Donnell, 2000a).

At the phylogenetic level, there are many differences among the members of the FSSC. Three clades have been identified in the FSSC based on translation elongation factor

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(TEF), internal transcribed spacer gene (ITS) and nuclear large subunit 28S rDNA (NLSU) phylogenies (O'Donnell *et al.*, 1998, 2008; O'Donnell, 2000b; Nalim *et al.*, 2011). Clade 1 includes two known species*, Fusarium illudens* and *Nectria plagianthi*, members of Clade 2 consists of pathogens that cause sudden death syndrome on soy-bean (Aoki *et al.*, 2003, 2005, 2012). A study done by Nalim *et al.*, (2011) showed that members of Clade 2 are paraphyletic. Clade 3 is known to contain the most common *Fusarium* spp. associated with plant diseases and include *Fusarium falciformis* and *Fusarium keratoplasticum* (O'Donnell, 2000a; Zhang *et al.*, 2006; O'Donnell *et al.*, 2008; Short *et al.*, 2013, 2014). The most haplotype-diverse species were also placed within Clade 3 (O'Donnell *et al.*, 2008; Nalim *et al.*, 2011; Short *et al.*, 2014).

The *Fusarium oxysporum* Species Complex (FOSC) is known as an anamorphic species and is a widespread fungus found world-wide (Kistler, 1997; Leslie and Summerell, 2006). It contains both pathogenic and non-pathogenic isolates (Gordon and Martyn, 1997). Pathogenic isolates of *F. oxysporum* usually cause Fusarium wilt on several agricultural crops and are divided into *formae speciales* (f. sp.) based on their host range, and may be further subdivided into pathogenic races (Hawksworth *et al.*, 1995; O'Donnell and Cigelnik, 1999). The close association with plant roots, and the ability of pathogenic isolates to colonise the root cortex and xylem vessels leads to characteristic wilting symptoms by limiting water movement through the plant (Beckman and Roberts, 1995). Pathogenic and non-pathogenic strains of *F. oxysporum* can be found in many native plant groups, in soils that have never been cultivated as well as in agricultural soils throughout the world (Gordon and Martyn, 1997; Gordon *et al.*, 1992). *F. oxysporum* isolates from uncultivated soil and native plants are known to be closely associated with plant roots, but are most often non-pathogenic to plants in native soils, even when high populations are present in some areas (Booth, 1971; Armstrong and Armstrong, 1978). *Fusarium oxysporum* is considered as a minor disease of citrus and only pathogenetic toward woody hosts under adverse conditions or when the plant is stressed by environmental conditions in South Africa (Labuschagne *et al.*, 1987).

The taxonomy of *F. oxysporum* was previously based on the morphology of the asexual reproductive structures. The limited variability of these characters led to a broad description of *F. oxysporum* (Snyder and Hansen, 1940), which did not reflect the inherent variability within the species complex (Kistler, 1997). There are to date more than 70 described *formae speciales* (f. sp) causing vascular wilt in over 100 plant species (Gordon and Martyn, 1997). Phylogenetic analyses have shown that many *forma speciales* are polyphyletic or paraphyletic, meaning that it is derived from more than one common evolutionary ancestor or ancestral group (O'Donnell *et al.*, 1998; Skovgaard *et al.*, 2001). Previous phylogenetic studies, based on the translation elongation factor 1-α (TEF) and the mitochondrial small subunit rDNA (*mtSSU*) loci showed that *F. oxysporum* consist of three clades, designated as

Clades 1, 2, and 3 (O'Donnell *et al.*, 1998; 2004). Subsequently, a fourth clade was defined within the FOSC, with the addition of clinical isolates found by O'Donnell *et al.* (2004). The four clades were further divided into two phylogenetic species, PS I and PS II (Laurence *et al.*, 2014). Phylogenetic species II (PS II) consists of Clades 2 to 4, and Phylogenetic species I (PS I) only consists of Clade 1 (Laurence *et al.*, 2014).

This study focused on the phylogenetic analyses of FOSC and FSSC isolates found in citrus orchard soils in South Africa, using DNA sequence data of the TEF and RNA polymerase second largest subunit (RPB2) gene regions. The isolates used in this study were collected from citrus root isolations in a previous study, where fungi associated with citrus replant disease were investigated. Preliminary Blast analyses of TEF sequences of the isolates showed that the isolates likely belonged to the FOSC and FSSC (Chapter 2).

MATERIALS AND METHODS

Isolate collection

An isolate collection of 13 *F. oxysporum* and 39 '*F. solani'* isolates (Table 1) from a previous study (Chapter 2) were used in this study. The isolates were obtained through a seedling bioassay with Carrizo citrange seedlings using soil from four different citrus orchards located in the Patensie, Addo, Hoedspruit and Letsitele citrus production areas in South Africa (Chapter 2).

DNA extraction

DNA of the selected *Fusarium* isolates was extracted from 3-week-old fungal cultures grown on 2% potato dextrose agar (Difco, Becton, Dickinson and Company) amended with 1 mL streptomycin sulphate solution (40 mg/L, Calbiochem, Merck) (PDA+s). The DNA extraction protocol of Osmundson *et al.* (2013) was used, with some modifications as described in a previous study (Chapter 2).

Polymerase chain reaction (PCR) and electrophoresis

Two gene regions, including the TEF and RNA polymerase II second largest subunit (RPB2) regions, were amplified and sequenced for the selected *Fusarium* isolates. The TEF 1-α and RPB2 sequences of the *Fusarium* isolates were obtained in a previous study (Chapter 2). A fragment length of approximately 700 bp was amplified for the TEF gene region. For the RPB2 region, two separate but adjacent and overlapping regions within the RPB2 gene were amplified, using two primer pairs in separate PCR reactions. The first primer pair consisted of the RPB2-5F2 primer (O'Donnell *et al.*, 2008; O'Donnell *et al.*, 2010) and the fRPB2-7R primer (Lui *et al.*, 1999). The second primer pair consisted of primers fRPB2-7F and fRPB2-11aR (Lui *et al.*, 1999). The length of the fragment amplified with the first primer pair (RPB2-5F;

fRPB-7R) was 1186 to 2336 bp and for the second primer pair (fRPB2-7F; fRPB2-a11R) it was 2317 to 3314 bp (primers indicated in Table 2). This resulted in a total RPB2 fragment length of approximately 3841 bp. The PCR reaction and conditions was followed in the same manner as described previously (Chapter 2). The PCR products were separated by gel electrophoresis on a 1.5% (w/v) SeaKem® LE agarose gel (Lorenza Rockland, ME USA) in TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5).

