DETECTION AND QUANTIFICATION OF SOILBORNE PATHOGENS IN GRAPEVINE NURSERIES

by

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SUMMARY

Black-foot disease (BFD) and crown and root rot (CRR) are important decline diseases of grapevines, worldwide. These diseases occur in both nurseries and vineyards. Black-foot disease is caused by species in the genera: *Dactylonectria*, *Ilyonectria*, *Campylocarpon*, *Cylindrocladiella* and *Thelonectria,* whereas CRR is caused by *Phytophthora*, *Pythium* and *Phytopythium* species. Plant stress, caused by improper planting holes, waterlogging, drought, nutrient deficiencies and high temperatures, is a major predisposing factor for BFD and CRR. The symptoms of these two diseases are similar and include necrotic root lesions, reduced root biomass, leaf chlorosis and ultimately, plant death.

The first aim of this study was to conduct a survey of BFD and CRR pathogens in nursery grafted plants over a 3 year period (2013-2015) as well as from weeds and rotation crops in the alternate season (2014). Furthermore, pathogen detection using quantitative real-time polymerase chain reaction (qPCR) was conducted on soil samples which were collected in close proximity to the sampled grapevine plants, weeds and rotation crops. Soil was collected up to a depth of 30 cm and 60 cm. The decline pathogens that were quantified in the soil with qPCR were *Dactylonectria* and *Ilyonectria* genera, *Phytophthora* species and *Pythium irregulare*. The predominant BFD pathogens isolated from grapevines were *Dactylonectria macrodidyma*, *Campylocarpon pseudofaciculare* and *Ca. fasciculare*. The predominant CRR pathogens were *Pythium irregulare* followed by *Phytopythium vexans.* Furthermore, *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *Py. irregulare*, *Py. ultimum* and *Py. heterothallicum* were isolated from Triticale roots. *Dactylonectria* was also isolated from the weed corn spurry, while *Py. irregulare* and *Py. ultimum* were isolated from numerous weeds and rotation crops. *Dactylonectria*, *Ilyonectria*, *Phytophthora* and *Py. irregulare* DNA was detected in all nurseries across all years at varying concentrations with Nursery E having the lowest concentrations. *Ilyonectria* and *Dactylonectria* mean soil DNA concentrations ranged from 0.04 $pg.\mu L^{-1}$ to 37.14 $pg.\mu L^{-1}$, while the *Py. irregulare* mean soil DNA concentrations ranged from 0.01 $pg.\mu L^{-1}$ to 3.77 $pg.\mu L^{-1}$. The *Phytophthora* mean soil DNA concentrations ranged from 0.01 $pg.\mu L^{-1}$ to 33.48 $pg.\mu L^{-1}$. This study successfully used existing SYBR green I qPCR assays to quantify BFD and CRR pathogens in grapevine nursery soil. During this study nursery soils were confirmed as a sources of infection for BFD and CRR pathogens, and that weeds may serve as alternative hosts to these pathogens. This is also the first report of *D. alcacerensis* and *D. pauciseptata* in South African nurseries.

The second aim was to describe a putative new *Phytopythium* species which was isolated from nursery grapevine roots and to determine if it is a pathogenic species. Six isolates of an unknown *Phytopythium* species were isolated from the roots of an asymptomatic

plant. Morphological observations and phylogenetic analyses, based on the ITS rDNA and cytochrome oxidase subunit 1 gene regions, proved that it was indeed a novel species. Together with an undescribed Japanese isolate, the six South African isolates were named *Phytopythium paucipapillatum* and displayed occasionally papillate, direct sporangial germination as well as sporangia which proliferates internally. The sporangia were globose, subglobose, ovoid, obovoid, limoniform to ellipsoid or distorted. The oogonia were small, globose, with some oogonia also displaying short papillae protruding from the surface. This putative new species was also insensitive to the isoxazole fungicide, hymexazol. A pathogenicity trial, on the rootstock 110-Richter, revealed that *Pp*. *paucipapillatum* was not pathogenic towards grapevines. In accordance with other studies regarding *Phytopythium* species, *Pp*. *paucipapillatum* may be a soil inhabitant.

OPSOMMING

Swartvoet en kroon- en wortelvrot is ernstige siektes wat ‘n afname in wingerd wêreldwyd te weeg bring. Hierdie siektes kom in beide kwekerye en wingerde voor. Swartvoet word veroorsaak deur spesies in die *Dactylonectria*, *Ilyonectria*, *Campylocarpon*, *Cylindrocladiella* en *Thelonectria* genera, terwyl kroon- en wortelvrot veroorsaak word deur *Phytophthora*, *Pythium* en *Phytopythium* spesies. Verskeie faktore kan stres op ‘n wingerd plaas, soos swak voorbereide plantgate, waterdeurdrenkte gronde, droogte, ‘n tekort aan voedingstowwe asook hoë temperature, wat die wingerd meer vatbaar maak vir swartvoet en kroon- en wortelvrot. Die simptome van hierdie twee siektes is soortgelyk en sluit nekrotiese wortelletsels, verminderde wortelmassa en blaarchlorose in, wat gesamentlik na die uiteindelike dood van die wingerd lei.

Die eerste doel van hierdie studie was om ‘n opname te doen van swartvoet en kroon- en wortelvrotpatogene in kwekery entplante oor 'n tydperk van 3 jaar (2013-2015), asook van onkruide en rotasiegewasse in die afwisselende seisoen (2014). Grondmonsters, wat in die nabyheid van wingerdplante, onkruide en rotasiegewasse geneem is tydens die steekproef, is ontleed met behulp van kwantitatiewe intyd polimerase kettingreaksies (kPKR) om die patogene wat daarin voorkom op te spoor. Die grondmonsters was tot op ‘n diepte van 30 cm en 60 cm geneem. Die siekteveroorsakende patogene wat gekwantifiseer is in die grond deur middel van hierdie tegniek sluit spesies in die *Dactylonectria* en *Ilyonectria* genera in, asook *Phytophthora* spesies en *Pythium irregulare*. *Dactylonectria macrodidyma*, *Campylocarpon pseudofaciculare* en *Ca. fasciculare* was die oorheerstende swartvoetpatogene wat vanuit wingerd geïsoleer was. Die mees algemene kroon- en wortelvrotpatogeen was *Pythium irregulare*, gevolg deur *Phytopythium vexans*. *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *Py. irregulare*, *Py. ultimum* en *Py. heterotallicum* is vanuit Triticale wortels geïsoleer. *Dactylonectria* is ook vanuit die onkruid ‘corn spurry’ geïsoleer, terwyl *Py. irregulare* en *Py. ultimum* vanuit verskeie ander onkruide en rotasiegewasse geïsoleer is. Wisselende konsentrasies van DNS van *Dactylonectria*, *Ilyonectria*, *Phytophthora* en *Py. irregulare* was gevind in alle kwekerye oor alle jare, met Kwekery E wat die laagste konsentrasies gehad het. Die gemiddelde grond DNS konsentrasies van *Ilyonectria* en *Dactylonectria* het gewissel tussen 0.04 pg.µL^{-1} en 37.14 pg.µL^{-1}, terwyl dit tussen 0.01 pg.µL^{-1} en 3.77 pg.µL^{-1} gewissel het vir *Py. irregulare*. Die gemiddelde grond DNS konsentrasies van *Phytophthora* was tussen 0.01 pg.µL^{-1} en 33.48 pg.µL^{-1}. Hierdie studie kon reeds betsaande SYBR groen I kPKR-toetse met sukses gebruik om swartvoet en kroon- en wortelvrotpatogene in wingerdkwekerygronde te kwantifiseer. Dit het bevestig dat kwekerye as bron van infeksie vir hierdie patogene dien,

terwyl dit ook daarop gedui het dat onkruide as alternatiewe gashere kan dien. Boonop is dit die eerste verslag van *D. alcacerensis* en *D. pauciseptata* in Suid-Afrikaanse kwekerye.

Die tweede doel van hierdie studie was om 'n moontlike nuwe *Phytopythium*-spesie te beskryf wat vanuit wingerdwortels geïsoleer is, en om die patogenisiteit daarvan te bepaal. Ses isolate van 'n onbekende *Phytopythium* spesie is geïsoleer vanuit die wortels van ‘n plant wat geen simptome getoon het nie. ‘n Morfologiese studie tesame met filogenetiese ontledings, wat gebaseer was op die ITS-rDNS en COI geenareas, het bewys dat dit inderdaad 'n nuwe spesie was. Hierdie ses isolate, tesame met ‘n onbeskryfde isolaat van Japan, is *Phytopythium paucipapillatum* genoem. Dit het soms papillate en direkte ontkieming van die sporangia vertoon, asook sporangia wat intern vermeerder. Die sporangia was bolvormige, subglobose, ovoid, obovoid, limoniform tot ellipsoid of verwring. Die oögonia was klein en bolvormige, terwyl sommige oögonia ook kort papilla vertoon het wat by die oppervlak uitsteek. Hierdie nuwe spesie is weerstandbiedend teen hymexazool, ‘n isoxazool swamdoder. ‘n Patogenesiteitstoets wat op die 110-Richter onderstok uitgevoer is, het egter daarop gedui dat *Pp. paucipapillatum* nie patogenies is tot wingerd nie. *Pp. paucipapillatum* is dus moontlik net 'n grondbewoner, wat ooreenstem met resultate van vorige studies op *Phytopythium* spesies.

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CONTENTS

CHAPTER 1

An overview of black foot disease and crown and root rot of grapevines

INTRODUCTION

Grapevine decline is a serious disease complex affecting young grapevines in all grapevine growing regions of the world (Úrbez-Torres *et al.*, 2014a). This disease complex often has huge economic implications for producers (Martin and Cobos, 2007). Several micro-organisms have been reported to be involved in the aetiology of grapevine decline (Gramaje and Armengol, 2011). These microbes include fungi (Marais 1979, 1980; Granett *et al.*, 1998; Rego *et al.*, 2000; Fourie and Halleen, 2001b; Halleen *et al.*, 2003; Gubler *et al.*, 2004, Gramaje and Armengol, 2011) and Oomycetes (Bumbieris, 1972; Marais, 1979, 1980; Latorre *et al.*, 1997; Stephens *et al.*, 1999; Spies *et al.*, 2011). Very often these diseases occur in mixed infections (Gramaje and Armengol, 2011; Úrbez-Torres *et al.*, 2014; Waite *et al.*, 2015) and the symptoms are indistinguishable from each other (Oliveira *et al.*, 2004). The symptoms usually start when new vines are planted in old vineyards (Rego *et al.*, 2000) or when host plants are subjected to stress conditions (Gubler *et al.*, 2004). Two of these decline diseases include black foot disease and crown and root rot.

For the purpose of this literature review, and the subsequent chapters, the focus will mainly be on black foot disease and crown and root rot. The aim of this literature review is to explore various aspects regarding black foot disease and root and crown rot and its main causal agents. The aetiology, epidemiology, symptoms, distribution, pathogen detection and disease management will be discussed.

BLACK FOOT DISEASE

Black foot disease (BFD) of grapevines (*Vitis vinifera* L.) is a serious soilborne disease occurring mainly in nurseries and young vineyards (Halleen *et al.*, 2006a) and is responsible for major damage and economic losses (Hall, 1994; Scheck *et al.*, 1998b; Oliveira *et al.*, 1998; Fourie and Halleen, 2001a; Mostert *et al.*, 2010; Gramaje and Armengol, 2011). Black foot disease occurs in most major grapevine producing countries around the world (Rego *et al.*, 2000; Halleen *et al.*, 2004; Petit and Gubler, 2005; Halleen *et al.*, 2006a; Alaniz *et al.*, 2007a; Abreo *et al.*, 2010). The symptoms of black foot disease include, vascular streaking (Scheck *et al.*, 1998a), black discolouration and gum inclusions of xylem vessels (Grasso and Magnano, 1975), reduced vigour of the vines, and sunken and necrotic root lesions (Halleen *et al.*, 2006a). In infected young vines, decline and death occurs rapidly, whereas in older vines, death may only occur after one year of decline (Gubler *et al.*, 2004).

Black foot disease of grapevines is caused by species of the genera *Dactylonectria* L. Lombard and Crous, *Ilyonectria* P. Chaverri and C. Salgado, *Campylocarpon* Halleen, Schroers and Crous and *Cylindrocladiella* Boesew. (Agustí-Brisach *et al.*, 2012; Agustí-Brisach and Armengol, 2013). Recently, a species from the genus *Thelonectria* P. Chaverri and C. Salgado has been added to the BFD complex (Carlucci *et al.*, 2017). Several species have been associated with black foot disease and its resulting decline of grapevines including nine species of *Dactylonectria*, five species of *Ilyonectria*, two species of *Campylocarpon,* and two species of *Cylindrocladiella* (Cabral *et al.*, 2012b; Agustí-Brisach *et al.*, 2012). The *Dactylonectria* species include *D. alcacerensis* A. Cabral, Oliveira and Crous*, D. estremocensis* (A. Cabral, Nascimento and Crous) L. Lombard and Crous*, D. hordeicola* L. Lombard and Crous*, D. macrodidyma* Halleen, Schroers and Crous*, D. novozelandica* (A. Cabral and Crous) L. Lombard and Crous*, D. pauciseptata* (Schroers and Crous) L. Lombard and Crous*, D. pinicola* L. Lombard and Crous*, D. torresensis* (A. Cabral, Rego and Crous) L. Lombard and Crous and *D. vitis* (A. Cabral, Rego and Crous) L. Lombard and Crous*, Ilyonectria* species include*, I. europaea* A. Cabral, Rego and Crous*, I. liriodendri* (Halleen, Rego and Crous) Chaverri and C. Salgado*, I. lusitanica* A. Cabral, Rego and Crous*, I. pseudodestructans* A. Cabral, Rego and Crous and *I. robusta* (A.A. Hildebr.) A. Cabral and Crous*.* The *Campylocarpon* species include *Ca. fasciculare* Schroers, Halleen and Crous and *Ca. pseudofasciculare* Halleen, Schroers and Crous. *Cylindrocladiella peruviana* (Bat., J.L. Bezerra and M.P. Herrera) Boesew., *Cy. parva* (P.J. Anderson) Boesew. and *Thelonectria blackeriella* M.L. Raimondo and A. Carlucci are recent additions to the black foot disease complex (Agustí-Brisach *et al.*, 2012; Agustí-Brisach and Armengol, 2013; Carlucci *et al.*, 2017)

Taxonomy of the species causing black foot disease

The genus *Cylindrocarpon* was first described in 1913 by Wollenweber as asexual morphs for species belonging to the genus *Nectria* section Willkommiotes Wollenw. (Wollenweber, 1913). Subsequently, the genus *Cylindrocarpon* was split into four groups based on the presence or absence of microconidia and chlamydospores (Booth, 1966). Group one contained the species *Cylindrocarpon magnusianum* (Sacc.) Wollenw., the anamorphs of *Neonectria* species and group two contained *C. cylindroides* Wollenw., the type species for the genus *Cylindrocarpon*. Group three contained the species *C. destructans* (Zinssm.) Scholten, which was the anamorph for *Neonectria radicicola* (Gerlach and L. Nilsson) Mantiri and Samuels and group four contained *Cylindrocarpon* species of the *Nectria mammoidea* W. Phillips and Plowr. group. Furthermore, all of the species in the *Nectria* group with *Cylindrocarpon* asexual morphs were then placed in the genus *Neonectria* (Rossman *et al.*, 1999; Brayford *et al.*, 2004).

Recently, the genus *Cylindrocarpon* (sexual morph: *Neonectria*) has been delineated into three novel genera *Ilyonectria* P. Chaverri and C. Salgado, *Thelonectria* P. Chaverri and C. Salgado, *Rugonectria* P. Chaverri and Samuels and *Neonectria* Wollenw. *sensu stricto.* All of the species associated with grapevine decline were grouped into the genus *Ilyonectria* (Chaverri *et al.*, 2011). Taxonomic studies by Cabral *et al.* (2012a) further delineated species formerly belonging to the *I. radicicola* (Gerlach and L. Nilsson) Chaverri and C. Salgado (=*'C'. destructans* = *D. destructans*) species complex based on morphology and phylogeny of the beta-tubulin, histone H3, translation elongation factor 1-alpha and ribosomal DNA Internal Transcribed Spacer (rDNA-ITS) gene regions. Species within this complex include *I. radicicola s.s.*, *I. vitis* A. Cabral, Rego and Crous, *I. robusta* and *I. europaea*, among others. Moreover, Lombard *et al.* (2013) recently described four new species isolated from the Proteaceae which formerly belonged to the *I. radicicola* complex. These species include *I. capensis* L. Lombard and Crous, *I. leucospermi* L. Lombard and Crous, *I. protearum* L. Lombard and Crous and *I. vredenhoekensis* L. Lombard and Crous and have, thus far, only been isolated from species in the Proteaceae in South Africa.

The species previously known as *'C'. macrodidymum* was also found to be a species complex. Therefore, Cabral *et al.* (2012c) delineated the species into five new species, based on multi-gene phylogenies, which include *I. macrodidyma s.s.*, *I. alcacerensis*, *I. novozelandica*, *I. estremocensis and I. torresensis.*

The most recent taxonomic study by Lombard *et al.* (2014), transferred the *I. macrodidyma* complex species to the new genus *Dactylonectria* Lombard and Crous. This change was based on the paraphyletic nature of the genus *Ilyonectria* in multi-gene phylogenies. The species in the new genus include *D. macrodidyma*, *D. alcacerensis*, *D. novozelandica*, *D. estremocensis* and *D. torresensis*. The species *I. anthuriicola* A. Cabral and Crous and *I. vitis* have also been transferred to this genus. It has been postulated that the species *D. pauciseptata* was closely related to *D. macrodidyma* and has therefore been placed within this genus (Schroers *et al.*, 2008; Lombard *et al.*, 2014). Two additional species were described within this genus namely, *D. pinicola* and *D. hordeicola*. Throughout this chapter the name '*Cylindrocarpon'* will be used when citing and referring to *Dactylonectria* and *Ilyonectria* species in literature published prior to 2011.

Aetiology

Grapevines are susceptible to many trunk and decline diseases, with BFD becoming an ever increasing problem for grape and grapevine producers (Cabral *et al.*, 2012a). As with many plant diseases, a whole range of factors contribute to the development of black foot disease. These include biotic factors (fungal species), environmental factors and factors leading to host

stress (Brayford, 1992). *Ilyonectria*, *Dactylonectria*, *Campylocarpon* and *Cylindrocladiella* species are soil inhabitants regarded as saprobes and weak pathogens and infect roots through wounds and natural openings (Agustí-Brisach and Armengol, 2013). *Ilyonectria* species have been found to inhabit asymptomatic grapevines, where it moves through the xylem vessels to the above- ground plant parts (Halleen *et al.*, 2003; Casieri *et al.*, 2009).