Sequencing of PCR products

The PCR products were purified using the MSB Spin PCRapase Kit (Invitek, Berlin, Germany), according to manufacturer's instructions. The PCR products sequenced (both directions) by the Central Analytical Facility (CAF) of Stellenbosch University. The sequencing reactions were conducted using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, United States) according to manufacturer's protocol. The sequencing primers were the same as those described in the previous section for amplifying each of the gene regions. The sequence products were treated with sodium dodecyl sulfate (SDS) and transferred onto Sephadex columns (Princeton Scientific) using a Tecan Freedom EVO 150 (Biorad, Germany) and centrifuged. The nucleotide order of samples was read in an ABI 3130xl DNA sequencer (Applied Biosystems, Foster City, California, United States) using a 50cm capillary array and POP-7 (Applied Biosystems, Foster City, California, United States).

For each gene region (TEF and RBP2), forward and reverse sequences for each of the FSSC and FOSC isolates were aligned and edited in Geneious R 9.1.8 (Biomatters Ltd., Auckland, New Zealand) and a consensus sequence was constructed. Consensus sequences were run through the Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information's nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Fusarium ID database (Geiser *et al*., 2004) to confirm identity. Several reference sequences representing different '*F. solani', F. oxysporum* and closely related species downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Genbank), were selected based on previous published phylogenetic trees. Several '*F. solani'* sequences from a phylogenetic study by Sandoval-Denis *et al.* (2017) related to *Fusarium* spp. on citrus were also used in this study. Sequences form each phylogenetic species within the FOSC representing the four clades were also included in the phylogenetic dataset.

Phylogenetic analysis of FSSC and FOSC isolates

Separate phylogenetic analyses were conducted for the FSSC and FOSC isolates. For each species complex, a single gene phylogeny of the TEF region was conducted using maximum

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parsimony (MP) analysis and maximum likelihood (ML) analyses. A concatenated phylogeny consisting of the combined datasets of TEF and RPB2 was conducted using only ML analysis.

Phylogenetic analyses of FSSC isolates

The FSSC phylogenetic analysis of only the TEF data set was carried out by first aligning the TEF sequence data set in MAFFT v7.017 (Katoh *et al.*, 2002; Katoh and Standley, 2013; http://mafft.cbrc.jp/alignment/server/), followed by editing in MEGA v7.0 (Kumar *et al.*, 2016). A MP analysis was conducted in PAUP v4. The heuristic search option with ten random taxon additions, tree bisections and reconstruction (TBR) was used as branch swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. A bootstrap analysis of 1 000 heuristic search replicates was performed to estimate the reliability of inferred phylogenies. A ML analysis for the same dataset was conducted using PhyML v3.0 (Guindon *et al.*, 2010; http://www.atgc-montpellier.fr/phyml/). The settings used for bootstrap were the same as that used for the MP analysis. The best-fit model was inferred using the Smart Model Selection (SMS) model test program (v1.8.1). The general time reversible model with gamma distribution and proportion invariable sites (GTR+G+I) was selected as the best-fit model for nucleotide substitution in MP analyses. Both the gamma distribution parameter and proportion of invariable sites were estimated. Bootstrap values were based on 1 000 repetitions and clades with bootstrap support ≥ 60% were considered significant with good support (Hillis and Bull, 1993; Figure 1).

The concatenated phylogenetic tree of the TEF and RPB2 regions for FSSC was conducted by first aligning and editing the data in the same way as described for the TEF data set. A ML analysis was conducted using PhyML v3.0 (Guindon *et al.*, 2010; http://www.atgc-montpellier.fr/phyml/) and bootstrap values were based on 1 000 repetitions and clades with bootstrap support ≥ 60% were considered significant (Hillis and Bull, 1993; Figure 2). The outgroup for both the concatenated and single gene phylogenetic tree analysis consisted of two isolates, *Nectria illudens* (NRRL 22090; O'Donnell, 2000a) and *Nectria plagianthi* (NRRL 22632; O'Donnell, 2000a) (Sandoval-Denis *et al.*, 2017).

Phylogenetic analysis of FOSC

The alignment, MP and ML analyses for the FOSC isolates were conducted as described for FSSC. The TEF dataset for FOSC isolates used the Tamura-Nei (TN93+G) substitution model, where the gamma distribution parameter was estimated and the proportion of invariable sites were fixed. The conditions used for bootstrapping were the same as those used for MP analysis and clades with bootstrap support ≥ 60% were considered significant and highly supported (Hillis and Bull, 1993; Figure 3). The outgroups for the TEF dataset

consisted of two isolates, *Fusarium commune* (NRRL 22903; Skovgaard *et al*., 2001) and *Fusarium circinatum* (NRRL 25331; O'Donnell, 2000b).

A multi-locus concatenated phylogenetic tree for the FOSC isolates was prepared in the same way as described for *F. solani*, using ML analysis. The outgroup consisted of two isolates, *Fusarium polyphialidicum* (NRRL 13459; O'Donnell *et al*., 2007) and *Fusarium concolor* (NRRL 25728; O'Donnell *et al*., 2010; Figure 4).

RESULTS

Phylogenetic analyses

The MP and ML analyses of the TEF datasets yielded similar phylogenetic tree topologies for both the FSSC and FOSC isolates and the concatenated multi-gene phylogenies were only analysed using ML. Bootstrap values for both analyses are indicated on the TEF trees (Figure 2 and 4).

Phylogenetic analyses of FSSC isolates

The multi-gene phylogeny of the FSSC isolates grouped into four clades including a *N. solani* clade (25 isolates), *N. croci* clade (one isolate), an unnamed *Fusarium* spp. clade (13 isolates) with *F. falciformis* as the most related known *Fusarium* spp., and another clade (one isolate) containing an unnamed *Fusarium* species (Figure 1). The latter citrus isolate (STEU 8454) was obtained from Patensie, and may represent a putative new species. The citrus *Fusarium* spp. (13 isolates; 91% bootstrap support) that were related to *F. falciformis* also represent a putative new species and were obtained from Hoedspruit and Letsitele production regions. Isolate STEU 8462 obtained from Addo, grouped with the ex-type *N. croci* sequence. The most widely distributed FSSC species from citrus, which occurred in all four production areas grouped with the ex-epitype strain (100% bootstrap support) of *N. solani* (NRRL 66304^{ET}) (Sandoval-Denis *et al*., 2017).

The TEF tree differed from the concatenated tree in a few instances. In the TEF tree, the clade containing the 13 FSSC isolates closely related to the *F. falciformis* reference sequences also had relative high bootstrap support (91%), but the *F. falciformis* reference sequences were unresolved (Figure 2). The sequences of STEU 8462 (Addo orchard) and the ex-type *N. croci* sequences were unresolved in the TEF phylogeny, but not in the multi-gene phylogeny. The clade containing the *N. solani* isolates differed in the TEF and concatenated trees, since in the TEF tree isolate STEU 8455 (Addo orchard) grouped with low bootstrap support (87%) with an unknown *Fusarium* spp. (NRRL46703), and not with the *N. solani* clade as in the concatenated tree. Furthermore, in the TEF tree STEU 8454 (Patensie orchard) grouped with the *N. solani* clade, unlike in the multi-gene tree (Figure 1 and 2).

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Phylogenetic analysis of FOSC

The 13 FOSC citrus isolates all grouped into phylogenetic species PS II within the FOSC, which was previously described by Laurence *et al.* (2014) (Figure 3 and 4). The isolates furthermore grouped into two of the four subclades described by O'Donnell *et al.* (1998; 2004).

The concatenated tree of FOSC did not provide bootstrap support for most of the clades and sub-clades as described by Laurence *et al.* (2014) and O'Donnell *et al.* (1998, 2004) (Figure 4). This can be since several gene regions were used to define the four clades described by Laurence *et al.* (2014). Due to the availability of TEF sequence data, a single gene phylogeny was conducted to compare the data with the multi-gene phylogeny for variability. For example, the clade 3 isolates within PSS II of the multi-gene phylogeny had low bootstrap support (81%) and included several sequences that did not group with this clade in the TEF phylogeny. Therefore, the TEF tree will be discussed in detail (Figure 3).

In the TEF phylogeny, two of the 13 citrus FOSC isolates (STEU 8492 and STEU 8508) grouped into clade 4 (99% bootstrap) within *F. oxysporum* PS II clade (99% bootstrap); both isolates were from Hoedspruit. The most closely related species to these two citrus isolates was *F. oxysporum* f. sp. *passiflorae* (BRIP28044) (Rooney-Latham and Blomquist, 2001; Cizislowski *et al.*, 2017). The remaining 11 of the 13 FOSC citrus isolates grouped into a well-supported (86% bootstrap) clade known as Clade 3 (88% bootstrap; O'Donnell, 1998, 2004) within the PS II clade (73% bootstrap) (Figure 3). A subclade with 65% bootstrap support contained three citrus STEU isolates all from Hoedspruit (STEU 8499, STEU 8512 and STEU 8494), along with some *Fusarium* spp. and *F. oxysporum* f.sp. sequences. There are good bootstrap support values (91%) that citrus isolates grouped within clade 3 of the FOSC and low bootstrap values was observed within clade 3 between the citrus isolates and representative isolates. The STEU 8516 (Letsitele orchard) isolate and STEU 8491 (Addo orchard), also within clade 3, grouped in two sub-clades that were distinct from the other citrus FOSC isolates within clade 3 (Figure 3).

DISCUSSION

Citrus is an important agricultural crop and is affected by a range of fungal pathogens, including *Fusarium* spp. that are known to be associated with a variety of symptoms, including dry root rot. Phylogenetic analyses were used in this study to evaluate the diversity and determine the phylogenetic species identity of *Fusarium* spp. isolates from the roots of citrus seedlings grown in soils obtained from old citrus orchards in South Africa. To our knowledge this is the first study to investigate the phylogenetic diversity of both the FSSC and FOSC associated with citrus in South Africa. The citrus seedlings from which isolations were made showed symptoms of citrus replant disease. Preliminary identification of the isolates in a previous study (Chapter 2) indicated that the isolates grouped within the FSSC and FOSC.

Phylogenetic analyses of the citrus associated FSSC and FOSC isolates from South Africa, confirmed that the isolates belonged to these two species complexes. The isolates furthermore represented some known and putative new species in the genera *Fusarium* and *Neocosmospora*. A concatenated multi-gene phylogeny of the FSSC isolates provided a better resolution of clades than the TEF phylogeny. In the multi-gene FSSC isolates showed citrus isolates (23 isolates) mostly belonged to *N. solani s.s*. The second largest group of FSSC citrus isolates (13 isolates) may represent a putative new species, with the most closely related known species being *F. falciformis*. The STEU 8454 sequence grouped with high bootstrap support in a clade related, but distinct from *N. croci.* The TEF phylogeny of the FOSC isolates provided a better resolution of clades than the multi-gene phylogeny. The 13 FOSC isolates from citrus all belonged to *F. oxysporum* PS II previously described by Laurence *et al*. (2014). Most of the citrus isolates (11), furthermore, belonged to FOSC Clade 3 described by O'Donnell *et al*. (1998, 2004), whereas only two isolates belonged to Clade 4 of O'Donnell *et al*. (1998, 2004).

The thirteen citrus FSSC isolates from South Africa most closely related to *F. falciformis*, may represent a putative new species. The isolates had a restricted occurrence and were mainly found in the Hoedspruit area (12 isolates), whereas only one isolate was obtained was from the Letsitele production area. The *F. falciformis* sequences (DQ247075, DQ24713) to which the citrus isolates were related to were from isolates that were isolated from sand and from a human in the USA. *Fusarium falciformis* has been associated with infections in both humans and in plants, but the severity and pathogenicity toward plants are unknown (Zhang *et al*., 2006). *Fusarium falciformis* has never before been associated with citrus roots.

Necospomara croci is a recently described species (Sandoval-Denis *et al*., 2017). This species along with FSSC isolates related to it, is associated with citrus in South Africa and Italy. In Italy, *N. croci* was also associated with citrus in Catania, and specifically orchards with dry root rot symptoms (Sandoval-Denis *et al*., 2017). *Necosmospora croci* belongs to FSSC Clade 3, which was described by O'Donnell *et al*. (1998, 2008). FSSC Clade 3*,* contains a group of important plant pathogens and human and animal opportunistic parasites (O'Donnell *et al*., 2008; Schroers *et al*., 2016). The morphological characteristics of *N. croci* are similar to those of the FSSC isolates. It can be distinguished from *N. solani* by the presence of a saffron diffusible pigment at 36°C and slower growth on artificial media (Schroers *et al*., 2016).

Most of the citrus associated FSSC isolates (23) from South Africa belonged to *N. solani.* These isolates were widely distributed and occurred in all four of the investigated production areas. This is the first study, subsequent to the transfer of *'F. solani'* to *Neocosmospora* to report this specie associated with citrus. *Neocosmospora solani* belongs to the FSSC clade 3 (O'Donnell, 2000a; O'Donnell *et al*., 2008; Sandoval-Denis *et al*., 2017).

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FSSC clade 3 is known to contain *Fusarium falciformis* and *Fusarium keratoplasticum* (Zhang *et al*., 2006; O'Donnell *et al*., 2008; Short *et al*., 2013, 2014).

The two citrus FOSC isolates from South Africa in Clade 4 (O'Donnell *et al*., 1998, 2004) was only obtained from the Hoedspruit and Letsitele areas, whereas the other 11 FOSC citrus isolates from Clade 3 were from all four production regions. The two clades indicate that the isolates are polyphyletic and define evolutionary events. The clades are not informative in terms of pathogenicity towards citrus. The citrus FOSC isolates were related to several *Fusarium oxysporum* and *F. oxysporum* f.sp. sequences in the TEF phylogeny. It is known that housekeeping genes such as TEF, cannot differentiate between non-pathogenic *F. oxysporum* isolates and pathogenic *F. oxysporum formae speciales*. Therefore, the grouping of some of the citrus isolates with specific *F. oxysporum formae speciales* sequences, does not provide an indication of their pathogenicity (Leslie and Summerell, 2006).