Fungal species associated with BFD

In Australia, '*Cylindrocarpon'* (= *Ilyonectria and Dactylonectria*) species have been isolated from young grapevines displaying typical symptoms of black foot disease such as poor growth, rapid decline and stunting (Edwards and Pascoe, 2004). Similarly, Rego *et al.* (2005), while conducting greenhouse pathogenicity trials on rooted cuttings, confirmed the development of black foot symptoms on grapevines (*Vitis vinifera* cv. Periquita) inoculated with '*Cylindrocarpon'* spp.

'Cylindrocarpon' liriodendri (= *Ilyonectria liriodendri*), previously identified as '*C. destructans'*, is thought to be one of the main causal agents of black foot disease (Halleen *et al.*, 2006b). Whitelaw-Weckert *et al.* (2007) confirmed the pathogenicity of '*C'. liriodendri* towards grapevines (cv. Chardonnay) in Australia. The '*C'. liriodendri* strain was initially isolated from *Vitis vinifera* cv. Pinot Noir displaying symptoms of general decline in growth, reduced shoot, leaf and root growth with dark brown roots and xylem tissue and rotting epidermal tissue (Whitelaw-Weckert *et al.*, 2007). Evaluating three '*C'. liriodendri* propagule types (chlamydospores, conidia and mycelia), at three concentrations on callused grapevine rootstock cuttings, revealed that '*C'. liriodendri* was more pathogenic towards the rootstock cuttings in comparison to '*C'. destructans* and that the disease severity increased with increasing concentrations of inoculum (Probst *et al.*, 2009). A recent study by Probst *et al.* (2012) reported that prior infection of rootstock 101-14 Mgt cuttings with *Phaeomoniella chlamydospora*, significantly increased the incidence of '*C'. liriodendri* infection as compared to the lower incidences of '*C'. macrodidymum* and '*C'. destructans*.

'Cylindrocarpon' macrodidymum (= *Dactylonectria* complex species) has also been implicated in the induction of black foot disease symptoms in grapevines (Halleen *et al.*, 2004; Halleen *et al.*, 2005). Furthermore, De Francisco *et al.* (2009) isolated '*C'. macrodidymum* together with '*C'. liriodendri,* more frequently in a vineyard survey to determine which fungi were associated with black foot diseased grapevines. A greenhouse trial to test the pathogenicity of '*C'. macrodidymum* and '*C'. liriodendri* towards seven different grapevine rootstocks revealed that both species significantly affected most rootstocks tested. The aforementioned species significantly reduced the root dry weight of rootstock 110-Richter and the shoot dry weight of rootstock 1103-Paulsen (Alaniz *et al.*, 2010b). In a different study,

grapevine seedlings (cv. Tempranillo) were inoculated with conidia from '*C'. macrodidymum* and '*C'. liriodendri.* After 10 days, typical black foot disease symptoms were observed with significant differences in virulence among the different isolates tested (Alaniz *et al.*, 2009b).

In a study conducted by Rego *et al.* (2000) in rootstock nurseries and young vineyards, '*C'. destructans* (= *I. destructans* complex) together with *Phaeoacremonium* spp. were isolated from symptomatic wood on grapevine stems. Halleen *et al.* (2004) also found '*C'. destructans* to be pathogenic towards grapevines with symptoms such as reduced root and shoot mass and typical black foot disease symptoms. A study conducted by Harvey and Hunt (2005), in New Zealand, on symptomatic grapevine roots, rootstocks, graft unions, bud wood and stems, found that of all vineyard pathogens isolated, 27% were identified as '*C'. destructans*. However, a subsequent study by Halleen *et al.* (2006b) revealed that isolates from grapevines in Europe and South Africa that were previously thought to be '*C'. destructans'*, were in fact *I. liriodendri.* This re-identification was based on rDNA-ITS and partial beta-tubulin gene phylogenies. This finding, effectively, ruled out the possibility of *'C'. destructans* being a causal agent of BFD in Europe and South Africa. In New Zealand (Mostert *et al.*, 2006), (Pathrose *et al.*, 2010), Uruguay (Abreo *et al.*, 2010) and Iraq (Haleem *et al.*, 2012), *'C'. destructans* has indeed been found to be one of the causal agents of black foot disease.

It was previously hypothesised that the newly described '*C'. pauciseptatum* (= *Dactylonectria pauciseptata*) may be a causal agent of black foot disease since it has been isolated from diseased grapevine roots. However, the pathogenicity of '*C'. pauciseptatum* towards grapevines was yet to be established (Schroers *et al.*, 2008). Subsequently, Alaniz and co-workers (2009a) confirmed that '*C'. pauciseptatum* does in fact cause root necrotic lesions on 110-Richter rootstock cuttings, albeit the need for further studies to elucidate its role in the aetiology of black foot disease. '*Cylindrocarpon' pauciseptatum*, together with '*C'. olidum* have also been isolated less frequently from grapevines displaying symptoms of black foot disease in Spain (De Francisco *et al.*, 2009). Recently, '*C'. pauciseptatum*, together with '*C'. destructans*, '*C'. macrodidymum*, '*C'. liriodendri* and '*C'. olidum* were isolated from roots of young and older grapevines displaying decline symptoms in Uruguay (Abreo *et al.*, 2010). Further evidence which supports the pathogenic status of '*C'. pauciseptatum* is contained in a report by Martin *et al.* (2011). In this report '*C'. pauciseptatum* was isolated from declining grapevines, showing low vigour and sparse foliage. A study done in Portugal revealed, through pathogenicity studies, that *'C'. pauciseptatum* does form part of the BFD complex (Cabral *et al.*, 2012b).

The role of *Campylocarpon* species in the aetiology of BFD was first introduced by Halleen *et al.* (2004). In this study the genus *Campylocarpon* was first described and its status as a grapevine pathogen, as part of the BFD complex in South Africa, was established. In a

subsequent study, Abreo *et al.* (2010) made six isolations of *Ca. pseudofasciculare,* together with *I. liriodendri*, *I. macrodidyma* complex and '*C'. pauciseptatum* from symptomatic rootstocks in Uruguay. Spain became the third country to report the occurrence of *Ca. fasciculare.* These isolations were made from eight-year-old, symptomatic grapevines (Alaniz *et al.*, 2011b). Reports of *Campylocarpon* isolations from symptomatic nursery grapevines have also been reported in Peru (Álvarez *et al.*, 2012) and Brazil (Correia *et al.*, 2012).

Cylindrocladiella peruviana and *Cy. parva* have recently been reported to be part of the BFD complex in Spain (Agustí-Brisach *et al.*, 2012). Although, *Cylindrocladiella* species have been associated with root rot of grapevines in South African nurseries (Van Coller *et al.*, 2005), this was the first report of *Cylindrocladiella* species being associated with black foot disease of grapevines. Furthermore, subsequent reports were published, in which *Cy. peruviana* was isolated from grapevines displaying black foot symptoms in Peru and *Cy. parva* was isolated from grapevines in New Zealand (Álvarez *et al.*, 2012; Jones *et al.*, 2012).

A recent study by Carlucci *et al.* (2017) added a new pathogen to the BFD complex. The pathogen, *Thelonectria blackeriella*, was isolated, together with *I. liriodendri* and *D. torresensis*, from young vines with decline and necrotic symptoms. Pathogenicity trials showed that these pathogens were able to cause black streaking of the vascular tissue of rootstock shoots.

Symptoms

In nurseries, the most visible symptoms include plants with reduced vigour, shortened trunks and internodes, sparse foliage and interveinal chlorosis (Halleen *et al.*, 2006a). Upon dissection of the roots and basal ends of rootstocks, black discolouration, gum inclusions of the xylem vessels and brown vascular streaking was observed (Grasso and Magnano di San Lio, 1975; Scheck *et al.*, 1998a; Rego *et al.*, 2000) (Fig. 1). A reduction in root biomass and root hairs, and sunken necrotic root lesions may also be observed on afflicted plants. In young vines (less than 5 years old) death can occur rapidly (Halleen *et al.*, 2006a).

Mature vines (5 years and older) display symptoms such as poor new growth, failure to form shoots and poor vegetative growth. Dark discolouration was also observed around the trunk at the ground level with necrotic bark and pith. Internally, the xylem vessels were occluded by thick walled tyloses and gum which are responsible for the aforementioned external symptoms (Sweetingham, 1983; Larignon, 1999; Fourie and Halleen, 2001b). Sometimes affected vines also display external symptoms such as the formation of a shallow root system which grows parallel to the soil surface and the formation of a second crown of roots which compensates for the lack of function of the original roots (Larignon, 1999; Fourie *et al.*, 2000). Mature grapevines would die gradually due to BFD (Gubler *et al.*, 2004).

Epidemiology

Dactylonectria and *Ilyonectria* species produce slimy macro- and microconidia, while *Cylindrocladiella* only produces microconidia (Petit *et al.*, 2011). These conidia are the primary infective propagules and are dispersed by water present in soil (Petit *et al.*, 2011; Agustí-Brisach and Armengol, 2013). Sexual reproduction may occur between compatible strains which results in the formation of perithecia which contain ascospores (Cabral *et al.*, 2012c). However, this has not been observed in nature. Information with regards to the life-cycle of BFD pathogens and the role of ascospores in the infection cycle is very limited. All BFD pathogens except *Campylocarpon* species are able to produce thick-walled chlamydospores which would allow these pathogens to persist in the soil for long periods (Booth, 1966; Halleen *et al.*, 2004; Agustí-Brisach and Armengol, 2013). An *in vitro* study by Agustí-Brisach and Armengol (2012) revealed that *I. liriodendri*, *D. macrodidyma* complex species and *D. pauciseptata* could sporulate at a very wide range of temperatures, pH and water potentials.

Environmental factors and host stress

Various factors can contribute to the development or exacerbation of black foot disease in grapevines. The most important of these factors are high temperatures, plant malnutrition, soil compaction, poor soil drainage, poor soil preparation and improper planting holes (Larignon, 1999; Fourie *et al.*, 2000; Fourie and Halleen, 2001b; Halleen *et al.*, 2004; Probst *et al.*, 2012).

High temperatures in summer have been cited as a contributing factor to the development of BFD symptoms. The reason for the latter can be attributed to an increase in the transpiration rate of plants at high temperatures and the inability of diseased plants with damaged roots to replenish the lost water (Larignon, 1999).

During the grapevine propagation process, grapevine cuttings are placed in cold storage prior to grafting and shipping (Gimenez-Jaime *et al.*, 2006). Probst *et al.* (2012) concluded that the storage of grapevine rootstock cuttings (cvv. 101-14 Mgt and 3309 Couderc) at cold temperatures for extended periods lead to host stress and the susceptibility of the grapevines to infection with '*C'. destructans* and '*C'. liriodendri.* All of the aforementioned factors lead to host stress and the accompanying increase in BFD susceptibility and severity.

Inoculum sources

It has often been hypothesised that infection with BFD pathogens can occur in rootstock and scion mother plants in the field and at various stages of the grapevine propagation process, such as grafting and callusing and through wounds when planting in nursery soil (Rego *et al.*, 2000, 2001; Rumbos and Rumbou, 2001; Halleen *et al.*, 2003; Fourie and Halleen, 2004; Gramaje and Armengol, 2011). Halleen *et al.* (2003) isolated low levels of *'Cylindrocarpon'*

spp. from asymptomatic rootstock-scion grafted plants prior to planting in the nursery. However, a significantly higher number of '*Cylindrocarpon*' spp. were isolated from roots and basal ends, after planting the grafted cuttings in the field, indicating that infections took place in the nursery soil. A subsequent study showed that *'Cylindrocarpon'* spp. were isolated from rootstock mother plants, but at low levels (0.17% on average) (Fourie and Halleen, 2004). The authors concluded that although the rootstock mother plants were not major inoculum sources, latent infections existed which may prove to be problematic at a later stage (Fourie and Halleen, 2004).

A study which revealed that nurseries may be a source of infection was done in Spain in 2006 (Aroca *et al.*, 2006). Among other grapevine trunk pathogens, *'Cylindrocarpon'* spp. were isolated from grafted vines and rooted rootstocks prior to planting in vineyards. Pathogen isolations were made from various points on the grapevine stubs. Most of the '*Cylindrocarpon*' isolations were made from the basal end and graft union, which meant that infections could have been caused by a latent infection in the mother plants or that infection occurred after planting the canes in infested nursery soil. These studies confirm that nurseries are points of infection for grafted vines.

Investigations into nursery soil as being a major inoculum source of BFD pathogens have been done and confirmed by several authors. Damm and Fourie (2005) detected *'C'. macrodidymum* in South African nursery soils, for the first time, using conventional polymerase chain reaction (PCR). Cardoso *et al.* (2012) tested nursery soil for *Ilyonectria* spp. using nested polymerase chain reaction and quantitative real-time PCR (qPCR) and confirmed the presence of these pathogens in soil. Similarly, Agustí-Brisach *et al.* (2013b), through multiplex nested PCR and qPCR, determined that *Ilyonectria* spp. were indeed present in nursery soil in Spain. These studies confirm that soil is an important inoculum source for BFD pathogens in grapevine nurseries.

Further studies with regard to the initiation of BFD infection during the grafting and callusing process and during transportation and storage of the grapevine canes were done. In Portugal, Cardoso *et al.* (2012) conducted a study to determine the source of inoculum in the grapevine propagation process in nurseries. In this study, samples were taken from plant material, cutting tools, hydration water from tanks and wells, callusing medium and soil. The air in cold rooms and storage rooms were also sampled. Molecular techniques (nested PCR) as well as conventional isolation by culturing were used to analyse the samples obtained. All the plant samples, cutting tools, and one cold room air sample tested positive for *Ilyonectria* species using both molecular and conventional techniques. The hydration and well water only tested positive with molecular analysis. Similarly, a study done by Agustí-Brisach *et al.* (2013a) determined that the *I. liriodendri* and the *D. macrodidyma* complex was indeed present in pre-

grafting hydration tanks, and on scissors, the omega grafting machine and peat used for callusing. Both of the aforementioned studies are in agreement and confirmed that grafting implements, aids and media are often contaminated with *Ilyonectria and Dactylonectria* conidia which may cause infection of grapevine propagation material.

Due to the soilborne nature of BFD pathogens, it has been found that weeds in nurseries and vineyards harbour BFD pathogens (Agustí-Brisach *et al.*, 2011b). This study revealed that *'C.' macrodidymum* was isolated from roots and xylem tissue of 26 out of 52 weed species found in nurseries and vineyards. Pathogenicity trials confirmed the cross-infection potential of *'C'. macrodidymum* to grapevines. Consequently, the authors concluded that weeds serve as an alternative host to BFD pathogens and may be a source of inoculum for grapevines.

Host range

Species of *Dactylonectria*, *Ilyonectria*, *Campylocarpon* and *Cylindrocladiella*, have a wide host range which include angiosperms and gymnosperms, and have been found in habitats such as tropical rainforest litter and arctic tundra soils (Brayford, 1992; Victor *et al.*, 1998; Chaverri *et al.*, 2011).

Hosts of BFD pathogens include *Olea europaea* L., *Actinidia chinensis* Planch. (Erper *et al.*, 2011; Úrbez-Torres *et al.*, 2014b), *Liriodendron tulipifera* L. (MacDonald and Butler, 1981), and *Panax quinquefolius* L. (Rahman and Punja, 2005). *Abies nordmanniana* (Steven) Spach., *Fragaria* spp., *Quercus* spp., *Festuca duriuscula* L., and *Picea glauca* (Moench) Voss have also been reported as hosts (Cabral *et al.*, 2012a). Several species, namely, *Persea americana* Mill. (Vitale *et al.*, 2012), Proteaceae spp. (Lombard *et al.*, 2013) and *Pinus radiata* D. Don (Agustí-Brisach *et al.*, 2011a) have been added to the list of hosts for *Ilyonectria* spp. and *D. pauciseptata*, respectively. *Dactylonectria alcacerensis*, *D. torresensis* and *I. robusta* have also recently been isolated from loquats (*Eriobotryo japonica* (Thunb.) Lindl. 'Algerie') in Spain (Agustí-Brisach *et al.*, 2016).

Distribution

Black foot disease was first reported from France in 1961 (Maluta and Larignon, 1991). However the name "Pied noir" only came into existence in 1969 (Badour, 1969). The new name, black foot disease, was then used by Scheck *et al.* (1998a). Subsequently, reports have been received from all the major grape growing regions of the world. Black foot disease has been reported from Tasmania, Portugal, Italy, USA, Chile, Greece, Spain, Australia, New Zealand (Halleen *et al.*, 2006a), Iran (Mohammadi *et al.*, 2009), Uruguay (Abreo *et al.*, 2010), Lebanon (Choueiri *et al.*, 2009),Turkey (Özben *et al.*, 2012), Brazil (Russi *et al.*, 2010), Iraq

(Halleem *et al.*, 2012), and Peru (Álvarez *et al.*, 2012). However, each country has its own BFD pathogen profile (Agustí-Brisach and Armengol, 2013).

BFD pathogen detection and quantification

Several assays have been developed to detect BFD pathogens using molecular techniques such as PCR and qPCR (Nascimento *et al.,* 2001; Alaniz *et al.*, 2009a; Mostert *et al.,* 2010; Probst *et al.,* 2010; Tewoldhemedhin *et al.*, 2011; Agustí-Brisach *et al.,* 2014). For the detection in soil, good quality DNA has to be extracted from the soil; which often proves difficult due to the co-extraction of PCR inhibitors such as humic acids, heavy metals and polyphenols (Wilson, 1997).

Soil DNA extraction

In an attempt to find a soil DNA extraction method with minimal inhibitors, Damm and Fourie, (2005) developed an inexpensive soil DNA extraction protocol using sodium dodecyl sulphate (SDS) buffer in combination with cell disruption with the FastPrep instrument (FP 120, Bio101, Savant, Farmingdale, New York) and a DNA clean-up step using polyvinylpolypyrollidone (PVPP) columns. Among four methods tested, the latter method was more sensitive and had the highest DNA yield. Using this method of DNA extraction, the authors were able to detect *Phaeomoniella chlamydospora* and *'Cylindrocapron*' sp*.* in grapevine nursery and vineyard soil with species- and genus-specific PCR. The identity of the *'Cylindrocapron*' sp*.* was confirmed as '*C*.' *macrodidymum* through sequencing of the PCR product.