The results from this study indicated that the *Fusarium* isolates collected from citrus soils in South Africa were phylogenetically much more diverse than previously thought. The thirteen isolates representing putative new species within the FSSC and closely related to *Fusarium falciformis* should be further investigated. The *N. croci*, *N. solani* isolate and the FSSC isolate related to it, will be of particular interest in pathogenicity assays, since it has been associated with citrus dry rot in Italy (Sandoval-Denis *et al*., 2017). Morphological studies of all of the citrus isolates and pathogenicity testing should be done to determine whether the isolates are pathogenic to citrus. Future studies should furthermore focus on other citrus production areas such as the Western Cape and Mpumalanga to identify species in these areas and compare it to the findings in this study. The current study only included isolates from the Eastern Cape and Limpopo province.

REFERENCES

Adesemoye, A., Eskalen, A., Faber, B., O'Connell, N., Kallsen, C. and Shea, T. 2011. Current knowledge on *Fusarium* dry rot of citrus. Citrograph, 29-33.

Alexopoulos, C.J., Mims, C.W. and Blackwell, M. 1996. Introductory Mycology. John Wiley and Sons, New York.

Aoki, T., O'Donnell, K. and Geiser, D.M. 2003. Sudden-death syndrome of soybean is caused by two morphologically and phylogenetically distinct species within the *Fusarium solani* Species Complex – *F. virguliforme* in North America and *F. tucumaniae* in South America. Mycologia, 95: 660-684.

Aoki, T., O'Donnell, K. and Scandiani, M.M. 2005. Sudden-death syndrome of soybean in South America is caused by four species of *Fusarium*: *Fusarium brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F. tucumaniae* and *F. virguliforme*. Mycologia, 46: 162-183.

Aoki, T., Scandiani, M.M. and O'Donnell, K. 2012. Phenotypic, molecular phylogenetic and pathogenetic characterisation of *Fusarium crassistipitatum* sp. nov., a novel soybean sudden death syndrome pathogen from Argentina and Brazil. Mycoscience, 53(3): 167-186.

Armstrong, G.M. and Armstrong, J.K. 1978. *Formae speciales* and Races of *Fusarium oxysporum* causing wilts of the Cucurbitaceae. Phytopathology, 68:19-28.

Beckman, C.H. and Roberts, E.M. 1995. On the nature and genetic basis for resistance and tolerance of wilt diseases on plants. Advances in Botanical Research 21: 35-77.

Booth, C. 1971. The genus *Fusarium*. Kew, United Kingdom, Commonwealth Mycological Institute, 1-237.

Cizislowski, E.J., Fraser-Smoth, S., Zander, M., O'Neill, W., Meldrum, R., Tran-Nguyen, L., Batley, J. and Aitken, E.A.B. 2017. Investigating the diversity of effector genes in the banana pathogen, *Fusarium oxysporum* f. sp. *cubense*, reveals evidence of horizontal gene transfer. Molecular Plant Pathology, 1-17.

Geiser, D.M., Jiménez-Gasco, M.D.M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A. and O'Donnell, K. 2004. *Fusarium*-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology, 110: 473-479.

Geiser, D.M., Aoki, T., Bacon, C.W., Baker, S.E., Bhattacharyya, M.K., Brandt, M.E., Brown, D.W., Burgess, L.W., Chulze, S., Coleman, JJ., Correll, J.C., Covert, S.F., Crous, P.W., Cuomo, C.A., De Hoop, G.S., Di Pietro, A., Elmer, W.H., Epstein, K., Frandsen, R.J., Freeman, S., Gagkaeva, T., Glenn, A.E., Gordon, T.R., Gregory, N.F., Hammond-Kosack, K.E., Hanson, L.E., Jímenez-Gasco Medel, M., Kang, S., Kistler, H.C., Kuldau, G.A., Leslie, J.F., Logrieco, A., Lu, G., Lysøe, E., Ma, L.J., McCormick, S.P. and

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

Zhang, N. 2013. One fungus, one name: defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. Phytopathology, 103(5): 400-408.

Gordon, T.R. Okamoto, D. and Milgroom, M.G. 1992. The structure and interrelationship of fungal population in native and cultivated soils. Molecular Ecology 1: 241-249.

Gordon, T.R. and Martyn, R.D. 1997. The evolutionary biology of *Fusarium oxysporum*. Annual Review of Phytopathology, 35: 111-128.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology, 59(3): 307-321.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N. 1995. Dictionary of the fungi. CAB International, Wallingford, United Kingdom.

Hillis, D.M. and Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182-192.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequences alignment based on fast Fourier transform. Nucleic Acids Research, 30: 3059-3066.

Katoh, K. and Standley, D.M. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution, 30(4): 772-780.

Kistler, H.C. 1997. Genetic Diversity in the Plant-Pathogenic Fungus *Fusarium oxysporum*. Phytopathology, 87(4): 474-479.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, 33(7): 1870-1874.

Labuschagne, N., Kotzé, J.M. and Putterill, J.F. 1987. Incidence of *Fusarium solani* and *F. oxysporum* in citrus roots and infection by *F. solani*. Phytophylactica, 19: 315-318.

Laurence, M.H., Summerell, B.A., Burgess, L.W. and Liew, E.C.Y. 2014. Genealogical concordance phylogenetic species recognition in the *Fusarium oxysporum* species complex. Fungal Biology, 118: 812-819.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual, 1st Edition. Blackwell Publishing, Iowa.

Lombard, L., van der Merwe, N.A., Groenewald, J.Z. and Crous, P.W. 2014. Lineages in *Nectriaceae*: re-evaluating the generic status of *Ilyonectria* and allied genera. Phytopathologia Mediterranea, 53(3): 1-18.

Lombard, L., van der Merwe, N.A., Groenewald, J.Z. and Crous, P.W. 2015. Generic concepts in *Nectriaceae*. Studies in Mycology, 80: 189-245.

Lui, Y.J., Whelen, S. and Hall, B.D. 1999. Phylogenetic relationships among Ascomycetes: Evidence from an RNA Polymerase II Subunit. Molecular Biology and Evolution, 16(12): 1799-1808.

Nalim, F.A., Samuels, G.J. and Wijesundera, R.L. 2011. New species from *the Fusarium solani* Species Complex derived from perithecia and soil in the old-World tropics. Mycologia, 103: 1302-1330.

O'Donnell, K. 2000a. Molecular Phylogeny of the *Nectria haematococca-Fusarium solani* Species Complex. Mycologia, 92(5): 919-938.

O'Donnell, K. 2000b. A multigene phylogeny of *Gibberella fujikuroi* species complex. Mycoscience, 41: 61-78.

O'Donnell, K. and Cigelnik, E. 1999. A DNA sequenced-based phylogenetic structure for the *Fusarium oxysporum* complex. Phytoparasitica, 27: 69.

O'Donnell, K., Kistler, C.K., Cigelnik, E. and Ploetz, R. 1998. Multiple evolutionary origins of fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Science, USA, 95: 2044-2049.

O'Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J., Frandsen, R.J.N., Lysøe, E., Rehner, S.A., Aoki, T., Robert, A.R.G., Crous, P.W., Groenewald, J.Z., Kang, S., and Geiser, D.M. 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. Fungal Genetics and Biology, 52: 20-31.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Magnon, K.C., Cox, P.A., Revankar, S.G., Sanche, S., Geiser, D.M., Juba, J.H., Van Burik, J.A.H. and Padhye, A. 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. Journal of Clinical Microbiology, 42(11): 5109-5120.

O'Donnell, K., Sarver, B.A., Brandt, M., Chang, D.C., Noble-Wang, J., Park, B.J., Sutton, D.A., Benjamin, L., Lindsley, M., Padhye, A., Geiser, D.M. and Ward, T.J. 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic Fusaria, including isolates from the multistate contact lens associated U.S. keratitis outbreaks of 2005 and 2006. Journal of Clinical Microbiology, 45(7): 2235-2248.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E. and Geiser, D.M. 2008. Mplecular phylogenetiv diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. Journal of Clinical Microbiology, 46(8): 2477-2490.

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

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Sarver, B.A.J., Arunmozhi Balajee, S., Schroers, HS., Summerbell, R.C., Robert, V.A.R.G., Crous, P.W., Zhang, N., Aoki, T., Hung, K., Park, J., Lee, YH., Kang, S., Park, B. and Geiser, D.M. 2010. Internet-Accessible DNA sequence database for identifying Fusaria from human and animal infections. Journal of Clinical Microbiology, 48(10): 3708-3718.

Osmundson, T.W., Eyre, C.A., Hayden, K.M., Dhillon, J. and Garbelotto, M.M. 2013. Back to basics: an evaluation of NaOH and alternative rapid DNA extraction protocols for DNA barcoding, genotyping, and disease diagnostics from fungal and oomycete samples. Molecular Ecology Resources, 13: 66-74.

Rooney-Latham, S. and Blomquist, C.L. 2001. First report of *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *passiflorae* on Passion fruit in North America. Plant Disease, 95(11): 1478-1478.

Rossman, A., Samuels, G.J., Rogerson, C.T. and Lowen, R. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales and Ascomycetes). Studies in Mycology, 42: 1-248.

Sandoval-Denis, M., Guarnaccia, V., Polizzi, G. and Crous, P.W. 2017. Symptomatic Citrus trees reveal a new pathogenic lineage in *Fusarium* and two new *Neocosmospora* species. Persoonia, 40: 1-25.

Schroers, H.J., Samuels, G.J., Zhang, N., Short, D.P., Juba, J. and Geiser, D.M. 2016. Epitypification of *Fusisporium (Fusarium) solani* and its assignment to a common phylogenetic species in the *Fusarium solani* species complex. Mycologia, 108(4): 806-819.

Short, D.P.G., O'Donnell, K., Thrane, U., Nielsen, K.F., Zhang, N., Juba, J.H. and Geiser, D.M. 2013. Phylogenetic relationships among members of the *Fusarium solani* Species Complex in human infections and the descriptions of *F. keratoplasticum* sp. nov. and *F. petroliphilum* stat. nov. Fungal Genetics and Biology, 53: 59-70.

Short, D.P.G., O'Donnell, K. and Geiser, D.M. 2014. Clonality, recombination, and hybridization in the plumbing-inhabiting human pathogen *Fusarium keratoplasticum* inferred from multilocus sequence typing. Evolutionary Biology, 14: 1-14.

Skovgaard, K., Nirenberg, H.I., O'Donnell, K. and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology, 91: 1231-1237.

Smith, IM., Dunez, J. and Philips, D.H. 1988. European handbook of plant diseases. Blackwell Scientific Publications, UK.

Snyder, W.C. and Hansen, H.N. 1940. The Species Concept in *Fusarium*. American Journal of Botany, 27(2): 64-67.

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

Zhang, N., O'Donnell, K., Sutton, D.A., Nalim, F.A., Summerbell, R.C., Padhye, A.A. and Geiser, D.M. 2006. Members of the *Fusarium solani* Species Complex that cause Infections in both Humans and Plants are common in the environment. Journal of Clinical Microbiology, 44(6): 2186-2190.

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TABLES AND FIGURES

Table 1. *Fusarium* and *Neocosmospora* isolates originating from four citrus production areas (Patensie, Addo, Hoedspruit and Letsitele) used in phylogenetic studies.

Strain number	Species name	Province	Production area
STEU 8448 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8449 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8450 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8451 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8452 [x]	*Fusarium* sp.	Limpopo	Letsitele
STEU 8453 *	*Fusarium* sp.	Eastern Cape	Patensie
STEU 8454 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8455 [#]	*Fusarium* sp.	Eastern Cape	Addo
STEU 8456 ^	*Neocosmospora solani*	Limpopo	Hoedspruit
STEU 8457 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8458 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8459 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8460 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8461 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8462 [#]	*Neocosmospora croci*	Eastern Cape	Addo
STEU 8463 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8464 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8465 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8466 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8467 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8468 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8469 ^	*Neocosmospora solani*	Limpopo	Hoedspruit
STEU 8470 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8471 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8472 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8473 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8474 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8476 [#]	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8477 ^	*Fusarium* sp.	Limpopo	Hoedspruit
STEU 8478 *	*Neocosmospora solani*	Eastern Cape	Patensie

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Strain number	Species name	Province	Production area
STEU 8479 *	*Neocosmospora solani*	Eastern Cape	Patensie
STEU 8480 #	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8481 ^	*Neocosmospora solani*	Limpopo	Hoedspruit
STEU 8482 #	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8483 #	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8484 #	*Neocosmospora solani*	Eastern Cape	Addo
STEU 8486 x	*Neocosmospora solani*	Limpopo	Letsitele
STEU 8487 ^	*Neocosmospora solani*	Limpopo	Hoedspruit
STEU 8488 ^	*Neocosmospora solani*	Limpopo	Hoedspruit
STEU 8489 #	*Fusarium oxysporum*	Eastern Cape	Addo
STEU 8491 #	*Fusarium oxysporum*	Eastern Cape	Addo
STEU 8492 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8494 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8499 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8508 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8510 *	*Fusarium oxysporum*	Eastern Cape	Patensie
STEU 8511 #	*Fusarium oxysporum*	Eastern Cape	Addo
STEU 8512 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8514 x	*Fusarium oxysporum*	Limpopo	Letsitele
STEU 8515 x	*Fusarium oxysporum*	Limpopo	Letsitele
STEU 8516 ^	*Fusarium oxysporum*	Limpopo	Hoedspruit
STEU 8517 x	*Fusarium oxysporum*	Limpopo	Letsitele

* Representing STEU isolates from Patensie production area

\# Representing STEU isolates from Addo production area

x Representing STEU isolates from Letsitele production area

^ Representing STEU isolates from Hoedspruit production area

Table 2. Primers, primer sequences and annealing temperatures used as putative molecular markers for the identification of *Fusarium* and *Neocosmospora* species.