Agustí-Brisach *et al.* (2013b) extracted total DNA from soil using a CTAB buffer for cell lysis followed by extraction using a commercial kit. However, before extracting DNA from the soil, freeze drying was used as a means to preserve the soil samples as well as to preserve the integrity of any microbial structures in the soil. Information regarding DNA extraction from soil for the testing of BFD pathogens is limited.

The use of polymerase chain reaction for detection

The detection and identification of BFD pathogens play a central role in disease management. Thus far, several PCR based techniques have been developed for the detection and identification of some BFD pathogens. A nested PCR protocol based on the ITS region was developed by Nascimento *et al.* (2001), for the detection of *'C' destructans* and *'C'. obtusisporum.* This protocol had limited success as no distinction could be made between the two species. A genus-specific PCR assay was developed by Dubrovsky and Fabritius (2007). The primers were designed to amplify a 395bp sequence of the ITS gene region.

A species-specific PCR protocol was then developed by Alaniz *et al.* (2009a) for '*C'. liriodendri, 'C'. macrodidymum* and *'C'. pauciseptatum.* Three primer pairs were designed to amplify species-specific fragments of the ITS region which could be used to detect the BFD pathogens from grapevine tissue as well as pure culture. Mostert *et al.* (2010) also developed a species-specific PCR assay for the detection of *'C'. liriodendri, 'C'. macrodidymum, Ca. fasciculare* and *Ca. pseudofasciculare*. These primers were designed to amplify certain fragments from the beta-tubulin gene region.

In the study by Outram *et al.* (2014), a PCR-RFLP protocol was developed to identify seven members of the *D. macrodidyma* species complex. The protocol involves the digestion of a histone H3 gene fragment amplified by the primers CYLH3F and CYLH3R (Crous *et al.*, 2004). The 500 bp fragment was initially digested by the restriction enzyme *Mnl*I to identify *D. hordeicola* (*Ilyonectria* sp. 1), *D. pinicola* (*Ilyonectria* sp. 2), *D. alcacerensis* and *D. macrodidyma*. A second digestion was carried out, using the enzyme *Hinf*I to identify *D. estremocensis*, *D. novozelandica* and *D. torresensis*. The study reported that *D. torresensis* was the only species to have polymorphic restriction sites.

Kernaghan *et al.* (2007) developed a qPCR assay for the detection of *'Cylindrocarpon' destructans* f.sp*. panacis* (CDP) in soils cultivated to *Panax quinquefolius* (ginseng). In these assays the authors made a significant correlation between the amount of CDP DNA and disease severity. The authors concluded that this qPCR assay can be used to determine DNA concentrations of root disease pathogens of any crop. In New Zealand, Probst *et al.* (2010) designed species-specific primers based on the beta-tubulin gene region, to be used in qPCR. The qPCR assay was designed to detect *'C'. macrodidymum* and *'C'. liriodendri* and was sensitive enough to detect the specific DNA at quantities of 3 pg which is equivalent to three conidia per gram of soil. Tewoldhemedhin *et al.* (2011) used the Cyl-R primer from Dubrovsky and Fabritius (2007) together with a newly designed primer, YT2F, to detect and quantify '*Cylindrocarpon*' species in apple roots. These '*Cylindrocarpon*' genus primers have also been used to test grapevine soils from mother blocks and nurseries in Spain (Agustí-Brisach *et al.,* 2014).

DNA sequencing

Traditionally, the ITS-rDNA (ITS1-5.8S-ITS2) region was sequenced to identify grapevine decline fungal species (White *et al.*, 1990; Úrbez-Torres *et al.*, 2015). Cabral *et al.* (2012a; b) found the ITS region to be the least informative as it could not discriminate between a few *Ilyonectria* species. More often multi gene phylogenies are used to identify species of and describe new species of *Ilyonectria*, *Dactylonectria*, *Campylocarpon* and *Cylindrocladiella* (Lombard *et al.*, 2014; 2015). Genes used in these phylogenies include: ITS, beta-tubulin,

histone H3, 28S large subunit and translation elongation factor 1-alpha gene regions. However, the histone H3 gene region, using primers CYLH3F and CYLH3R (Crous *et al.*, 2004), was reported to have the highest resolution as it could resolve all species in the *I. radicicola* and *D. macrodidyma* species complexes. To identify *Cylindrocladiella* spp., Van Coller *et al.* (2005) used phylogenetic analysis of the ITS, beta-tubulin and the histone H3 partial gene regions. Using a combined phylogeny of the aforementioned gene regions, the authors were able to identify all the isolates. Similarly, a study by Lombard *et al.* (2012), used phylogenetic analysis of the ITS, beta-tubulin, histone H3, 28S large subunit and translation elongation factor 1-alpha gene regions to positively identify cryptic species within the *Cylindrocladiella* species complex.

DNA macroarray

Úrbez-Torres *et al.* (2015), developed a DNA macroarray for the simultaneous detection of young vine decline (YVD) pathogens in plants prior to planting. The macroarray was designed to use reverse dot-blot hybridisation and contained oligonucleotides based on the beta-tubulin gene region. The macroarray detected 61 species of which 37 were YVD pathogens. The black foot pathogens that were detected by the macroarray included *D. macrodidyma*, *D. torresensis*, *D. pauciseptata*, *Ca. fasciculare*, *Ca. pseudofasciculare*, *I. europea*, *I. liriodendri*, *I. radicicola* and *I. robusta*.

Management of black foot disease

The management of BFD in nurseries mainly involves the use of cultural practices, as there are no registered fungicides in South Africa (Van Zyl, 2011). Before planting grapevines in vineyards, producers should ensure that the vines have been certified disease free by the certification scheme. However, BFD pathogens can be present in apparently healthy rooted vines.

The limiting of predisposing factors which may lead to host stress has traditionally been used to control BFD (Halleen *et al.*, 2006b). The prevention of soil compaction and the planting of vines in sufficiently large planting holes for proper root development have been cited as a mitigating factor in the control of BFD in vineyards (Larignon, 1999; Fourie *et al.*, 2000).

In vitro studies done by Rego *et al.* (2005) investigated the effect of 14 fungicides on mycelial growth and spore germination of *'C'. desctructans* (*I. destructans* complex). Mycelial growth was inhibited by prochloraz, benomyl and by mixtures of cyprodinil and fludioxonil, and carbendazim and flusilazole. The fungicides tebuconazole and difenoconazole were less effective. Spore germination was reduced by the cyprodinil and fludioxonil mixture, azoxystrobin, tryfloxistrobin and tolyfluanide. Furthermore, *in vitro* studies conducted by

Halleen *et al.* (2007), investigated the effect of the fungicides benomyl, flusilazole, imazilil and prochloraz manganese chloride on *'C'. liriodendri*, *'C'. macrodidyma*, *Ca. fasciculare* and *Ca. pseudofasciculare*, and found that these fungicides were the most effective against these pathogens. The study by Rego et al. (2009) investigated the use of the fungicides cyprodinil+fludioxonil, pyraclostrobin+metiram, fludioxonil and cyprodinil to prevent infections by *Ilyonectria* and *Dactylonectria*, among other wood fungi. The rootstock and scion cuttings were soaked in a fungicide bath prior to grafting. The grafted plants were then planted in an open field nursery. Cyprodinil+fludioxonil was successful in reducing the incidence of *Ilyonectria* and *Dactylonectria* infection and reducing disease severity. Another study on the effects of fungicides on BFD pathogens was conducted by Alaniz *et al.* (2011a). Potted assays were used to determine the effect of these fungicides on *'C'. liriodendri* and *'C'. macrodidyma* during the rooting phase. The fungicides captan, carbendazim, copper oxychloride, dodecyl dimethyl ammonium chloride, hydroxyquinoline sulphate, imazalil and prochloraz were tested, with all proving to be effective in reducing disease severity, except for imazilil which was not effective against '*C'. macrodidymum*. Even though these studies yielded positive results against BFD pathogens, it should be extended to field trials where a wider range of BFD pathogens are tested.

Two reports have been published on the successful use of hot water treatment (HWT) of grapevines as a control measure for BFD. Halleen *et al.* (2007) conducted HWT (50°C for 30min) on uprooted nursery vines. No BFD pathogens were isolated from the hot water treated vines whereas positive isolations were made from the untreated controls. Also, Gramaje *et al.* (2011) determined the effect of HWT on grapevines at 41 to 49°C for 30, 45 and 60 minutes. Temperature above 45 and 48°C for at least 45 minutes was needed to inhibit conidial germination and mycelial growth, respectively.

Biofumigation as control measure for BFD has also been investigated. A study done by Bleach *et al.* (2010) investigated the use of mustard (*B. juncea* (L.) Czern., canola (*B. napus* L.) and oats (*Avena sativa* L.) which were ploughed into the soil as biofumigants against '*Cylindrocarpon'* spp. Grapevines were then planted in the biofumigated soil for nine months. The mustard treatment proved to be most effective by reducing disease incidence by 11% and 43% in rootstock 101-14 Mgt and Teleki 5C, respectively. Mustard meal worked into the soil also proved to be effective against '*Cylindrocarpon'* spp. A study by Barbour *et al.* (2014) showed that during *in vitro* tests, brown mustard and the mustard cultivar Caliente 199 was able to reduce the mycelial growth and conidial germination of *Dactylonectria* species. Furthermore, soil box assay studies also showed that the amount of conidia and chlamydospores isolated from soil, mixed with the mustard plants, was reduced. Another study

by Whitelaw-Weckert *et al.* (2014) also showed the potential of mustard biofumigants on BFD pathogens.

Studies to determine rootstock susceptibility to BFD pathogens have been done. In California, Gubler *et al.* (2004) reported that the rootstocks *Vitis riparia*, O39-16 and Freedom were resistant to *'C'. destructans.* However, in New Zealand, Jaspers *et al.* (2007) determined that the most commonly used rootsocks Riparia Glorie, Schwarzman, Kober 5BB, 140-Ruggeri, Couderc 3309 and Millardet et de Grasset 420A, were all susceptible to BFD at varying levels. Similarly, in Spain, Alaniz *et al.* (2010) evaluated the rootstocks 110-Richter, 1103-Paulsen, 140-Ruggeri, Couderc 161-49, Couderc 196-17, Fercal and SO4 against the BFD pathogens *'C'. liriodendri* and *'C'. macrodidymum.* All of the rootstocks tested were susceptible to the aforementioned pathogens, with 110-Richter being the most susceptible. Information on rootstock susceptibility and tolerance to the wider BFD pathogen range is lacking and more studies need to be conducted in this regard.

Biological control as a means to control BFD has been investigated. A study investigating the use of *Trichoderma harzianum* Rifai as a dip, soil amendment or drench against grapevine trunk pathogens showed positive results by stimulating root growth and reducing the amount of '*Cylindrocarpon*' isolations by 42.9% (Fourie *et al.*, 2001c). The authors concluded that the root stimulating effect of *T. harzianum* treatments would make the grafted cutting more tolerant to replant shock and infection by pathogens. In a semi-commercial nursery trial, Fourie and Halleen (2006) soaked grafted cuttings in a commercial formulation of *T. harzianum* before planting in an open field. However, the results were inconsistent between the two seasons tested. Similarly, a study done by Halleen *et al.* (2007) where the cut basal ends of grafted cuttings were dipped in commercial formulations of *T. harzianum* before planting in the nursery field proved to be inconsistent. Further research is needed with regard to biological control of BFD.

A recent study by Halleen and Fourie (2016) attempted to use an integrated approach to BFD management. This study used several treatments such as the fungicides benomyl and carbendazim, the quaternary ammomium compound Sporekill, a Trichoderma biological control agent, Trichoflow, chlorine dioxide and HWT before grafting and after uprooting of the grapevines. Some of the treatments were in combinations of the above mentioned treatments. This study revealed that HWT (30 min at 50°C), after uprooting the grapevine plants, was the most effective way of reducing BFD infections. Furthermore, a combination of benomyl, HWT and Sporekill before grafting, as well the application of Trichoflow after grafting and before planting in the soil proved to be effective in reducing BFD incidence in grapevine roots.

CROWN AND ROOT ROT OF GRAPEVINES

Aetiology

Crown and root rot have several causal agents, among them several species of *Oomycetes* spanning three genera. These species belong to the genera *Phytophthora* de Bary, *Pythium* Pringsheim and *Phytopythium* Abad, de Cock, Bala, Robideau, Lodhi and Lévesque . In an earlier study, *Pythium ultimum* Trow and six *Phytophthora* species were isolated from grapevines in California. However, the *Phytophthora* species were not identified to species level (Chiarappa, 1959). Also in California, Gubler *et al.* (2004), isolated *Ph. cambivora* (Petri) Buisman from grapevines, as well as an unidentified *Pythium* species which was highly virulent on young vines.

In South Africa, several species have been identified as causal agents in grapevine nurseries and vineyards (Marais 1979; 1980). Using morphological characteristics, these species were identified as *Phytophthora cryptogea* Pethybr. and Laff., *Ph. cactorum* (Lebert and Cohn) J. Schröt., *Ph. parasitica* Dastur (= *Ph. nicotianae* Breda de Haan), *Ph. megasperma* Drechsler, with *Ph. cinnamomi* Rands being the most prevalent and virulent vineyard pathogen. Moreover, several *Pythium* species were also isolated namely, *Pythium sylvaticum* W.A. Campb. and F.F. Hendrix, *Py. irregulare* Buisman, *Py. rostratum* E.J. Butler and *Py. aphanidermatum* (Edson) Fitzp*. Pythium ultimum* was found to be the predominant and most virulent species present in nurseries.

In the most recent study by Spies *et al.* (2011), *Phytophthora*, *Pythium* and *Phytopythium* species were isolated from nursery and vineyard grapevines displaying decline symptoms. In nurseries, the most infections were caused by *Phytopythium vexans* de Bary followed by *Py*. *ultimum* var. *ultimum* and *Py*. *irregulare*. In the vineyards, *Py*. *irregulare* caused the most infections, followed by *Pp*. *vexans*, *Py*. *heterothallicum* W.A. Campb. and F.F. Hendrix, *Ph*. *cinnamomi*. For the first time, through pathogenicity testing, *Pp*. *vexans* and *Phytophthora niederhauserii* Z.G. Abad and J.A. Abad were confirmed as grapevine decline pathogens.

Taxonomy of CRR pathogens

The genera *Phytophthora*, *Pythium* and *Phytopythium* belong to the subphylum Oomycota (class: Oomycetes; family: Pythiaceae) in the Kingdom Chromista or Stramenopila, which are heterotrophic, filamentous, eukaryotes (Schroeder *et al.*, 2013). There are several fundamental differences between the Oomycetes and true fungi (Kingdom Eumycota). The cell walls of the Oomycetes consists of cellulose and beta-glucans, instead of chitin (as in true fungi). Moreover, the Oomycetes produce haploid heterogametagia (oogonia and antheridia),

through meiosis, which then fuse to form sexual spores (oospores). In contrast to the gametangia, the vegetative mycelia are diploid. Oomycetes also produce coenocytic (non-septate, multinucleate) hyphae instead of septate hyphae. The motile zoospores produced by Oomycetes are biflagellate, consisting of both a tinsel and whiplash flagellum (Erwin and Ribeiro, 1996; Hardham, 2005, Rossman and Palm, 2006). The mitochondria were also found to have tubular christae instead of the flattened christae found in true fungi (Rossman and Palm, 2006). Due to the aforementioned factors, the Oomycetes are morphologically and phylogenetically much closer related to diatoms and brown algae than to true fungi (Hardham, 2005; Schroeder *et al.*, 2013).

The genus *Phytopythium* was described in 2010 (Bala *et al.*, 2010) and contains species that formerly belonged to the genus *Pythium* clade K (Lévesque and de Cock, 2004). Species that formerly belonged to the genera *Halophytophthora* (Marano *et al.*, 2014) and Phytophthora (Baten *et al.*, 2014), together with newly described species, now also form part of the *Phytopythium* genus (Baten *et al.*, 2014; 2015; Marano *et al.*, 2014; Thines, 2014; de Cock *et al.*, 2015). *Phytopythium* species share traits with both *Phytophthora* (internal proliferation) and *Pythium* species (zoospore discharge from a vesicle) and is described as an intermediate species (de Cock *et al.*, 2015).

Morphological species are usually classified based on certain morphological features. *Phytophthora* species are classified according to sporangial shape, papillation, caducity and proliferation, as well as the reproductive strategy (homo- or heterothallic) and optimum growth temperature (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996). *Pythium* species are classified using sporangial type, shape, oogonial ornamentation, the presence or absence of hyphal swellings, antheridial attachment, pleroticity, and whether chlamydospores are present (Van der Plaats-Niterink, 1981; Dick, 1990; Schroeder *et al.*, 2013). The classification for *Phytopythium* species is usually a combination of the above criteria (de Cock *et al.*, 2015).

Symptoms

Root and crown rot symptoms include black to brown root necrosis, while the above ground plant parts show typical decline symptoms such as stunted growth, leaf chlorosis, wilting, die-back, poor fruit-set and shoot growth (Marais, 1979; Latorre *et al.*, 1997; Browne and Latorre, 2015). It has been reported that some *Phytophthora* spp. may even infect the crowns and cause cankers which may girdle grapevine trunks. This girdling may then lead to plant collapse (Latorre *et al.*, 1997). The infected vines may be isolated or occur in small groups (Latorre *et al.*, 1997).

Reproduction

The asexual and sexual phases in the life-cycles of *Phytophthora*, *Pythium* and *Phytopythium* species are similar, with a few exceptions (Erwin and Ribeiro, 1996).

Asexual reproduction

Asexual reproduction in *Phytophthora*, *Pythium* and *Phytopythium* occurs mainly by the dispersal of motile zoospores produced in sporangia. Zoospores are the main infective propagule which is uninucleate and lacks cell walls (Erwin and Ribeiro, 1996). Each zoospore contains two flagella (whip and tinsel) which causes it to move in a helical path. Zoospores movement is driven by geotaxis, chemotaxis and electrotaxis. These zoospores also contain vesicles which secrete glycoprotein rich substances which it uses to adhere to actively growing root tissue such as the meristematic tissue, root caps or germinating seeds. (Zentmyer, 1961; Hardham *et al.* 1991; Morris and Gow, 1993).