Target area	Primers	Sequence	Annealing Temp (°C)	References
TEF	EF1 / EF2	ATGGGTAAGGARGACAAGAC / GGARGTACCAGTSATCATGTT	55	O'Donnell *et al.*, 1998
RPB2	RPB2-5F2 / RPB2-7R	GGGGTGACCAGAAGAAGGC / CCCATRGCTTGYTTRCCCAT	55	O'Donnell *et al.*, 2008; 2010 Lui *et al.*, 1999
	RPB2-7F / RPB2-11aR	ATGGGYAARCAAGCYATGGG / GCRTGGATCTTRTCRTCSACC	55	Lui *et al.*, 1999

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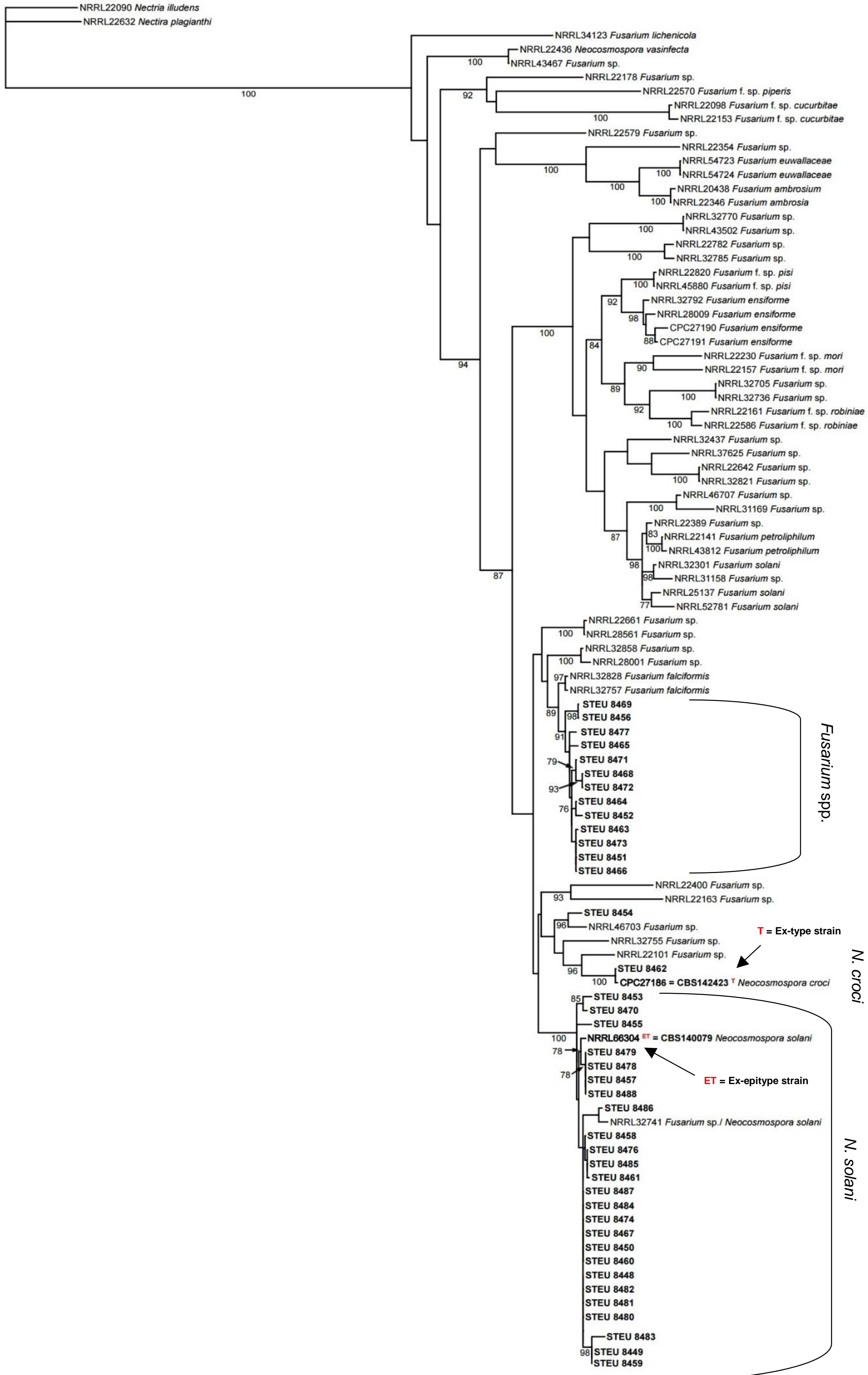

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Figure 1: Maximum likelihood (ML) phylogenetic tree of *Fusarium* and *Neocosmospora* species which was based on the translation elongation factor 1-alpha (TEF) and RNA polymerase II second largest subunit (RPB2) sequence data. Bootstrap support values were calculated from 1000 replicates and bootstrap support of 60% and higher are shown. *Nectria illudens* and *Nectria plangianthi* was used as the outgroups. Isolates obtained in this study are indicated in bold.

STEU 8448
STEU 8449
STEU 8450
STEU 8454
STEU 8458
STEU 8459
STEU 8460
STEU 8467
STEU 8469
STEU 8474
STEU 8476
STEU 8480
STEU 8481
STEU 8482
STEU 8483
STEU 8484
STEU 8485
STEU 8486
STEU 8487
NRRL32741 Neocosmospora solani
STEU 8461
STEU 8456
STEU 8457
STEU 8478
STEU 8479
STEU 8488
NRRL66304 ET = CBS140079 Neocosmospora solani
ET = Ex-epitype strain
N. solani
86/90
80
76
NRRL32755 Fusarium sp.
NRRL22400 Fusarium sp.
87
STEU 8455
NRRL46703 Fusarium sp.
NRRL 22163 Fusarium sp.
86
STEU 8464
STEU 8452
STEU 8451
STEU 8453
STEU 8463
STEU 8466
STEU 8471
STEU 8473
91
90
STEU 8468
STEU 8470
STEU 8472
STEU 8477
STEU 8465
Fusarium spp.
100
NRRL32858 Fusarium sp.
NRRL28001 Fusarium sp.
NRRL22101 Fusarium sp.
98
NRRL22661 Fusarium sp.
NRRL28561 Fusarium sp.
NRRL32828 Fusarium falciformis
NRRL32757 Fusarium falciformis
STEU 8462
CPC27186 = CBS142423 T Neocosmospora croci
T = Ex-type strain
N. croci
88
94
100
NRRL22436 Neocosmospora vasinfecta
NRRL22436 Neocosmospora vasinfecta
NRRL43467 Fusarium sp.
93/98
NRRL20438 Fusarium ambrosium
76/80
NRRL22346 Fusarium ambrosia
100
96/99
NRRL54723 Fusarium euwallaceae
NRRL54724 Fusarium euwallaceae
NRRL22354 Fusarium sp.
NRRL22579 Fusarium sp.
NRRL34123 Fusarium lichenicola
85
NRRL32792 Fusarium ensiforme
NRRL22820 Fusarium f. sp. pisi
NRRL45880 Fusarium f. sp. pisi
75
CPC27190 Fusarium ensiforme
NRRL28009 Fusarium ensiforme
CPC27191Fusarium ensiforme
83/90
NRRL22230 Fusarium f. sp. mori
NRRL22157 Fusarium f. sp. mori
80/88
NRRL22161 Fusarium f. sp. robiniae
NRRL22586 Fusarium f. sp. robiniae
97
NRRL22782 Fusarium sp.
NRRL32785 Fusarium sp.
63
97/99
NRRL32770 Fusarium sp.
NRRL43502 Fusarium sp.
LEMM110739 Fusarium solani
95
NRRL22642 Fusarium sp.
NRRL32821 Fusarium sp.
NRRL32437 Fusarium sp.
NRRL37625 Fusarium sp.
NRRL31158 Fusarium sp.
NRRL32301 Fusarium solani
90
96
LEMM111347 Fusarium solani
NRRL22141 Fusarium petroliphilum
NRRL43812 Fusarium petroliphilum
99
NRRL22389 Fusarium sp.
NRRL25137 Fusarium solani
NRRL52781 Fusarium solani
100
NRRL46707 Fusarium sp.
NRRL31169 Fusarium sp.
NRRL32705 Fusarium sp.
NRRL32736 Fusarium sp.
100
NRRL22178 Fusarium sp.
100
NRRL22098 Fusarium f. sp. cucurbitae
NRRL22153 Fusarium f. sp. cucurbitae
NRRL22570 Fusarium f. sp. piperis
NRRL22090 Nectria illudens
NRRL22632 Nectria plagianthi

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Figure 2: Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic tree of *Fusarium* and *Neocosmospora* species which was based on translation elongation factor 1-alpha (TEF) sequence data. Bootstrap support values were calculated from 1000 replicates and bootstrap support of 60% and higher are shown. *Nectria illudens* and *Nectria plangianthi* was used as the outgroups. Isolates obtained in this study are indicated in bold.

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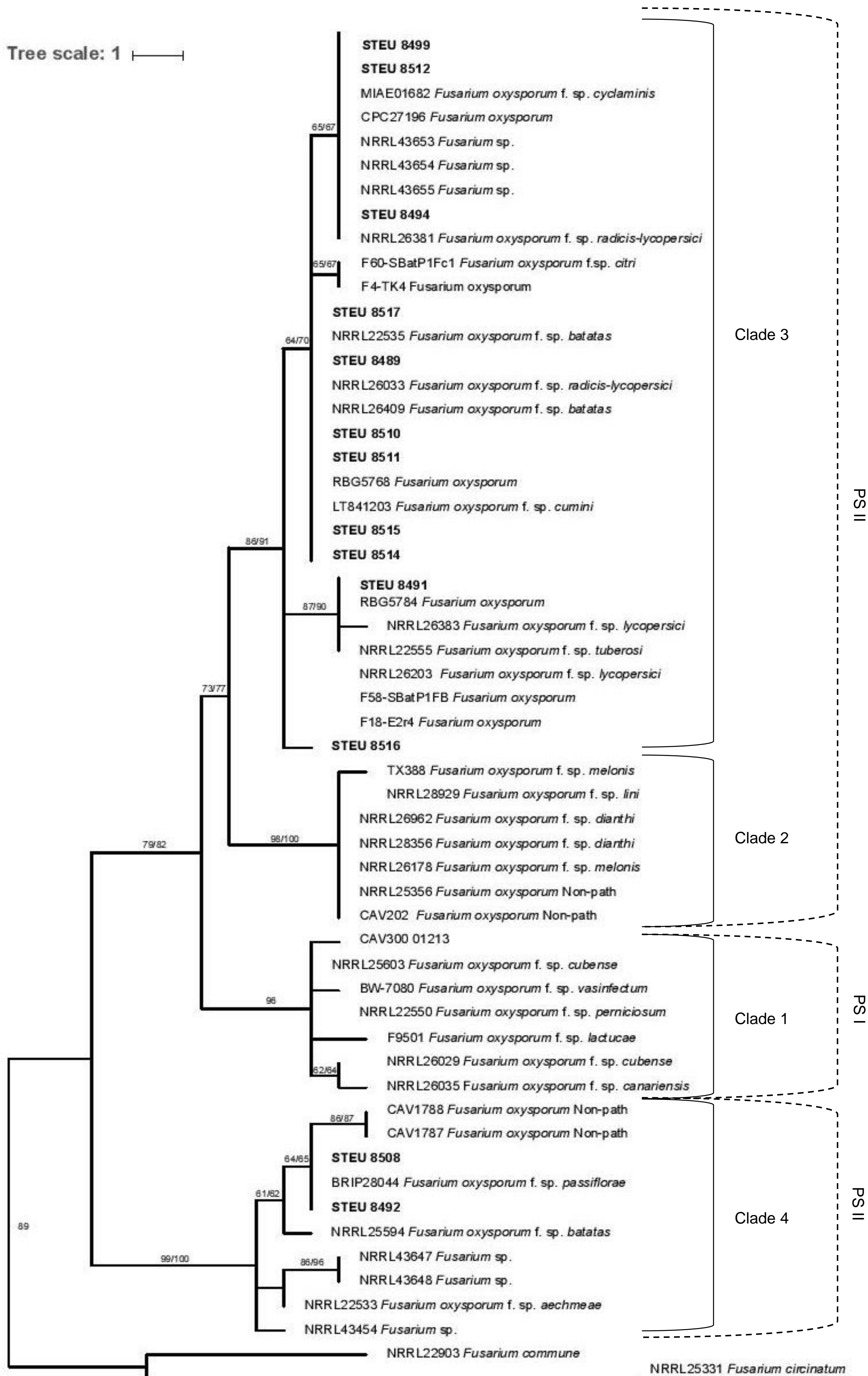