After the zoospores become attached to the root tissue, the process of encystment is initiated. The zoospore become immobile, sheds the flagella and develops an outer cell wall (Hardham *et al.* 1991; Walker and Van West, 2007). The zoospore cysts may then germinate within two to three hours and may form appressoria-like structures which it uses to invade root tissue (Marais and De la Harpe, 1982; Hohl, 1991; Kamoun, 2003; Hardham, 2007). The invasion of the root tissue is facilitated by cell wall degrading enzymes produced and secreted by the penetrating hyphae (Van der Plaats-Niterink, 1981; Hardham, 2007). Once the plant has been invaded and colonised, new sporangia may develop on the plant surface which will release more zoospores and cause additional infections (Erwin and Ribeiro, 1996).

In *Phytophthora* species, the zoospores are produced and matured in the sporangia (Erwin and Ribeiro, 1996). However, in *Pythium* and *Phytopythium* species undifferentiated cytoplasm is released into evanescent vesicles through a discharge tube. After which, the individual zoospores develop and mature and are released once the vesicle has ruptured. Moreover, not all *Pythium* species are able to produce or rarely produce zoospores, such as *Pythium ultimum* var. *ultimum*, *P. heterothallicum* and *P. irregulare* (Van der Plaats-Niterink, 1981).

In addition to the production of zoospores, sporangia may germinate directly, giving rise to vegetative hyphae which can infect root tissue (Van der Plaats-Niterink, 1981; Hill *et al.*, 1998). The production of thick-walled survival structures such as chlamydospores and hyphal swellings have been observed in some *Phytophthora* and *Pythium* species, respectively (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996). These survival propagules may persist in soil and plant tissue for extended periods of time (Zentmyer and

Mircetich, 1966). Chlamydospores and hyphal swellings also germinate directly, resulting in the development of mycelia or sporangia (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996).

Sexual reproduction

Sexual reproduction in *Phytophthora*, *Pythium* and *Phytopythium* species results in the production of oospores. Oospores are formed by the fusion of antheridia and oogonia, during which genetic material is transferred from the antheridium to oogonium (Dick, 1969). The *Pythium*, *Phytophthora* and *Phytopythium* species may be either homothallic or heterothallic. Homothallic species are able to produce oospores in single culture, whereas heterothallic species requires an opposite mating type for oospore production. When conditions are favourable, the oospores will germinate (Erwin and Ribeiro, 1996).

Epidemiology

Waterlogging is a major predisposing factor for diseases caused by Oomycetes (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996). High soil water content may be problematic for several reasons. Free water in soil allows for the movement of pathogens in soil, especially in soil with larger pores which allows for the unfettered movement of water (Marais and Hattingh, 1985; Kinal *et al.*, 1993; Erwin and Ribeiro, 1996). Furthermore, excess water allows for the diffusion of root and seed exudates into the soil, which may cause propagules to germinate and chemotactically attract motile zoospores to roots (Nelson, 1990; Zentmyer, 1961; Erwin and Ribeiro, 1996). Waterlogging conditions may also deprive plant roots from oxygen leading to increased susceptibility to infection by pathogens (Drew and Lynch, 1980; Wilcox and Mircetich, 1985). It has also been noted by Gardner and Hendrix (1973) that *Pythium* species has higher saprophytic activity during anaerobic conditions and therefore, excess water may increase *Pythium* inoculum in soil. Soil with higher clay content has also been found to be a contributing factor to Oomycete disease severity due to its high water retention ability (Workneh *et al.*, 1993; Martin and Loper, 1999).

Phytophthora, *Pythium* and *Phytopythium* are known to produce propagules for long-term survival such as thick-walled oospores and chlamydospores. Oospores are the main and most persistent survival structure for *Phytophthora*, *Pythium* and *Phytopythium* species and may persist in soil for many years (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996).

The sources of inoculum for CRR pathogens include water-bodies such as rivers, dams and reservoirs (Von Broembsen, 1984; Ristaino and Gumpertz, 2000; Erwin and Ribeiro, 1996). In addition, soil, dust and infected nursery material may also disseminate

Oomycete propagules (Marais, 1980; Weste and Marks, 1987; Erwin and Ribeiro, 1996; Spies *et al.*, 2011).

Distribution

Root and crown rot and its associate pathogens have a worldwide distribution. This disease has been reported from the USA (California) (Chiarappa, 1959), Australia (McGechan, 1966; Bumbieris, 1972), India (Agnihothrudu, 1968), South Africa (Marais, 1979; 1980; Spies *et al.*, 2011), New Zealand and Spain (Brown and Latorre, 2015). *Phytophthora cinnamomi* has been found to be the most virulent pathogen in all affected locations (Browne and Latorre, 2015).

Detection and quantification of CRR pathogens

Culture based methods together with molecular techniques are used for the detection and identification of CRR pathogens. Techniques such as the use of bait plants (apples, pears, avocados and grass blades, etc.) are used to isolate CRR pathogens from soil and water substrates (Erwin and Ribeiro, 1996). Selective culture media such as PARP is often used to isolate the Oomycete pathogens as it inhibits the growth of co-isolated fungi and bacteria (Jeffers and Martin, 1986). The isoxazole fungicide, hymexazol, is also often added to the PARP culture medium for the specific isolation of *Phytophthora*, and certain *Phytopythium* species, as it inhibits the growth of *Pythium* species (Tsao and Guy, 1977). However, there are certain *Phytophthora* species which are sensitive to hymexazol and may be difficult to isolate by direct plating onto the selective medium (Jeffers and Martin, 1986; Erwin and Ribeiro, 1996).

Various DNA-based assays have been developed for the identification of CRR pathogens. A genus-specific PCR protocol, based on the COI and COII gene regions, was developed by Martin and Tooley (2004) for the genus *Phytophthora*. This diagnostic protocol required the downstream use of the restriction enzymes, *Msp*I, *Rsa*I, *Taq*I and *Alu*I to identify the *Phytophthora* to species level. Similarly, Drenth *et al.* (2006) developed a genus-specific PCR protocol for the *Phytophthora* genus which would amplify the ITS region. This assay also uses the restriction enzymes *Msp*I, *Rsa*I, *Taq*I to further identify the individual *Phytophthora* species. Schena *et al.* (2008) developed a genus-specific PCR assay to detect *Phytophthora* pathogens, which includes *Ph. cinnamomi*, from leaves, soil and water in forest and natural ecosystems. This assay was based on the ras-related protein gene Ypt1 and used a nested approach which enabled the identification to species level.

Species-specific assays have also been developed for the detection of Oomycete pathogens on various crops. An assay was developed by Kageyama *et al.* (1997) for the detection of *P. ultimum.* The primers used in this assay were designed to amplify the ITS

region. Also, an ITS PCR assay was developed by Cullen *et al.* (2007) for the detection of *P. ultimum* which causes storage rot on potato tubers. Similarly, Klemsdal *et al.* (2008), reported on an assay which detected *Pythium* species known to cause cavity spot on carrots. This assay which was also based on the ITS region was developed to detect five *Pythium* species which included *Py. sylvaticum*. A conventional PCR assay was developed for the detection of *Ph. cryptogea* on *Gerbera jamesonii*. The PCR primers were based on the *Ypt*1 gene region was found to be specific for *Ph. cryptogea* (Minerdi *et al.*, 2008).

Quantitative real-time PCR (qPCR) is a sensitive, rapid and high-throughput method of detection and DNA quantification (Lievens *et al.*, 2006). A qPCR assay using SYBR Green I chemistry was developed by Schroeder *et al.* (2006). This assay was able to quantify pathogen DNA in soil, namely, *Py. heterothallicum*, *Py. irregulare*, *Py. sylvaticum and Py. ultimum,* among others. The above-mentioned assay was designed to amplify the ITS rDNA region. Atallah and Stevenson (2006) developed an assay to detect and quantify DNA from five Oomycete, fungal and bacterial pathogens which included *Py. ultimum*. The primers used in this assay to detect *Py. ultimum* was based on the ribosomal polymerase B1 (rpb1) gene region and was a SYBR Green I assay. *Pythium ultimum* has also been the target of a SYBR Green I assay developed by Lievens *et al.* (2006). This assay aimed to detect *Py. ultimum* in tomato plants and soil and was designed to amplify the partial ITS2 region of the ITS rDNA. Kernaghan *et al.* (2008) developed an assay to quantify the *Panax quinquefolius* L. damping-off pathogens, *Py. irregulare* and *Py. ultimum* in soil. The assay primer design was based on the ITS region and employed SYBR Green I chemistry. A qPCR assay designed specifically for the quantification of grapevine CRR pathogens was developed by Spies *et al.* (2011). In this study, a genus-specific qPCR assay, adapted from the conventional PCR assay from Schena *et al.* (2006), was developed for *Phytophthora*. This assay used SYBR Green I chemistry and was designed to target the Ypt1 gene. A SYBR Green I assay based on the ITS region was developed for quantification of *Py. irregulare*. Moreover, two Taqman probe assays were developed for *Py. ultimum* and *Pp. vexans*. These assays were used to detect and quantify the CRR pathogens in grapevine roots (Spies *et al*., 2011).

Management of CRR

The management of CRR involves various strategies which include cultural practices, chemical control and hot water treatment. Excessive soil moisture allows for the dispersal of Oomycete pathogens and may have a negative impact on CRR, and should therefore be avoided. Flooding, over-irrigation and soil compaction in nurseries and vineyards should be avoided (Erwin and Ribeiro, 1996; Gubler *et al.*, 2004; Browne and Latorre, 2015). The useful effect of hot water treatment on *Ph. cinnamomi* in dormant vines and canes has also been

demonstrated (Von Broembsen and Marais, 1978). This study recommended the hot water treatment of canes and dormant vines at 50°C for 15 minutes.

Studies with regard to host resistance to CRR is limited. In South Africa, a study by Marais (1988) found that the rootstock 143B Mgt was highly tolerant to *Phytophthora*, whereas rootstocks 99-Richter and 110-Richter were highly susceptible to infection. The study by Gubler *et al.* (2004) showed that rootstocks 9033 Couderc, Kober 5BB and St. George were susceptible to infection by *Pythium* and *Phytophthora*.

Chemical control of *Pythium* and *Phytophthora* using fosetyl-Al and metalaxyl has been studied. Fosetyl-aluminium is an alkyl phosphonate, systemic fungicide (Van Zyl., 2011) and acts by strengthening the host response to infection (Cohen and Coffey, 1986). The study by Marais and Hattingh (1986) found fosetyl-Al to be effective as grapevine foliar spray against *Ph. cinnamomi*. Furthermore, in the same study, the soil fumigants methyl bromide, metham sodium and dazomet were found to reduce the root rot in nursery soil, but was unable to prevent the recurrence of the pathogen in the soil. Fosetyl-Al is the only fungicide registered for use against soilborne pathogens of grapevines in South Africa (Van Zyl, 2011). The phenylamide fungicide metalaxyl has broad spectrum activity (Kato *et al.*, 1990). The study by Marais and Hattingh (1986) also found that metalaxyl soil drenches were effective and was recommended due to its specificity in targeting *Pythium* and *Phytophthora*. However, resistance to metalaxyl in *Pythium* and *Phytophthora* may arise (Mazzola *et al.*, 2002).

CONCLUSION

Black foot disease and crown and root rot continue to be challenges for grapevine nurseries and vineyards globally. The soilborne nature and the fact that these diseases are caused by fungal and oomycete complexes makes it hard to manage. Nursery vines are only visually inspected for fungal and Oomycete disease symptoms and not tested for plant certification, therefore symptomless infections of these pathogens can occur. Additionally, studies with regard to the management of these soilborne diseases are limited. Therefore emphasis should be placed on the detection of these pathogens as a first step in attempting to manage these diseases at nursery level. Furthermore, the ever changing taxonomy of the BFD and CRR pathogens poses a challenge for the detection and identification of these organisms. Molecular techniques such as PCR and qPCR have proven to be important in this regard.

AIM AND OBJECTIVES

Grapevine nurseries are important initial points of infection for BFD and CRR. Therefore the aim of the following study was to detect BFD and CRR pathogens in nursery grapevines or

rotation crops and quantify these pathogens in grapevine nursery soils over a 3 year period. The objectives were:

1. Quantify BFD and CRR pathogens in nursery soils over 3 years in five nurseries.
2. Isolate BFD and CRR pathogens from grapevines, rotation crop plants and weeds over a 3 year period from the same fields in the five nurseries.
3. Describe a new *Phytopythium* species isolated from grapevine roots and determine its pathogenic status towards grapevines.

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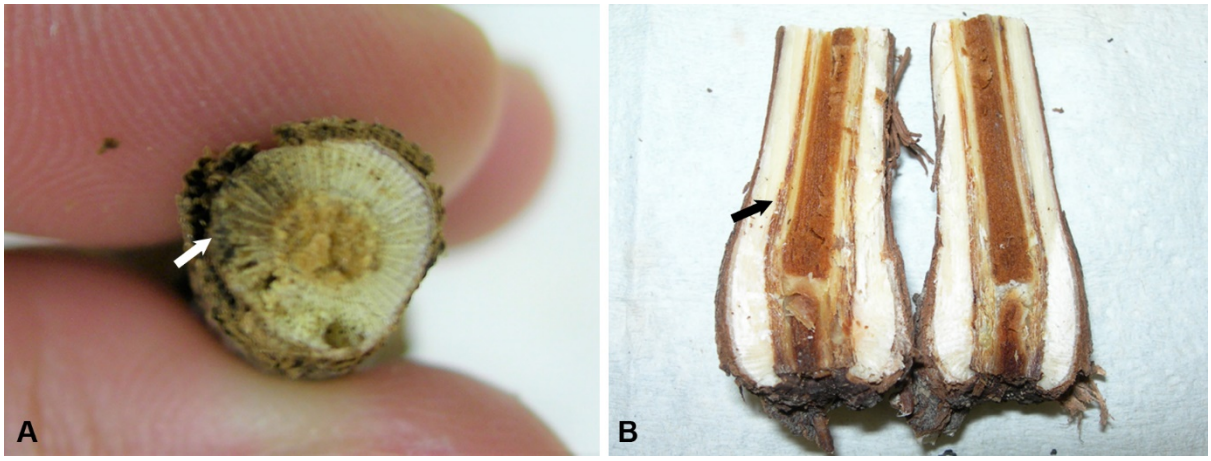

Figure 1. The internal symptoms of black foot disease in grapevine rootstocks. A) Brown discolouration (arrow) and, B) streaking of the vascular tissue (arrow) in the basal ends can be observed.

CHAPTER 2

Detection and quantification of black foot and crown and root rot pathogens in grapevine nursery soils in the Western Cape of South Africa

ABSTRACT

Black foot disease (BFD) and crown and root rot (CRR) are important soilborne diseases which affects young grapevines in both nurseries and vineyards. A 3 year survey (2013-2015) of five open-field grapevine nurseries was conducted in the Wellington and Piketberg areas of the Western Cape Province. The survey involved the isolation of BFD and CRR pathogens from grafted rootstocks (10 plants per nursery, per year) that were rooted in soil for 1 year. In 2013 and 2015, grapevines were sampled, while in 2014, rotation crops and weeds (10 plants each) were sampled. The rotation crops included white mustard, lupins, canola, Triticale and forage radish. The weed species sampled included Johnson grass, rye grass, winter grass, Cape marigold and corn spurry. Soil samples (10 samples per nursery) were also collected in close proximity to the sampled plants to the depths of 30 cm and 60 cm (10 samples per depth). Isolations were made from the grapevines, rotation crops and weeds. Pathogens detection and quantification in the soil was conducted using quantitative real-time polymerase chain reaction. The predominant BFD pathogens isolated from grapevines were *Dactylonectria macrodidyma*, *Campylocarpon pseudofaciculare* and *Ca. fasciculare*. The predominant CRR pathogens were *Pythium irregulare* followed by *Phytopythium vexans.* Furthermore, *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *Py. irregulare*, *Py. ultimum* and *Py. heterothallicum* were isolated from Triticale roots. *Dactylonectria* was also isolated from the weed corn spurry, while *Py. irregulare* and *Py. ultimum* were isolated from numerous weeds and rotation crops. *Ilyonectria* and *Dactylonectria* mean soil DNA concentrations ranged from 0.04 pg.µL^{-1} to 37.14 pg.µL^{-1}, while the *Py. irregulare* mean soil DNA concentrations ranged from 0.01 pg.µL^{-1} to 3.77 pg.µL^{-1}. The *Phytophthora* mean soil DNA concentrations ranged from 0.01 pg.µL^{-1} to 33.48 pg.µL^{-1}.The qPCR protocols were used successfully to detect and quantify BFD and CRR pathogens in grapevine nursery soil. This is the first report of *D. pauciseptata* and *D. alcacerensis* in South African grapevine nurseries.

INTRODUCTION

Black foot disease (BFD) and crown and root rot (CRR) are important diseases which affect young grapevines in both nurseries and vineyards. Both of the aforementioned diseases result in the eventual decline of grapevines (Agustí-Brisach *et al.*, 2013; Spies *et al.*, 2011).

Black foot disease is a disease complex caused by species from four genera namely, *Ilyonectria* P. Chaverri and C. Salgado, *Dactylonectria* L. Lombard and Crous, *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew. and *Thelonectria* P. Chaverri and C. Salgado. The following BFD pathogens have been isolated from grapevines in South Africa: *I. liriodendri* (Halleen, Rego and Crous) Chaverri and C. Salgado, *D. macrodidyma* (Halleen, Schroers and Crous) L. Lombard and Crous, *D. novozelandica* (A. Cabral and Crous) L. Lombard and Crous, *D. torresensis* (A. Cabral, Rego and Crous) L. Lombard and Crous, *Ca. fasciculare* Schroers, Halleen and Crous and *Ca. pseudofasciculare* Halleen, Schroers and Crous (Halleen *et al.*, 2004; 2006; Cabral *et al.*, 2012c; Carlucci *et al.*, 2017). In Spain, *Cylindrocladiella parva* (P.J. Anderson) Boesew. and *Cy. peruviana* (Bat., J.L. Bezerra and M.P. Herrera) Boesew. have been found to be associated with BFD (Agustí-Brisach *et al.*, 2012). In South Africa, these *Cylindrocladiella* species were isolated from grapevines (Van Coller *et al.*, 2005), but their role as BFD pathogens has yet to be established.

Crown and root rot of grapevines is caused by species in three Oomycete genera, namely, *Pythium* Pringsh., *Phytopythium* Abad, de Cock, Bala, Robideau, A.M. Lodhi and Lévesque and *Phytophthora* de Bary. The *Pythium* species include *Pythium aphanidermatum* (Edson) Fitzp., *Py*. *heterothallicum* W.A. Campb. and F.F. Hendrix, *Py. irregulare* Buisman, *Py. rostratum* E.J. Butler, *P. sylvaticum* W.A. Campb. and F.F. Hendrix *and Py. ultimum* Trow (Marais 1979; 1980; Gubler *et al.* 2004; Spies *et al.*, 2011). The *Phytophthora* species identified as causal agents were *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., *Ph. cambivora* (Petri) Buisman, *Ph. cinnamomi* Rands, *Ph. cryptogea* Pethybr. and Laff., *Ph. megasperma* Drechsler, *Ph. niederhauserii* Z.G. Abad and J.A. Abad and *Ph. nicotianae* Breda de Haan . *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, Lodhi and Lévesque is a highly virulent grapevine pathogen and is the only *Phytopythium* species known to be a causal agent of CRR (Marais 1979; 1980; Gubler *et al.* 2004; Spies *et al.*, 2011). All of the above mentioned Oomycete pathogens, except *Ph. cambivora*, have been found to cause CRR in South Africa.

Symptoms of BFD include brown discolouration and streaking of the vascular tissue, gum inclusions of the xylem vessels, reduced grapevine vigour, sunken necrotic root lesions. This leads to shortened internodes and reduced root biomass. Death in young vines occur rapidly whereas decline and death in mature vines occur at a slower rate (Grasso and Magnano, 1975; Scheck *et al.*, 1998; Gubler *et al.*, 2004; Halleen *et al.*, 2006). The outward symptoms of BFD are often indistinguishable from other grapevine trunk diseases like Petri disease. Crown and root rot symptoms in grapevines often manifest as brown/black discoloured rot of roots, stunted growth, chlorosis and wilting. Die-back and decline may also

be observed. Stem cankers may also be caused by certain *Phytophthora* species (Chiarrappa, 1959, Marais, 1979, Zentmyer, 1980, Latorre *et al.*, 1997, Fourie and Halleen, 2001).

Various methods for the detection and quantification of BFD pathogens have been developed. A nested polymerase chain reaction (PCR) assay based on the internal transcribed spacers 1 and 2 and 5.8S rRNA (ITS) gene was developed by Nascimento *et al.*, 2001) and species-specific PCR assay was developed by Alaniz *et al.* (2009). In addition, a species-specific PCR assay, based on the β-tubulin region, have been developed for *'C'. liriodendri* (= *I. liriodendri*), *'C'. macrodidymum* (= *D. macrodidyma* complex species), *Ca. fasciculare* and *Ca. pseudofasciculare* (Mostert *et al.*, 2010). Quantitative real-time PCR (qPCR) is a sensitive, rapid and high-throughput method of detection and quantification (Hardegger *et al.*, 2000). A qPCR assay, based on the ITS rDNA region, was developed for the detection and quantification of the '*Cylindrocarpon'* genus (= *Ilyonectria* and *Dactylonectria* species) (Tewoldemehdin *et al.*, 2011). For this SYBR Green I assay, the reverse primer designed by Dubrovsky and Fabricius (2007) was used, while a new forward primer was developed. Moreover, this qPCR assay was used in a subsequent study in Spain by Agustí-Brisach *et al.* (2014), to detect the level of '*Cylindrocarpon'* pathogens in grapevine nursery soils.

DNA-based assays have also been developed for the detection of CRR pathogens. Several genus-specific PCR assays for the genus *Phytophthora* have been developed (Martin and Tooley, 2004; Drenth *et al.*, 2006; Schena *et al.*, 2008). Also, species-specific PCR assays have been developed for the detection of Oomycete pathogens on various crops. These species-specific assays for the detection of *Py. ultimum (*Kageyama *et al.*, 1997; Cullen *et al.*, 2007), *Py. sylvaticum* (Klemsdal *et al.*, 2008) and *Ph. cryptogea* (Minerdi *et al.*, 2008) have been developed. Quantitative real-time PCR assays, using different chemistries, have been developed for the following Oomycete pathogens: *Py. ultimum* (Atallah *et al.*, 2006; Lievens *et al.*, 2006; Kernaghan *et al.*, 2008), *Py. heterothallicum*, *Py. irregulare*, *Py. sylvaticum and Py. ultimum* (Schroeder *et al.*, 2006) and *Py. irregulare* (Kernaghan *et al.*, 2008). Moreover, during a study on CRR in grapevine nurseries and vineyards, qPCR assays were developed (Spies *et al.*, 2011). A genus-specific qPCR assay was developed for *Phytophthora*. Species-specific assays were also developed for the detection and quantification of CRR pathogens. SYBR Green I assays were developed for *Py. irregulare,* while Taqman probe assays were developed for *Py. ultimum* and *Pp. vexans.* These assays were used to detect and quantify the CRR pathogens in grapevine roots (Spies *et al.*, 2011).

Three important studies have recently widened the understanding on possible inoculum sources of BFD pathogens in grapevine nurseries. The first study done by Agustí-Brisach *et al.* (2013) revealed that each step in the grapevine propagation process, such as the hydration tanks, scissors and grafting machines, may serve as a point of infection for BFD

pathogens. Using PCR and real-time quantitative PCR (qPCR), this study found *Ilyonectria* species to be present in most of the soil sampled from nurseries and rootstock mother fields in Spain. Similarly, Cardoso *et al.* (2012) attempted to detect *Ilyonectria* species in nurseries in Portugal. Samples taken from plant material, cutting tools, hydration water from tanks and wells, callusing medium and soil tested positive for BFD pathogens. The CRR pathogens are known soil inhabitants, but may also thrive in waterbodies such as rivers, dams and irrigation systems (Erwin and Ribeiro, 1996; Ristaino and Gumpertz, 2000).

In South Africa, the management options for BFD is limited to the use of cultural practices as no registered fungicides are available (Van Zyl, 2011). Several studies have been conducted which proposes methods for BFD control. Limiting the predisposing stress factors such as improper planting holes and soil compaction have been proposed (Larignon, 1999; Fourie *et al.*, 2000). Also, two studies were done which reported on the successful use of hot water treatment of nursery grapevines (Halleen *et al.*, 2007; Gramaje *et al.* 2011). Biological control using *Trichoderma harzianum* Rifai has also been studied (Fourie *et al.*, 2001; Halleen *et al.*, 2007). Several studies were conducted to test various fungicides against BFD pathogens (Rego *et al.*, 2005; Halleen *et al.*, 2007; Rego *et al*., 2009 Alaniz *et al.*, 2011). Although some fungicides showed some promise, further trials are needed to confirm field efficacy.

Only three studies have been done on the management of crown and root rot on grapevines (Williams and Hewitt, 1948; Von Broembsen and Marais, 1978; Marais and Hattingh, 1986). Cultural practices such as preventing soil compaction and water logging conditions have been suggested (Utkhede, 1992; Gubler *et al.*, 2004). The study by Von Broembsen and Marais (1978) also showed that hot water treatment (50°C for 30 min) is able to reduce the *Ph. cinnamomi* propagules in grapevine rootstocks. In South Africa, the fungicide fosetyl-Al is registered for use against soilborne disease of grapevines. However, metalaxyl is registered for use against downy mildew (Van Zyl, 2011). Soil fumigation using methyl bromide, metham sodium and dazomet has also been shown to reduce *Phytophthora* and *Pythium* populations in grapevine nursery soil (Marais and Hattingh, 1986; Stephens *et al.*, 1999). The use of methyl bromide has recently been phased out in South Africa (UNEP, 2017). A study by Marais (1988) determined that the rootstock 143B Mgt had the highest tolerance to *Phytophthora* while the rootstocks 99-Richter and 110-Richter were highly susceptible.

The planting of cover crops in perennial cropping systems and nurseries is common practice. Cover crops are planted for various reasons such as to prevent soil erosion from winter rain (Baumgartner *et al.*, 2005), soil temperature regulation (Fourie and Freitag, 2010), to suppress weeds (Blaser *et al.*, 2006), to facilitate nitrogen fixation (Parkin *et al.*, 2006), carbon sequestration (Reicosky and Forcella, 1998) and due to the fact that certain cover

crops possess fungicidal, bactericidal, nematicidal and insecticidal properties (Brown and Morra, 1997; Kruger *et al.*, 2013). Many different cover crops are planted such as legumes, C_3 and C_4 grasses and *Brassica* species (Vukicevich *et al.*, 2016). The *Brassica* species are of particular importance as they are known to suppress soilborne pathogens and play an important role in biofumigation (Brown and Morra, 1997). The *Brassica* species produce allelochemicals known as glucosinolates which are broken down into volatile compounds, isothiocyanates, which possesses pesticidal activity. A study by Gamliel and Stapleton (1993) demonstrated that cabbage residue amended soil had an effect on *Py. ultimum*. Similarly, a study by Mattner *et al.* (2008) showed that isothiocyanates released during biogfumigation was able to suppress '*C*'. *destructans*, *Py. ultimum* and *Ph. cactorum*. Two studies have also shown the beneficial effect of mustard meal on reducing BFD inoculum in soil (Bleach *et al.,* 2010; Barbour *et al.,* 2014). The weed suppressive effect of cover crops is also an important aspect as weeds may reduce crop yields due to allelopathy and competition (Mohler, 2001). Some weed species are also known to serve as alternative hosts to insect pests, plant pathogens and nematodes (Mohler, 2001; Agustí-Brisach *et al.*, 2011).

Several grapevine nursery surveys have been conducted worldwide for the detection of black foot pathogens (Alaniz *et al.*, 2007; Dubrovsky and Fabritius, 2007; Petit *et al.*, 2011; Agustí-Brisach *et al.*, 2013; Úrbez-Torres *et al.*, 2014. In South Africa, the last nursery surveys for BFD and CRR were conducted by Halleen *et al.* (2003) and Spies *et al.* (2011), respectively. Soil has been confirmed as a major inoculum source for BFD pathogens and CRR pathogens (Halleen *et al.*, 2003; 2007; Chaverri *et al.*, 2011). Therefore, the objectives of this study were to: i) quantify BFD and CRR pathogens in five grapevine nursery soils in the Western Cape over a 3 year period; and ii) to assess the level of infection of grapevines, rotation crop and weeds sampled in close proximity of the soil sampling.

MATERIALS AND METHODS

Plant and soil sampling

In 2013, 2014 and 2015 plant material and soil samples were collected in five open field nurseries in the Western Cape. Four of the nurseries were situated in Wellington (nurseries A-D), while the fifth nursery was located in Piketberg (nursery E) (Fig. 1). Due to the crop rotation system used in these nurseries, rooted grapevine plants were sampled in 2013 and 2015, while in 2014, rotation crops and weeds were sampled. Sampling of these plants were done in a zig-zag pattern (W-shape) across the fields. Each year 10 plants were sampled per nursery, one plant per site. No distinction was made between the cultivars sampled and therefore various rootstocks were sampled. All the sampled grapevines were visually healthy. The sampling was done at approximately the same sites every year. Nursery E employs a 3

year crop rotation system and as a result, rotation crops and weeds were sampled in 2015, instead of rooted grapevine plants. Approximately three rotation crop plants and/or weeds were sampled at each site. Additionally, soil samples were taken at the site where the plants were collected. Soil samples were taken with a soil auger approximately 10cm from the grapevine plants at depths of 0-30 cm and at 30-60 cm and placed in separate bags. The soil samples were then placed at -20°C until processing. A subsample of each soil sample was sent for soil and particle analysis at Bemlab (Strand, South Africa) and the Central Analytical Facilities at Stellenbosch University, respectively (Table 1). Soil types were assessed from soil maps for fields for which it is was available or aerial examination of field photos by Dr. Freddie Ellis (soil scientist, Department of Soil Science, Stellenbosch University). Soil wetness classes given on the available soil maps was used to determine the soil wetness index of Lambrechts *et al.* (1978). For those areas where no detailed soil maps were available Dr. Ellis used his knowledge of the soils of the area to derive at a wetness class that were then used in the study.

Isolations from rootstocks, rotation crops and weeds

Isolations were made from the roots and basal ends for crown and root rot (Oomycete) as well as black foot disease (fungal) pathogens. After thoroughly rinsing the roots under running water, 10 root pieces were plated onto PARP medium (Jeffers and Martin, 1986) amended with Switch fungicide (cyprodonil 375 $g.kg^{-1}$and fludioxonil 250 $g.kg^{-1}$, Syngenta) for the isolation of *Pythium* and *Phytopythium* species and 10 root pieces onto PARPH medium for the isolation of *Phytophthora* species. The roots were then surface sterilised in 70% ethanol for 1 minute and left to air dry. Isolations were then made from the roots and basal ends (5 pieces of root and 5 pieces of basal end tissue) onto potato dextrose agar (PDA, Biolab, Randburg) (Fig. 2) amended with streptomycin (0.04 $g.L^{-1}$). For the rotation crops and weeds, isolations were only made from the roots onto PDA. The plates were incubated at 25°C for 1 week and any growth was transferred onto clean PDA plates.

The weed species were identified using the guides by Henderson and Musil (1987); Stirton, (1987); Henderson (1995); Bromilow (2001) and Henderson (2001).

DNA extraction from mycelia and grapevine soil

DNA was extracted from fungal mycelia in pure culture using a modified CTAB DNA extraction protocol by Lee *et al.* (1990). The modifications were as follows: 1) harvested mycelia was macerated using 0.5 g of glass beads which were shaken at 30 Hz in a Retsch MM301 mixer/miller (Retsch, GmbH and Co., Haan, Germany) for 5 minutes; 2) two chloroform-isoamylalcohol steps were done instead of one to enhance the purification of the DNA. The

DNA concentrations were determined using a NanoDrop UV spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The soil samples were removed from the freezer and allowed to thaw after which the soil was thoroughly mixed and left to air-dry in sterile Petri dishes for 2 days. The dried soil aggregates were crushed using a sterile spatula. The soil DNA extractions were done using the NucleoSpin Soil kit (Macherey-Nagel GmbH and Co., Germany) according to the manufacturer's instructions. The SL1 lysis buffer was used together with the enhancer SX. The DNA was extracted from 0.5 g of soil per sample. Two DNA extractions were carried out on each soil sample (2 x 0.5g) at each depth (0-30 cm and 30-60 cm) resulting in four DNA extractions per site (10 sites per nursery). The DNA was eluted in 100 µL of buffer SE. All soil DNA samples were diluted five times in sterile deionised PCR grade water before use in qPCR.

Species-specific polymerase chain reaction (PCR)

The DNA was diluted to 25 ng μL^{-1} and was subjected to species-specific PCR using primer pairs CymaF1 and CymaR2, CyliF1 and CyliR1, CafaF1 and CafaR1, and CapsF1 and CapsR1 which are based on the beta-tubulin gene region and was used to screen for the *Dactylonectria macrodidyma* complex, *Ilyonectria liriodendri*, *Campylocarpon fasciculare* and *Ca. pseudofasciculare*, respectively (Mostert *et al.*, 2010) (Table 2). The PCR reactions were set up separately for each primer pair using 1x NH_4 buffer (Bioline, USA Inc., Taunton, MA), 1.5 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics, Randburg, South Africa), 0.2 mM of each dNTP, 0.4 mM of each primer and 0.5 U of BIOTAQ (Bioline). The PCR reaction was conducted in an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) using a touchdown cycling programme with an initial denaturation temperature of 94°C for 5 min and then 94°C for 45 s with 5 cycles at 66°C for 30 s, 5 cycles at 62°C for 30 s and 20 cycles at 60°C for 30 s, with an extension step at 72°C for 60 s and a final extension step at 72°C for 6 min. The PCR products were resolved on a 1.5% agarose gel (Lonza, Rockland, ME, USA) stained with ethidium bromide and viewed on a UV transilluminator (Syngene, Cambridge, UK).

PCR and sequencing of ITS and histone H3

DNA samples that were identified as *D. macrodidyma* complex were further subjected to sequencing of the histone H3 gene region to resolve the individual species in the *D. macrodidyma* species complex. The primers CYLH3F and CYLH3R were used to amplify 500 bp of the partial histone H3 gene according to Crous *et al.* (2004). The PCR consisted of 1x NH_4 buffer (Bioline), 1 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V

(Roche), 0.2 mM of each dNTP, 0.25 µM of each primer and 0.5 U of BIOTAQ (Bioline). PCR of the ITS region was conducted on all the remaining samples that could not be identified using the species-specific PCR approach as well as to identify all the Oomycete isolates. A subset of the samples that could be positively identified using the species-specific PCR was also subjected to the ITS PCR to test the robustness of the primers. For the fungal samples, the universal fungal primers ITS1 and ITS4 were used (White *et al.*, 1990) to amplify 550 bp of ITS region. The PCR for the amplification of *Phytophthora*, *Pythium* and *Phytopythium* (Oomycete) DNA was carried out using the primer pair ITS4 (White *et al.*, 1990) and ITS6 (Cooke and Duncan, 1997). The reactions for both fungal and Oomycete ITS PCR consisted of 1x NH_4 buffer (Bioline), 2 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche), 0.2 mM of each dNTP, 0.2 µM of each primer, 0.5 U of BIOTAQ (Bioline) and 50 ng of target DNA. However, the Oomycete PCR consisted of 2.5 mM of $MgCl_2$. The PCR was conducted in an Applied Biosystems 2720 thermal cycler with an initial denaturation temperature of 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, 72°C for 90 s and a final extension step at 72°C for 5 min. The PCR product was run on a 1.5% agarose gel stained with ethidium bromide. The PCR product was subjected to a post-PCR clean-up using the MSB Spin PCRapace kit (STRATEC Molecular GmbH, Berlin, Germany). For both gene regions the PCR product was sequenced in the forward and reverse directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions.

All the samples were sequenced on an ABI 3130XL Genetic Analyzer by the Central Analytical Facilities at Stellenbosch University. The resulting histone and ITS sequences were edited, aligned and consensus sequences were generated using Geneious Pro 5.33 (Biomatters Ltd., Auckland, New Zealand) (Kearse *et al.*, 2012). The consensus sequences were then compared to sequences on GenBank using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative real-time polymerase chain reaction

The total soil DNA was used for DNA quantification of black foot and root and crown rot pathogens according to the qPCR protocol by Tewoldemedhin *et al.* (2011) for *Dactylonectria* and *Ilyonectria* species and Spies *et al.* (2011) for *Phytophthora* and *Pythium irregulare*. A five-fold DNA dilution series were made from DNA of reference cultures of *D. macrodidyma* (SL281), *Phytophthora cinnamomi* (STE-U 7392) and *Pythium irregulare* (STE-U 6752). The following concentrations 0.8 ng.µL^{-1}, 0.16 ng.µL^{-1}, 0.032 ng.µL^{-1}, 0.0064 ng.µL^{-1}, 1.28 pg.µL^{-1}, 0.256 pg.µL^{-1}, 51.2 fg.µL^{-1}, 10.2 fg.µL^{-1} and 2.04 fg.µL^{-1} were used to set up a standard curve

for DNA quantification. The concentration standards were done in triplicate and the soil DNA samples were done in duplicate.