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Figure 3: Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic tree of *Fusarium* species which was based on translation elongation factor 1-alpha (TEF) sequence data. Bootstrap support values were calculated from 1000 replicates and bootstrap support of 60% and higher are shown. *Fusarium circinatum* and *Fusarium commune* was used as the outgroups. Isolates obtained in this study are indicated in bold

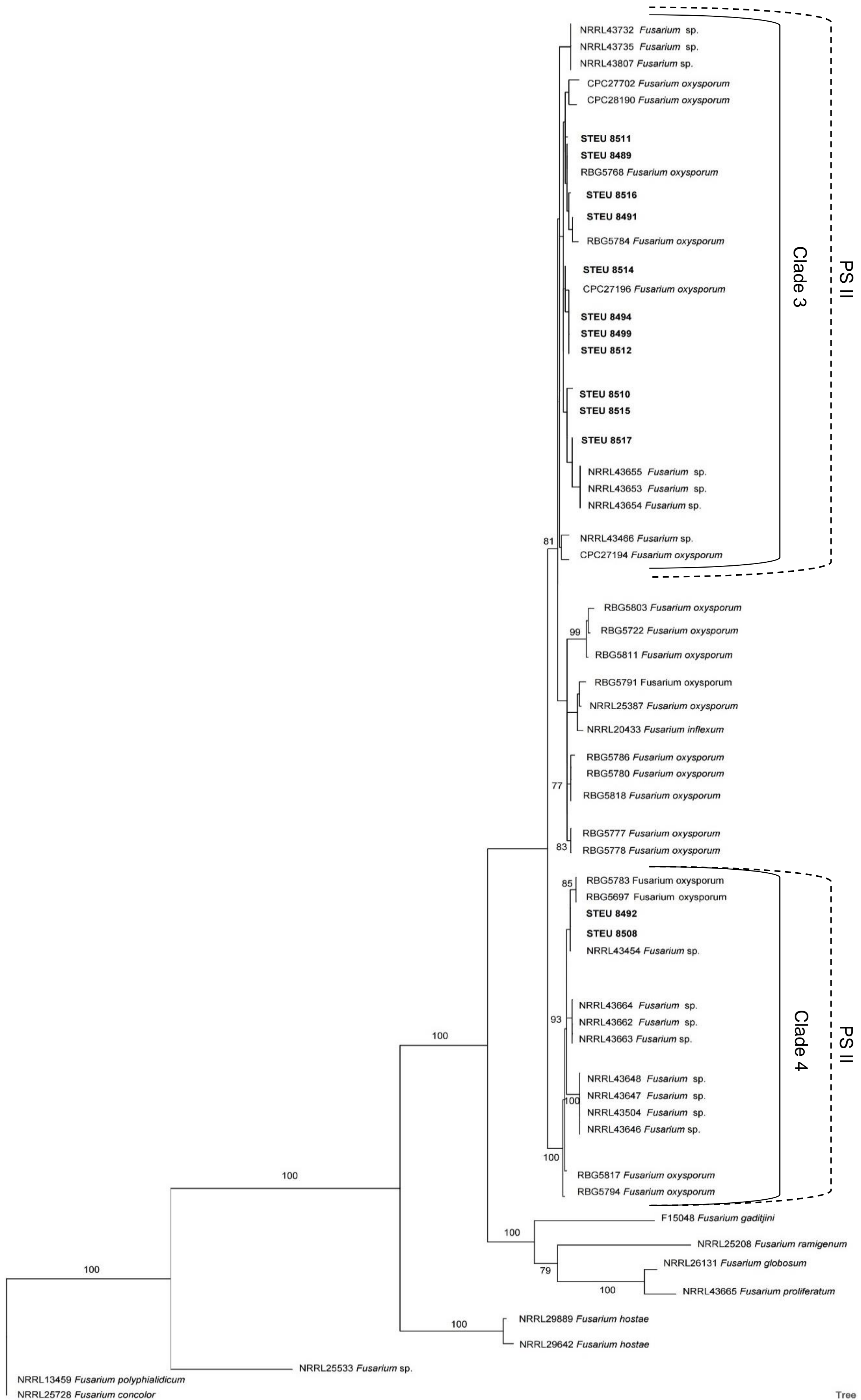
NRRL43732 Fusarium sp.
NRRL43735 Fusarium sp.
NRRL43807 Fusarium sp.
CPC27702 Fusarium oxysporum
CPC28190 Fusarium oxysporum
STEU 8511
STEU 8489
RBG5768 Fusarium oxysporum
STEU 8516
STEU 8491
RBG5784 Fusarium oxysporum
STEU 8514
CPC27196 Fusarium oxysporum
STEU 8494
STEU 8499
STEU 8512
STEU 8510
STEU 8515
STEU 8517
NRRL43655 Fusarium sp.
NRRL43653 Fusarium sp.
NRRL43654 Fusarium sp.
NRRL43466 Fusarium sp.
CPC27194 Fusarium oxysporum
Clade 3
PS II
RBG5803 Fusarium oxysporum
RBG5722 Fusarium oxysporum
RBG5811 Fusarium oxysporum
RBG5791 Fusarium oxysporum
NRRL25387 Fusarium oxysporum
NRRL20433 Fusarium inflexum
RBG5786 Fusarium oxysporum
RBG5780 Fusarium oxysporum
RBG5818 Fusarium oxysporum
RBG5777 Fusarium oxysporum
RBG5778 Fusarium oxysporum
RBG5783 Fusarium oxysporum
RBG5697 Fusarium oxysporum
STEU 8492
STEU 8508
NRRL43454 Fusarium sp.
NRRL43664 Fusarium sp.
NRRL43662 Fusarium sp.
NRRL43663 Fusarium sp.
NRRL43648 Fusarium sp.
NRRL43647 Fusarium sp.
NRRL43504 Fusarium sp.
NRRL43646 Fusarium sp.
RBG5817 Fusarium oxysporum
RBG5794 Fusarium oxysporum
Clade 4
PS II
F15048 Fusarium gaditjini
NRRL25208 Fusarium ramigenum
NRRL26131 Fusarium globosum
NRRL43665 Fusarium proliferatum
NRRL29889 Fusarium hostae
NRRL29642 Fusarium hostae
NRRL25533 Fusarium sp.
NRRL13459 Fusarium polyphialidicum
NRRL25728 Fusarium concolor
81
99
77
83
85
93
100
100
100
100
100
100
79
100
100
100
Tree

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Figure 4: Maximum likelihood (ML) phylogenetic tree of *Fusarium* species which was based on the translation elongation factor 1-alpha (TEF) and RNA polymerase II second largest subunit (RPB2) sequence data. Bootstrap support values were calculated from 1000 replicates and bootstrap support of 60% and higher are shown. *Fusarium polyphialidicum* and *Fusarium concolor* was used as the outgroups. Isolates obtained in this study are indicated in bold.