The qPCR assay for the detection of *Ilyonectria* and *Dactylonectria* species consisted of the following; 1x KAPA SYBR FAST qPCR master mix (contains SYBR Green I and $MgCl_2$ at a concentration of 2.5 mM) (KAPA Biosystems, Cape Town, South Africa) 0.3 µM of each genus specific primers YT2F and Cyl-R, and 2 µL of five times diluted DNA. The $MgCl_2$ concentration was adjusted to 4.5 mM by the addition of extra $MgCl_2$ (Bioline, USA Inc., Taunton, MA) and the final reaction volume was adjusted to 20 µL using sterile deionised PCR grade water (Bioline). The no template controls received 2 µL of sterile deionised water instead of DNA. The qPCR was carried out at an initial denaturation temperature of 95°C for 10 min, and 60 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s with a final extension step at 72°C for 30 s. In addition, melt curve analysis was included in the run at temperatures between 65 to 95°C with 1.0°C increments at 5 second intervals.

Phytophthora species in the soil samples were detected and quantified using the primers published by Schena *et al.* (2006) as optimised for use with SYBR Green I by Spies *et al.* (2011). This protocol uses a genus-specific primer pair Yph1F and Yph2R. Each qPCR reaction consisted of 1x KAPA SYBR FAST qPCR master mix (with 2.5 mM $MgCl_2$), 0.3 mM of each primer Yph1F and Yph2R, 2 µL of five times diluted DNA. Each reaction was adjusted to 20 µL using sterile deionised PCR grade water (Bioline). No template controls were included in each run. The qPCR cycling conditions consisted of an initial denaturation at 95°C for 10 min, then 50 cycles at 95°C for 10s, 62°C for 15s and 72°C for 30s. Melt curve analysis was included in each run at temperatures between 65 to 95°C with 1.0°C increments at 5 second intervals.

The protocol used for the detection and quantification of root rot pathogen, *Pythium irregulare* was developed by Spies *et al.* (2011). Each reaction consisted of 1x KAPA SYBR FAST qPCR master mix (contains SYBR Green I and $MgCl_2$ at a concentration of 2.5 mM) (KAPA Biosystems), 0.3 µM of primer PirF1 and 0.9 µM of PirR3, and 2 µL of five times diluted DNA. The $MgCl_2$ concentration was adjusted to 3 mM by the addition of extra $MgCl_2$ (Bioline) and the final reaction volume was adjusted to 20 µL using sterile deionised PCR grade water (Bioline). No template controls and concentration standards were included in each run. The cycling conditions for each run consisted of an initial denaturation of 95°C for 10 min, then 50 cycles at 95°C for 10 s, 65°C for 5 s and 72°C for 20 s. Melt curve analysis was included in each run as described above.

DNA concentration standards were included in triplicate in each run to enable DNA quantification after importing a saved standard curve. The qPCR analyses were done on the RotorGene 6000 real-time rotary analyser (Qiagen Inc., Valencia, CA, USA).

A subset of the *Phytophthora* (10 samples) and *Py.irregulare* (10 samples) qPCR products were sequenced using the sequencing reaction protocol described above with the same primers that were used in the qPCR reaction.

qPCR inhibition testing

Nursery soil was sterilised by autoclaving (121°C at 103.4 kPa for 20 minutes) three times, on three consecutive days. Soil DNA extractions were carried out on the sterile soil using the Nucleospin soil kit (Macherey-Nagel GmbH and Co.). Four dilutions: 5x, 10x, 100x and 1000x, were made of each extracted DNA sample and one sample was left undiluted. Each dilution and the undiluted samples were spiked with 10ng of *I. liriodendri* DNA. The spiked DNA extractions were then tested using the qPCR assay described above. The quantitation cycle (C_q) values were recorded and subjected to analysis of variance using SAS (V9.3, SAS Institute, Cary, NC).

Statistical analyses

The experimental design was a completely randomised design with 10 replicates (sites). The treatment design was a combined split plot design. Five nurseries were studied and combined after homogeneity of nursery variances were verified (Brown and Forsythe, 1974). Means for two technical and biological repeats were calculated for each depth and site. The data were transformed using the natural logarithm function prior to analysis. The data was continuous data then subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Observations over time (year) were combined in a split-plot analysis of variance with year as sub-plot factor (Cramer *et al.*, 1989). Shapiro-Wilk test was performed on the standardized residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

RESULTS

Soil sampling and analysis

The soil analysis revealed that the texture of the soil sampled from nurseries A, B and D were coarse sand, and nurseries C and E had loamy coarse sand and loamy medium sand, respectively. There were no differences in soil texture at both 0-30 cm and 30-60 cm depths

except for nursery E, which had loamy medium sand at depth 0-30cm and loamy coarse sand at a depth of 30-60cm (Table 1). Soils of nursery B were classified as mainly Wasbank (Wa) soil form (Soil Classification Working Group, 1991), those of nursery C mainly Westleigh and Kroonstad soil forms and nursery D Cartref soil form. All the above soil forms are known to show hydromorphic features during some time of the year with Westleigh and Kroonstad soils the most of the listed ones. Of the different elements tested, only boron were low in nurseries B and D. The resistance measured of the soil of 30 to 60 cm depth for nurseries A and D were low, indicating excess of salts (resistance levels of below 300 Ohm is seen as problematic). Soils of the nurseries A, B, C and D is prone to wetness and their soil wetness index ranged from 3-6 in contrast with nursery E with an index of below 3.

Species identification

Black foot disease species identification were confirmed by comparing sequences of the histone H3 gene region and CRR species with ITS-rDNA sequences with reference sequences from GenBank (Addendum A, Table 1). In total the number of black foot pathogens isolated were 176. In 2013, 2014 and 2015 there were 86, 18 and 72 black foot isolates for the respective years. Addendum A, Table 2 provides a list of black foot species identified. The predominant BFD pathogens isolated were *Dactylonectria macrodidyma*, *Campylocarpon pseudofasciculare* and *Ca. fasciculare*. In 2013, 2014 and 2015, the number of CRR isolates obtained were 19, 19 and 70, respectively with the total number of 108 isolates (Addendum A, Table 2). The predominant CRR pathogens were *Pythium irregulare* followed by *Pp. vexans.*

Species-specific polymerase chain reaction

Successful amplification was obtained by using the species-specific PCR. The *I. liriodendri*, *D. macrodidyma* complex, *Ca. fasciculare* and *Ca. pseudofasciculare* isolates were positively identified using the assay. This assay was also able to amplify the DNA of all species belonging to the *D. macrodidyma* complex found in the Western Cape. This was confirmed after sequencing the histone H3 gene region of these isolates. No non-specific amplification was observed.

Grapevine infections

In 2013, the black foot pathogens were the predominant species isolated from roots and basal ends of grapevine rootstocks. Among these, *Dactylonectria macrodidyma* was the predominant species isolated from 22 plants in four out of five nurseries (Table 3). This was followed by *Campylocarpon fasciculare* which was isolated from 6 plants in three out of five nurseries. *Phytopythium vexans* was the most predominant Oomycete species infecting 5

plants in two nurseries. Nursery B had the most infected plants (22 plants), as well as the only *Phytophthora* species, followed by nursery C (21 infected plants). No pathogens were obtained from nursery E. Fungal isolates were first stored at 4°C and thereafter plated again for identification. Due to this process, several of the putative pathogen isolates did not grow again after storage and could not be identified. The total number of black foot isolates could therefore have been more from the 2013 isolations.

In 2015, *Campylocarpon pseudofasciculare* was the predominant fungal pathogen isolated from 20 plants across four nurseries. This was followed by *Campylocarpon fasciculare* which infected 11 plants in four nurseries. The predominant Oomycete pathogen was *Pythium irregulare* (20 infected plants) followed by *Pp.* vexans (15 infected plants). Again, nursery B had the highest number of infected plants (32 plants) followed by nursery C (23 plants). Nurseries A had the lowest number of infected plants (11 plants).

Five *Dactylonectria* species, one *Ilyonectria* species, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare.* The Dactylonectria and Ilyonectria species included *D. macrodidyma*, *D. novozelandica*, *D. torressensis*, *D. alcacerensis*, *D. pauciseptata* and *I. liriodendri*. The root and crown rot pathogens that were isolated from grapevines included five *Pythium* species, three *Phytopythium* species and one *Phytophthora* species. These species were *Pythium irregulare*, *Py. sylvaticum*, *Py. ultimum*, *Py. heterothallicum*, *Py. rostratum*, *Pp. vexans*, and *Ph. niederhauserii.*

Isolations from rotation crops and weeds

In 2014, rotation crops and weeds were sampled in the five nurseries. The rotation crops sampled include Canola (nursery A, C), white mustard (nurseries A, B, C), forage radish (nursery C), Triticale (nursery D) and lupins (nursery E). The weeds sampled included Johnson grass, rye grass, winter grass, Cape marigold and Corn spurry (Table 4). In nursery A, no pathogens were isolated from either rotation crops or weeds. In nursery B, no pathogens were isolated from the rotation crops, however, one isolate of *D. macrodidyma* was obtained from the weed, Corn spurry. In nursery C, one isolate of *Py. irregulare* was obtained from forage radish, while one and two isolates of *Py. irregulare* were obtained from the weeds, winter grass and rye grass, respectively. Two *Py. ultimum* isolates were also obtained from rye grass and Cape marigold. Nursery D had the greatest diversity of pathogens isolated from the Triticale rotation crop, while no pathogens were isolated from the weeds. Four isolates of *Py. irregulare* as well as 2 isolates of *Py. ultimum* were obtained from Triticale roots. In nursery E, four *Py. irregulare* isolates were obtained from the weed Johnson grass and three unknown weed species. No pathogens were isolated from the lupin rotation crop. In 2015, weeds were also sampled from nursery E. Ten weed plants were infected with *Py. irregulare*, while 1 plant

each *Py. ultimum* and *Py. heterothallicum*. In addition, two plants were infected with *Pp. vexans* (Table 3).

qPCR inhibition testing

Quantitative PCR amplification was successful and amplified the spiked *I. liriodendri* DNA with an efficiency of 88.4% and the standard curve had a correlation coefficient of 0.993 to the concentration standards. The C_q values were recorded for each sample at the various soil DNA dilutions. The C_q values across all dilutions were similar indicating no to very little qPCR inhibition.

Quantitative real-time polymerase chain reaction for pathogens from soil

Ilyonectria and *Dactylonectria, P. irregulare* and *Phytophthora* species DNA were detected and quantified in grapevine soil samples.

The fluorescence obtained during the SYBR Green I assays for *Ilyonectria* and *Dactylonectria* species reached 100%. The efficiencies for the standard curves ranged between 80-86% with R^2 values of 0.99. The minimum and maximum amount of *Ilyonectria* and *Dactylonectria* DNA detected across all nurseries was 0.04 pg.µL^{-1} and 37.14 pg.µL^{-1}. The melting temperatures ranged between 85-87°C. The DNA melting temperature for the standard DNA (*D. macrodidyma*) was 86.5°C.

The fluorescence obtained during the *Phytophthora* assay reached 100% with standard curve reaction efficiencies between 80-100% with R^2 values of 0.99. The melting temperatures for the *Phytophthora* species ranged between 82-88°C. The DNA melting temperature for the standard DNA (*Ph. cinnamomi*) was 85°C. The minimum and maximum amount of *Phytophthora* DNA detected in a nursery was 0.01 pg.µL^{-1} and 29.53 pg.µL^{-1}.

The assay for the detection of *P. irregulare* reached a fluorescence of 100% with standard curve reaction efficiencies between 78-100% with R^2 values of 0.99. The melting temperature for the *P. irregulare* amplicons was 79.5°C. The DNA melting temperature for the standard DNA (*P. irregulare)* was 79.5°C.The minimum and maximum amount of DNA detected in a nursery was 0.01 pg.µL^{-1} and 3.77 pg.µL^{-1}.

The qPCR results of *Dactylonectria* and *Ilyonectria* species (Addendum A, Tables 3 and 4) showed that these species were present in all the samples tested except for one site in nursery A in 2013. The minimum and maximum amount of *Ilyonectria* and *Dactylonectria* DNA detected across all nurseries was 0.04 pg.µL^{-1} and 37.14 pg.µL^{-1}. In only one sample (nursery A, site 6, 2015) in the depth of 30-60 cm, *Dactylonectria* and *Ilyonectria* species were not detected. There was no significant differences between the top and lower layer of soil (*P*

= 0.2058) and quantities were therefore combined (Table 5). Significant nursery and year interaction was observed ($P < 0.001$). In 2015, nursery A had a significantly higher DNA concentration than all the other nurseries and years. Following this, nurseries B, C and D in 2015 and nurseries A and C in 2014 had concentrations that did not differ significantly from each other. Nursery E had the lowest concentration which did not significantly differ from nurseries B, C and D in 2013 and nurseries B and D in 2014.

Pythium irregulare (Addendum A, Tables 5 and 6) were detected in most of the samples tested except from six sites in nursery A, nine sites in nursery B and four sites in nursery D in 2013. Furthermore, in 2014, *P. irregulare* was not detected in one site each in nurseries B, C and D. In 2015, no detections were made from one site in nursery A. The minimum and maximum amount of *Phytophthora* DNA detected in a nursery was 0.01 pg.µL-[1] and 29.53 pg.µL^{-1}. There was no significant differences between the top and lower layer of soil ($P = 0.3970$) and quantities were therefore combined (Table 5). Significant nursery and year interaction was observed ($P < 0.001$). Higher concentrations was observed in 2015 for nurseries A, B, C and D, however, these mean concentration of nursery C were not significantly different from that obtained for this nursery in 2013. The remaining sampling of the nurseries in 2013 and 2014 had equally lower DNA concentrations.

Phytophthora species were detected in most of the samples tested, except in three sites of nursery A in 2013 (Addendum A, Tables 7 and 8). There was no significant differences between the top and lower layer of soil ($P = 0.2516$) and was therefore combined (Table 5). Significant nursery and year interaction was observed ($P < 0.001$). In 2015, nurseries A and C had significantly higher mean *Phytophthora* spp. concentrations, followed by nursery B. A middle group was formed by nurseries D and E in 2015 and A, B, C and D in 2014. These were all significantly higher than all the nurseries in 2013 and D and E in 2014.

In general, higher concentrations were measured from the 2015 sampling, except nursery for E. For nursery E *Phytophthora* species were also higher in the 2015 sampling. Nursery E had the lowest DNA concentrations for all three pathogen types tested. Nursery A had significant higher DNA concentrations for *Ilyonectria* and *Dactylonectria* over all three years for all five nurseries except for nursery C in 2014. For *Py. irregulare* nursery A only had significantly higher DNA concentration in 2015 for nurseries B, C and E. *Phytophthora* DNA concentrations was significantly higher for nursery A in 2014 in comparison with nurseries B and E, and in 2015 with nurseries B, D and E.

The sequences obtained from the *Phytophthora* qPCR product matched a published *Phytophthora* sp. sequence in GenBank. Similarly, the *Py. irregulare* sequences were homologous to published *Py. irregulare* sequences in GenBank.

DISCUSSION

This was the first study to quantify black foot and root and crown rot pathogen DNA in grapevine nursery soils in South Africa. The DNA of black foot and crown and root rot pathogens were detected with qPCR in the soil of all five nurseries in 2013, 2014 and 2015. Isolations from nursery grapevines confirmed the presence of these pathogens in the 2013 and 2015 sampling from four of the five nurseries.

A wide diversity of black foot pathogens were identified in this study. Five *Dactylonectria* species, one *Ilyonectria* species, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*. This is the first report of *Dactylonectria alcacerensis* and *D. pauciseptata* on grapevines in South Africa. *Dactylonectria* species were frequently isolated from nursery vines and is in accordance with other studies conducted in Spain (Alaniz *et al.*, 2007; Agustí-Brisach *et al.*, 2014). .

Of the root and crown rot pathogens, five *Pythium* species, three *Phytopythium* species and one *Phytophthora* species were isolated. *Pythium irregulare*, *Py. sylvaticum*, *Py. ultimum*, *Py. heterothallicum*, *Py. rostratum*, *Pp. vexans*, and *Ph. niederhauserii* were isolated and are known grapevine pathogens (Marais 1979, 1980; Spies *et al.* 2011). *Phytopythium helicoides* and *Py. litorale* were also isolated from grapevines in nurseries and vineyards (this study, Spies *et al.* 2011), but their pathogenicity toward this crop has not been determined. *Pythium sylvaticum* was isolated from nursery vines for the first time since 1980 (Marais, 1980). *Pythium vexans* was only isolated from grapevines which confirms its affinity for grapevines and its status as a grapevine pathogen (Spies *et al.*, 2011). Only one *Phytophthora* isolate (*Ph. niederhauserii*) was obtained from nursery B in 2013. This is in accordance with Spies *et al.* (2011) who also only found *Ph. niederhauserii* in one nursery in the Wellington area. Spies *et al.* (2011) surveyed nurseries in Clanwilliam, Malmesbury, Vredendal and two locations in Wellington. The low occurrence of *Phytophthora* species in nursery vines can be attributed to the application of fosetyl-Al and metalaxyl based fungicides for the control of downy mildew. It is also known that *Phytophthora* species are difficult to isolate by direct plating as they tend to display weak growth in the presence of saprophytes (Erwin and Ribeiro, 1996) and this could also contribute to the low occurrence of this pathogen. Another reason for the difficulty in isolating *Phytophthora* from infected roots is due to the disintegration of the necrotic tissue during the rinsing process (Bumbieris, 1972). Alternative approaches for the detection of *Phytophthora* in the soil can be attempted such as dilution plating and soil baiting with the appropriate baiting material (Erwin and Ribeiro, 1996). Dilution plating and soil baiting can also be used to test the viability of any propagules present in the soil. A direct approach to detection and quantifying of pathogenic fungi and Oomycetes is through the use of qPCR on root DNA samples such as the studies by Spies *et al.* (2011) and Tewoldemedhin *et al.* (2011).

Of the different rotation crops investigated, only Triticale (5 plants) and forage radish (1 plant) harboured black foot and crown and root rot pathogens. Canola, white mustard and lupins did not have any of these pathogens. The potential of biofumigation through *Brassica* plants or *Brassica* products of black foot pathogens have been shown (Bleach *et al.* 2010; Barbour *et al.* 2014; Whitelaw-Weckert *et al.* 2014). *Brassica* species release glucosinolates when incorporated into soil which is then broken down into volatile isothiocyanates, and these compounds in turn can suppress pathogenic fungi (Brown and Morra, 1997). However, studies have shown that biofumigant treatments may not have an effect on some *Pythium* species (Stephens *et al.*, 1999; Mazzola *et al.*, 2001). Only one forage radish plant tested positive for *P. irregulare.* This plant was sampled from nursery C which had loamy coarse sand and the highest clay content. According to Brown and Morra (1997), clay and organic matter in soil may absorb the glucosinolates rendering it less effective against pathogens. The interaction of *Brassica* plants and clay and organic matter content require further investigation. The effect of rotation crops on the occurrence of soilborne pathogens need to be tested over a period of time longer than 3 years.

The weeds that harboured pathogens in the current study included corn spurry, Cape marigold and three grasses; rye grass, winter grass and Johnson grass. A study in Spain found that 26 weed species including grasses (Poaceae) and flowering weeds in the Asteraceae carried *Dactylonectria macrodidyma* (Agustí-Brisach *et al.*, 2011). They also demonstrated that the *D. macrodidyma* isolated from weeds could induce typical black foot symptoms on grapevines. The current study showed that both black foot pathogens and *Pythium* species can occur on weeds. Several *Pythium* species were also obtained from weed and grass samples in Japan (Uzuhashi *et al.*, 2010). A study by French-Monar *et al.* (2006) showed that *Phytophthora capsici* was able to use Solanaceous weeds, in a vegetable field, as an alternative host. Weeds can be a source of inoculum, but the relevance of the different weeds in terms of disease incidence need to be determined before strategies can be recommended for the removal of weeds. In addition, weeds may allow for the survival of pathogens through seasons when a host crop is unavailable (rotation or fallow periods).

This study has demonstrated that qPCR is a sensitive, rapid and high-throughput method of soil-borne pathogen detection and quantification. The established protocols by Spies *et al.* (2011) and Tewoldemedhin *et al.* (2011) used in this study, were successfully adapted for the detection and quantification of pathogens in soil. In general, over the three years of sampling, there was an increase in the mean DNA concentrations across all pathogens. The reason for this observation is not clear. A study done by Halleen *et al.* (2003) suggested that there might be inoculum build-up of BFD pathogens during a 2 year rotation period. A period longer than 3 years is necessary to be able to see to what extent there is a

build-up of inoculum in the soil. The presence of the pathogen DNA in the soil as well as in grapevine plants shows that pathogen inoculum persist in the soil during the crop rotation year. The DNA concentrations in the crop rotation year (2014) for *Dactylonectria* and *Ilyonectria* species as well as *Phytophthora* species were equal or higher than in the first grapevine year (2013) investigated. Together with the fact that the DNA concentrations for these pathogens were higher in the second grapevine year investigated (2015) indicate that the pathogens survive successfully in the soil or in the roots and basal ends of specific weeds and rotation crops. It is known that *Dactylonectria*, *Ilyonectria* and *Phytophthora* species form chlamydospores, which are hardened survival structures allowing these fungi to persist in soil (Erwin and Ribeiro, 1996; Halleen *et al*., 2004). *Pythium* and certain *Phytophthora* species are homothallic and can produce oospores that can survive in the soil (Van der Plaats-Niterink, 1981; Erwin and Ribeiro, 1996). It is interesting to note that in nursery E which had an additional non-grapevine year in 2015, the DNA concentrations remained similar to the previous two years except in the case of *Phythophthora* that showed an increase for 2015. It would seem that a 3 year crop rotation system in which the third year is a fallow period could hold some benefit. Even though pathogens were not isolated from canola and white mustard, nurseries B and C which planted these rotation crops had higher numbers of grapevines infected with black foot and root and crown rot pathogens.

It is also interesting to note that nursery A generally had low numbers of infected plants, but high levels of black foot pathogen DNA were detected across all years in soil, and high levels of *Phytophthora* and *Pythium irregulare* DNA were found in 2015. Soil analyses from this nursery revealed higher levels of phosphorous, potassium and manganese in this nursery compared to the other nurseries. It is possible that these levels of nutrients are more adequate for the growth of grapevines, leading to healthier nursery plants that are more resistant to pathogen infection, despite the presence of pathogens in the soil.

The soil characteristics determined by the laboratory tests were all considered to be within the normal ranges, except for boron in nurseries B and D and low resistance levels for the deeper layer of soil for nurseries A and D (Dr F. Ellis, personal communication). Therefore, stress as a result of nutrient deficiencies or salinity were deemed to be negligible. The soil wetness index is an important characteristic which indicates the potential of a soil to become waterlogged. The Wellington area is known for its low lying alluvial soils which are prone to wetness and also had higher soil wetness index than the Piketberg sampling area. Nursery E, in Piketberg is situated on the slopes of a hill and makes use of ridging as a method to improve soil drainage. The soil type and cultural practice of ridging most definitely would contribute to lower pathogen levels as measured in the soil and absence of pathogens in the vines of this nursery. Other factors that could also contribute to this could be that nursery E is situated in

an area that does not have a long history of grapevine nursery cultivation as well as the 3 year rotation system applied in this nursery. Another contributing factor could be due to the use of lupin as a rotation crop as these leguminous plants are known to produce phytochemicals known as saponins. Saponins are produced by many plant families as a deterrent for pest and pathogens and are released into the soil. The composition of the saponins produced by a plant affects how effectively a plant can respond to certain pest and pathogens (Moses *et al.*, 2014). It would be interesting to ascertain the effect of lupins on BFD and CRR pathogens and whether it would lower disease occurence.

It is difficult to ascertain the biological relevance of the DNA concentrations determined with qPCR. The question remains as to how soil DNA concentration will correlate to disease severity. Various factors also influence this equation and cannot be answered by only the presence of the pathogen in the soil. Disease will not necessarily develop with the host and pathogen present, but also needs specific environmental conditions. In the current scenario, conditions that would favour disease would be any form of stress that the plant might experience such as water logging or drought.

Nurseries are known to be sources of inoculum for black foot disease (Halleen *et al.* 2003, Agustí-Brisach *et al.*, 2014) and root and crown rot pathogens (Spies *et al.* 2011). All the grapevines that were sampled appeared healthy despite being infected with BFD and CRR pathogens. This confirms reports by Halleen *et al.* (2003) that symptomless plants harboured BFD pathogens. This study also highlights the need for fungal pathogen testing before grapevines are certified disease-free. The results of the current study confirm the presence of decline pathogens in the soils of grapevine nurseries in South Africa. Triticale and forage radish harboured grapevine pathogens and would need to be taken into account in decision making over which rotation crop to plant in nurseries with a history of black foot and crown and root rot. Furthermore, a more extensive study should be conducted on the suitability of different rotation crops currently used in grapevine nurseries in South Africa and especially the contribution of *Brassica* crops to biofumigation. In the absence of registered fungicides against BFD pathogens the only effective control method is hot water treatment of rooted grapevine plants (Halleen and Fourie, 2016). The fungicide fosetyl-Al is still the most effective means to control CRR pathogens, however, hot water treatment of nursery stock may also be beneficial. Proper water drainage systems such as ridging and avoidance of soil compaction should also be implemented.

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Table 1. Selected characteristics of the soil samples taken at 0-30 cm and 30-60 cm depths from five grapevine nurseries A-E.

Nursery	Soil texture	Depth (cm)	pH	Stones (%)	Carbon (%)	Resistance (Ohm)	P Bray II (mg/kg)	K (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	B (mg/kg)	Fe (mg/kg)	Clay (%)
A	Coarse sand	0-30	6,9	18	1	1050	808	114	9.56	37.5	42.0	0.41	188.61	6,18
A	Coarse sand	30-60	7,1	18	0,84	280	53	241	7.76	33.7	38.1	0.44	178.04	6,07
B	Coarse sand	0-30	6	6	0,66	3300	207	52	14.61	15.5	19.3	0.12	92.67	6,31
B	Coarse sand	30-60	6,1	6	0,66	1370	196	80	18.55	19.1	23.3	0.14	97.22	6,83
C	Loamy coarse sand	0-30	6,3	9	0,6	2610	188	54	3.34	40.6	16.4	0.25	269.73	10,46
C	Loamy coarse sand	30-60	6,2	11	0,74	1920	135	48	4.53	34.1	22.0	0.50	875.51	10,61
D	Coarse sand	0-30	6	11	0,74	620	117	56	4.53	21.4	25.9	0.20	76.47	5,43
D	Coarse sand	30-60	5,8	13	0,71	390	133	55	5.86	25.1	33.2	0.14	93.67	5,04
E	Loamy medium sand	0-30	6,6	1	0,22	1340	56	89	1.70	1.4	7.8	0.43	121.07	8,29
E	Loamy coarse sand	30-60	6,6	1	0,17	1340	44	78	1.31	1.1	5.7	0.65	88.69	8,04

Table 2. Species-specific primers used to amplify the partial beta-tubulin gene region for the detection of *Dactylonectria macrodidyma* complex, *Ilyonectria liriodendri*, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*.

Primer	Pathogen	Sequence (5'-3')	Tm (°C)	Amplicon size	Reference
CymaF1	*Dactylonectria macrodidyma* complex	CTGGGACATGATGGCTAATATGACTT	56.6	304bp	Mostert *et al.*, 2010, this study
CymaR2		GGTGGTGTGAGTTTCGTGC	56.7		Mostert *et al.*, 2010, this study
CyliF1	*Ilyonectria liriodendri*	CTCCTCTTCAACGATCCGACGTGCC	63.0	192bp	Mostert *et al.*, 2010, this study
CyliR1		GGGGCAGAGCAGATTTCG	56.2		Mostert *et al.*, 2010, this study
CafaF1	*Campylocarpon fasciculare*	CAACACAGCTCACGACAGCAG	58.7	350bp	Mostert *et al.*, 2010, this study
CafaR1		CTGTGTAGTCCAATATTAGTTGTTGTG	53.9		Mostert *et al.*, 2010, this study
CapsF1	*Campylocarpon pseudofasciculare*	AACACAGCTCGCGGCAGC	61.9	339bp	Mostert *et al.*, 2010, this study
CapsR1		GAGGCCTGTRTTGTGTAAGTTTAGTTGC	58.8		Mostert *et al.*, 2010, this study

Table 3. The number of plants infected by black foot and crown and root rot pathogens in five nurseries sampled in 2013, 2014 and 2015.

		Number of infected plants[1]														
		2013					2014[2]					2015				
		Nursery					Nursery					Nursery				
Disease	**Pathogen**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E[3]**
Crown and root rot	*Pythium irregulare*	-	1	-	-	-	-	-	4	4	4	3	2	3	2	10
	Pythium ultimum	-	-	-	-	-	-	-	2	1	-	-	2	-	1	1
	Pythium heterothallicum	-	-	-	-	-	-	-	-	2	-	-	2	10	1	1
	Pythium rostratum	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pythium sylvaticum	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
	Phytopythium helicoides	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-
	Phytopythium litorale	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-
	Phytopythium vexans	-	3	2	-	-	-	-	-	-	-	2	4	6	1	2
	Phytophthora niederhauserii	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Black foot	*Dactylonectria alcacerensis*	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-
	Dactylonectria macrodidyma	5	7	7	3	-	-	1	-	3	-	1	5	2	3	-
	Dactylonectria novozelandica	-	1	4	-	-	-	-	-	2	-	1	1	-	2	-
	Dactylonectria pauciseptata	-	3	-	-	-	-	-	-	2	-	-	2	-	1	-
	Dactylonectria torresensis	1	1	-	-	-	-	-	-	-	-	-	-	1	-	-
	Ilyonectria liriodendri	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-
	Campylocarpon fasciculare	-	3	2	1	-	-	-	-	-	-	2	6	2	1	-
	Campylocarpon pseudofasciculare	-	-	3	-	-	-	-	-	-	-	1	7	8	4	-

[1] At least 10 plant were sampled per field per year (approximately 50 plants per year)

[2] Rotation crops and weeds sampled in 2014. The rotation crops for nurseries: A, canola, white mustard; B, white mustard; C, canola, white mustard, forage radish; D, triticale and E, lupins.

[3] Nursery E uses a 3 year crop rotation system and as a result no grapevines were planted in 2015. Only weeds and rotation crops were sampled.

Table 4. Pathogens isolated from weeds and rotation crop species sampled in four of the grapevine nurseries in 2014.

Nursery	Site[1]	Plant	Common name	Genus and species	Family	Weed or rotation crop	Pathogen
B	9	3	corn spurry	*Spergula arvensis*	Caryophyllaceae	weed	*D. macrodidyma*
C	1	2	rye grass	*Lolium temulentum*	Poaceae	weed	*P. irregulare*
		5	winter grass	*Poa annua*	Poaceae	weed	*P. irregulare*
	2	3	forage radish	*Raphanus sativus*	Brassicaceae	rotation crop	*P. irregulare*
		4	ryegrass	*Lolium temulentum*	Poaceae	weed	*P. irregulare*
	4	1	Cape marigold	*Arctotheca calendula*	Asteraceae	weed	*P. ultimum var. ultimum*
		4	ryegrass	*Lolium temulentum*	Poaceae	weed	*P. ultimum*
D	1	1	Triticale	*x Triticosecale*		rotation crop	*D. pauciseptata*
	4	1	Triticale	*x Triticosecale*		rotation crop	*P. irregulare*
	5	2	Triticale	*x Triticosecale*		rotation crop	*P. irregulare* *D. pauciseptata*
		2	Triticale	*x Triticosecale*		rotation crop	*P. ultimum var. ultimum* *P. heterothallicum* *P. irregulare* *D. novozelandica*
	8	1	Triticale	*x Triticosecale*		rotation crop	*D. macrodidyma* *D. novozelandica*
	9	1	Triticale	*x Triticosecale*		rotation crop	*D. macrodidyma*
	10	1	Triticale	*x Triticosecale*		rotation crop	*P. irregulare* *P. heterothallicum* *D. macrodidyma*
E	3	1	Johnson grass	*Sorghum halepense*	Poaceae	weed	*P. irregulare*
		2	Unknown weed				*P. irregulare*
	6	1	Unknown weed			weed	*P. irregulare*
	8	1	Unknown weed			weed	*P. irregulare*

[1]In total ten sites were sampled per nursery field

Table 5. Mean *Dactylonectria* and *Ilyonectria*, *Pythium irregulare* and *Phytophthora* DNA concentrations in soil, determined with quantitative real-time PCR analyses of five grapevine nurseries sampled over three years.

		Mean soil DNA concentration (pg.µL^{-1})[1]		
Nursery	**Year**	***Dactylonectria* and *Ilyonectria***	***Pythium irregulare***	***Phytophthora***
A	2013	2.10[2] (1.13[3]d)[4]	0.07[2] (0.07[3]ef)[5]	0.11[2] (0.10[3] f)[6]
	2014	4.82 (1.76 bc)	0.04 (0.04 f)	1.45 (0.90 de)
	2015	10.14 (2.41 a)	0.83 (0.61 a)	10.47 (2.44 a)
B	2013	0.33 (0.29 ef)	0.01 (0.005 f)	0.17 (0.15 f)
	2014	0.49 (0.40 ef)	0.10 (0.09 ef)	2.45 (1.24 c)
	2015	6.04 (1.95 bc)	0.50 (0.41 bc)	4.96 (1.78 b)
C	2013	0.80 (0.59 e)	0.21 (0.19 de)	0.13 (0.12 f)
	2014	4.17 (1.64 c)	0.02 (0.02 f)	2.14 (1.14 cd)
	2015	6.01 (1.95 bc)	0.39 (0.33 cd)	7.74 (2.17 a)
D	2013	0.33 (0.29 ef)	0.08 (0.08 ef)	0.16 (0.15 f)
	2014	0.55 (0.44 ef)	0.04 (0.04 f)	0.92 (0.65 e)
	2015	6.38 (2.00 b)	0.64 (0.50 ab)	2.97 (1.38 c)
E	2013	0.30 (0.26 f)	0.08 (0.08 ef)	0.04 (0.04 f)
	2014	0.25 (0.23 f)	0.06 (0.06 ef)	0.17 (0.16 f)
	2015	0.28 (0.24 f)	0.07 (0.07 ef)	2.24 (1.18 cd)

[1] Values followed by the same letter do not differ significantly (P>0.05) according to Fisher's least significant difference test
[2] Back transformed DNA concentrations
[3] Log transformed DNA concentrations
[4] LSD for log transformed DNA concentrations = 0.3137
[5] LSD for log transformed DNA concentrations = 0.1454
[6] LSD for log transformed DNA concentrations = 0.3129

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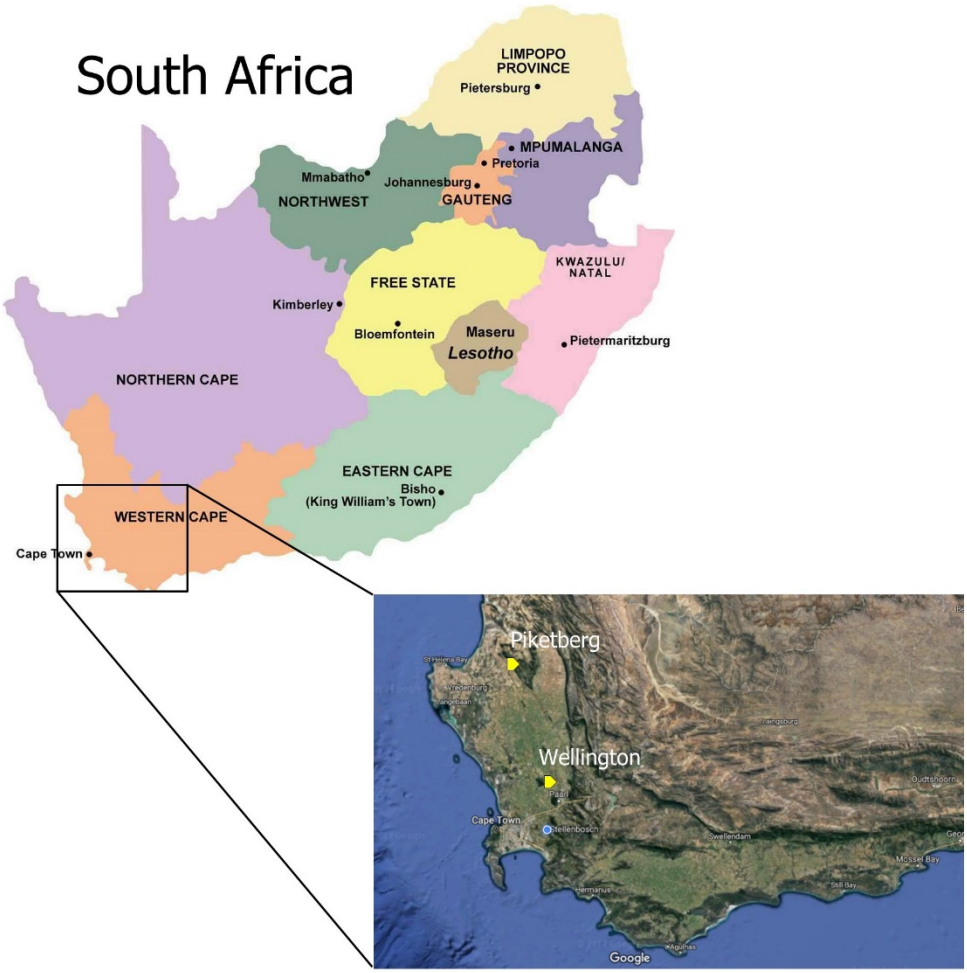

Figure 1. Sampling for rooted grapevine cuttings was done in Wellington (4 nurseries) and Piketberg (1 nursery), both in the Western Cape province of South Africa. Maps adapted from Wikipedia (Creative commons license) and Google Maps.

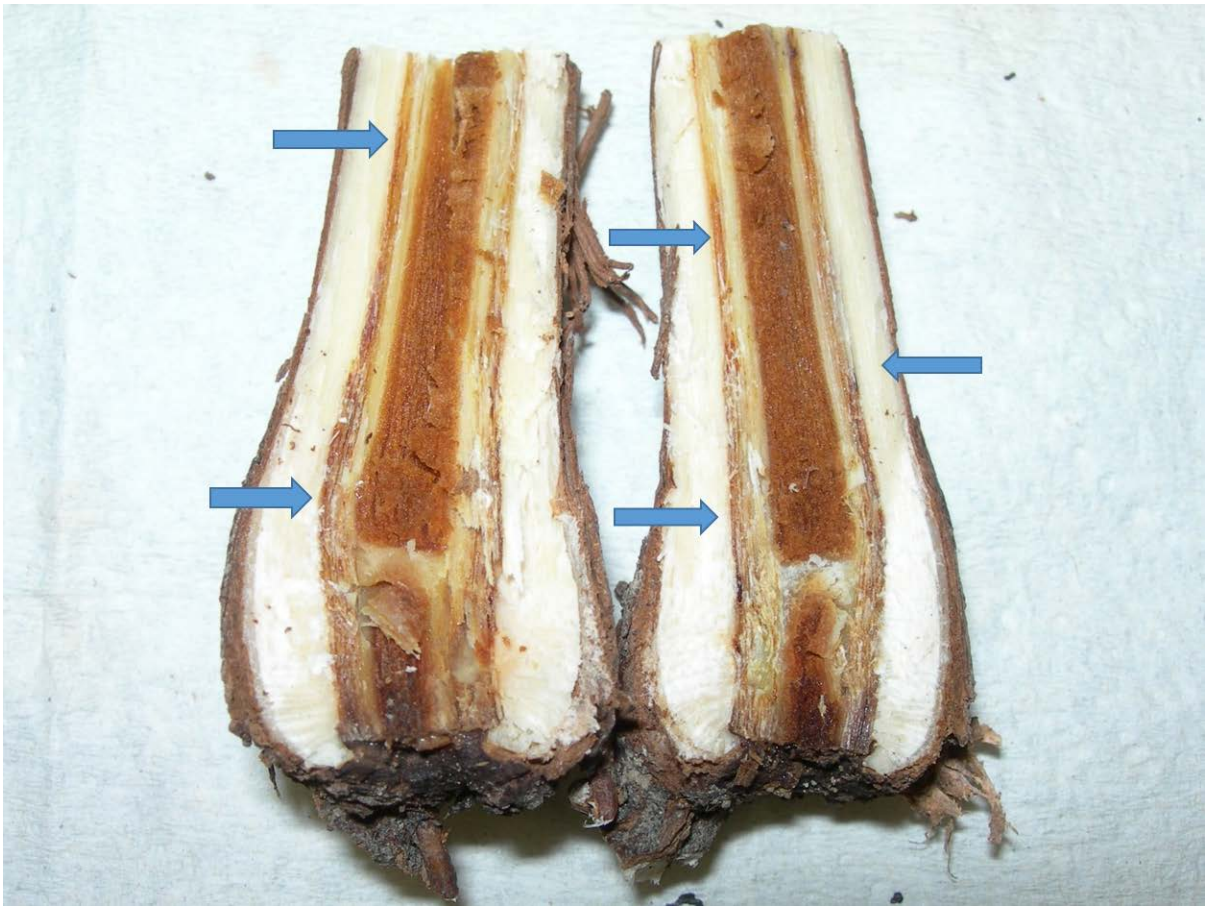

Figure 2. The sites in the grapevine rootstock basal end from which black foot disease pathogens were isolated (indicated by the arrows). Brown discoloration and streaking can be observed in the xylem tissue. Isolations were made onto potato dextrose agar amended with streptomycin.

ADDENDUM A

Table 1. Sequence similarities with published reference sequences obtained from GenBank for the specific species identifications. Sequence comparisons were made to histone H3 gene and ITS rDNA (ITS1-5.8S-ITS2) reference sequences in GenBank.

Species and isolate	Sequence identities (bp) and percentage similarity	Accession numbers
Dactylonectria alcacerensis (SL21-15)	456/462 (99%)	JF735630[1]
D. macrodidyma (SL23-15)	459/463 (99%)	JF735646[1]
D. novozelandica (SL73)	460/464 (99%)	JF735632[1]
D. pauciseptata (SL207-15)	441/443 (99%)	JF735582[1]
D. torresensis (SL252)	457/463 (99%)	JF735668[1]
Ilyonectria liriodendri	495/498 (99%)	KF512000[2]
Campylocarpon fasciculare (SL129)	476/476 (100%)	AY677298 [2]
Ca. pseudofasciculare (SL55)	494/500 (99%)	KF447567[2]
Phytophthora niederhauserii (SL239)	829/834 (99%)	JF900372[2]
Pythium irregulare (SL43-15)	973/985 (99%)	KU211360[2]
Py. ultimum (SL66-15)	877/882 (99%)	AB355596[2]
Py. heterothallicum (SL14-276)	729/730 (99%)	FJ415955[2]
Py. sylvaticum (SL52-15)	948/950 (99%)	KF806441[2]
Py. rostratum (SL268)	902/937 (96%)	AY598696[2]
*Phytopythium helicoides (*SL180)	695/698 (99%)	KT223574[2]
Pp. litorale (SL183-15)	826/838 (99%)	HQ643386[2]
Pp. vexans (SL70-15)	822/829 (99%)	HQ643400[2]

[1] histone H3 Genbank accessions
[2] ITS rDNA (ITS1-5.8S-ITS2) Genbank accessions

Table 2. The number of black foot disease pathogen and crown and root rot isolates obtained during the 3 year (2013-2015) nursery survey. Isolates originate from the roots and basal ends of 1-year-old grafted grapevines. In 2014, the isolations were made from rotation crops and weeds.

		Number of isolates per year			
Disease	**Species**	**2013**	**2014**	**2015**	**Total**
Black foot disease	*Dactylonectria alcacerensis*	1	0	1	**2**
	D. macrodidyma	59	4	12	**75**
	D. novozelandica	7	2	4	**13**
	D. pauciseptata	4	3	5	**12**
	D. torresensis	4	0	3	**7**
	Ilyonectria liriodendri	2	0	1	**3**
	Campylocarpon fasciculare	6	0	16	**22**
	Ca. pseudofasciculare	3	0	30	**33**
Crown and root rot	*Pythium irregulare*	1	15	30	**46**
	Py. ultimum	0	4	5	**9**
	Py. heterothallicum	0	0	4	**4**
	Py. rostratum	1	0	0	**1**
	Py. sylvaticum	0	0	2	**2**
	Phytopthium helicoides	5	0	0	**5**
	Pp. litorale	6	0	0	**6**
	Pp. vexans	5	0	29	**34**
	Phytophthora niederhauserii	1	0	0	**1**

Table 3. *Ilyonectria* and *Dactylonectria* mean DNA concentrations in grapevine soil at 0-30 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds).

	Mean DNA concentration (pg.ul^{-1})				
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	5.26[1]	0.39	0.6	0.12	0.07
2	15.32	0.41	0.94	0.04	0.3
3	1.59	0.35	2.78	0.04	0.05
4	0.00	0.65	1.57	0.09	0.13
5	37.14	0.28	0.6	0.64	0.04
6	0.62	0.19	0.36	3.02	0.5
7	0.80	0.09	1.18	0.88	0.37
8	9.75	0.16	0.66	0.33	1.43
9	0.67	0.49	0.44	0.19	0.05
10	0.85	0.70	0.65	0.36	0.06
Mean[2]	**7.20 a ± 11.66[3]**	**0.37 b ± 0.20**	**0.98 b ± 0.73**	**0.57 b ± 0.90**	**0.30 b ± 0.28**
2014					
1	5.99	0.64	1.81	0.5	0.35
2	4.22	0.53	2.05	0.54	0.32
3	4.8	0.38	4.27	0.43	0.1
4	7.14	0.47	3.79	1.22	0.53
5	12.65	0.39	17.24	1	0.12
6	7.04	0.63	11.39	0.61	0.08
7	7.73	0.4	3.22	2.15	0.24
8	6.08	1.26	8.82	0.06	0.25
9	3.28	0.95	11.02	0.42	0.39
10	4.88	1.32	21.11	1.11	0.74
Mean[4]	**6.38 a ± 2.61**	**0.70 b ± 0.36**	**8.47 a ± 6.70**	**0.80 b ± 0.59**	**0.31 b ± 0.21**
2015					
1	7.67	3.38	4.82	6.36	0.18
2	11.5	5.15	4.85	4.28	0.34
3	21.53	17.59	4.95	3.14	0.13
4	8.75	4.94	10.69	6.6	0.27
5	4.6	3.17	5.63	5.31	0.12
6	20.99	3.89	8.11	5.69	0.27
7	21.02	5.14	6.77	3.76	0.09
8	11.92	3.54	5.37	8.76	0.13
9	8.02	3.39	3.74	15.71	0.78
10	6.71	3.95	4.91	4.7	0.73
Mean[5]	**12.27 a ± 6.50**	**5.41 b ± 4.34**	**5.98 b ± 2.05**	**6.43 b ± 3.64**	**0.30 c ± 0.25**

[1] Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.544
[3] Standard deviation calculated from means of the 10 sites.
[4] LSD = 0.3618
[5] LSD = 0.334

Table 4. *Ilyonectria* and *Dactylonectria* mean DNA concentrations in grapevine soil at 30-60 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds).

Mean DNA concentration (pg.ul^{-1})					
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	8.01[1]	0.18	1.04	0.04	0.48
2	0.39	0.37	0.58	0.18	0.42
3	0.3	0.74	1.46	0.09	0.06
4	0.43	0.24	0.58	0.27	0.2
5	1.54	0.13	1.14	0.22	0.06
6	1.66	0.54	0.27	0.12	0.41
7	1.82	0.29	1.12	1.04	0.05
8	3.44	0.35	0.93	0.37	0.64
9	1.07	0.12	0.18	0.08	1.51
10	0.65	0.2	0.42	0.3	0.08
Mean[2]	**1.93 a ± 2.34[3]**	**0.32 bc ± 0.20**	**0.77 b ± 0.43**	**0.27 c ± 0.29**	**0.39 bc ± 0.45**
2014					
1	3.84	0.35	0.9	0.31	0.3
2	2.66	0.53	1.01	0.45	0.34
3	2.27	0.16	6.45	0.28	0.04
4	6.18	0.3	1.78	0.6	0.26
5	6.01	0.37	4.69	0.34	0.3
6	3.7	0.13	1.7	0.72	0.11
7	4.25	0.19	2.4	1.12	0.12
8	2.02	0.21	1.5	0.05	0.23
9	6.75	0.49	5.66	0.13	0.15
10	2.99	0.63	3.99	0.21	0.27
Mean[4]	**4.07 a ± 1.71**	**0.34 c ± 0.17**	**3.01 b ± 2.03**	**0.42 c ± 0.32**	**0.21 c ± 0.10**
2015					
1	7.37	11.49	7.41	15.55	0.16
2	15.18	3.45	5.64	1.62	0.25
3	23.1	13.46	7.76	3.18	0.12
4	26.76	5.67	7.85	5.99	0.16
5	23.23	5.45	8.55	3.12	0.15
6	0	5.79	6.69	9.63	0.14
7	12.13	3.94	7.9	11.73	0.1
8	11.73	13.33	3.22	3.17	0.05
9	6.53	9.09	7.07	27.25	1.29
10	4.4	13.5	3.24	11.17	0.64
Mean[5]	**13.04 a ± 8.94**	**8.52 a ± 4.13**	**6.53 a ± 1.91**	**9.24 a ± 7.85**	**0.31 b ± 0.38**

[1]Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.3009
[3] Standard deviation calculated from means of the 10 sites.
[4] LSD = 0.2643
[5] LSD = 0.5558

Table 5. *Pythium irregulare* mean DNA concentrations in grapevine soil at 0-30 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds).

Mean DNA concentration (pg.ul^{-1})					
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	0.14[1]	0.05	0.18	0.14	0.61
2	0.3	0	0.45	0	0.05
3	0	0	0.19	0.03	0.04
4	0	0.04	0.1	0.17	0.05
5	0	0	0.16	0.14	0.03
6	0	0	0.03	0.07	0.07
7	0.14	0	0.12	0.15	0.04
8	0	0	0.13	0.11	0.04
9	0.15	0	0.02	0	0.09
10	0.19	0.01	0.59	0	0.08
Mean[2]	**0.09 ab ± 0.11[3]**	**0.01 b ± 0.02**	**0.20 a ± 0.18**	**0.08 b ± 0.07**	**0.11 ab ± 0.18**
2014					
1	0.02	0.13	0.02	0.04	0.06
2	0.01	0.1	0.05	0	0.24
3	0.01	0.06	0.02	0.06	0.01
4	0.15	0.1	0.01	0.03	0.06
5	0.03	0.06	0.01	0.02	0.01
6	0.01	0.57	0.01	0.09	0.03
7	0.03	0.11	0.01	0.04	0.02
8	0.31	0.11	0	0.01	0.05
9	0.05	0.23	0.04	0.01	0.09
10	0.03	0.01	0.03	0.02	0.19
Mean[4]	**0.07 b ± 0.10**	**0.15 a ± 0.16**	**0.02 b ± 0.02**	**0.03 b ± 0.03**	**0.08 ab ± 0.08**
2015					
1	0.81	1.28	0.42	0.27	0.08
2	0.56	0.32	0.37	1.06	0.15
3	1.62	1.2	0.35	0.1	0.07
4	0.43	0.34	0.57	0.2	0.18
5	0.74	0.22	0.55	1.02	0.02
6	1.07	0.47	0.15	0.19	0.06
7	0.84	0.26	0.5	1.25	0.07
8	0.99	0.07	0.11	0.09	0.1
9	2.2	0.24	0.11	2.33	0.11
10	0.98	0.04	0.05	0.14	0.03
Mean[5]	**1.02 a ± 0.52**	**0.44 b ± 0.44**	**0.32 bc ± 0.20**	**0.67 b ± 0.74**	**0.09 c ± 0.05**

[1]Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.0915
[3] Standard deviation calculated from means of the 10 sites.
[4] LSD = 0.0677
[5] LSD = 0.2237

Table. 6. *Pythium irregulare* mean DNA concentrations in grapevine soil at 30-60 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds)

Mean DNA concentration (pg.ul^{-1})					
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	0.15[1]	0	0.3	0.03	0.01
2	0	0	0.88	0.06	0.16
3	0	0	0.27	0.01	0.01
4	0.06	0.01	0.14	0.26	0.1
5	0.06	0	0.11	0.12	0.06
6	0	0	0.07	0.04	0.03
7	0.02	0	0.17	0	0.01
8	0.06	0	0.15	0.23	0.03
9	0.18	0	0.17	0.01	0.16
10	0.04	0	0.21	0.03	0
Mean[2]	**0.06 b ± 0.06[3]**	**0.001 b ± 0.0032**	**0.25 a ± 0.23**	**0.08 b ± 0.09**	**0.06 b ± 0.06**
2014					
1	0.07	0.01	0.01	0.06	0.06
2	0.01	0.14	0.02	0.01	0.15
3	0.01	0.06	0.02	0.16	0.01
4	0.06	0.03	0.01	0.01	0.03
5	0.02	0.12	0.02	0.04	0.07
6	0.01	0	0.08	0.13	0.02
7	0.01	0.12	0.02	0.02	0.01
8	0.01	0.03	0	0.02	0.02
9	0.05	0.01	0.08	0.05	0.05
10	0.03	0.06	0.04	0.04	0.11
Mean[4]	**0.03 a ± 0.02**	**0.058 a ± 0.05**	**0.03 a ± 0.03**	**0.05 a ± 0.05**	**0.05 a ± 0.05**
2015					
1	0.15	1.83	1	1.54	0.06
2	1.51	0.19	0.61	0.18	0.04
3	3.77	0.87	0.89	0.34	0.08
4	1.58	0.4	0.82	0.09	0.12
5	0.8	0.71	0.78	0.27	0.04
6	0	0.18	0.12	1	0.01
7	0.13	0.31	0.54	2.4	0.03
8	0.57	0.21	0.1	0.06	0.02
9	0.42	1	0.28	3.76	0.02
10	0.29	1.17	0.09	0.36	0.08
Mean[5]	**0.92 a[3] ± 1.14**	**0.687 a ± 0.54**	**0.52 a ± 0.35**	**1.00 a ±1.23**	**0.05 b ± 0.03**

[1]Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.0798
[3] Standard deviation calculated from means of the 10 sites.
[4] LSD = 0.036
[5] LSD = 0.3266

Table 7. *Phytophthora* mean DNA concentrations in grapevine soil at 0-30 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds).

Mean DNA concentration (pg.ul^{-1})					
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	0.2[1]	0.16	0.09	0.19	0.01
2	0.04	0.17	0.28	0.01	0.05
3	0.05	0.12	0.07	0.07	0.02
4	0	0.12	0.05	0.1	0.05
5	0.01	0.13	0.04	0.1	0.01
6	0	0.05	0.02	0.2	0.11
7	0.1	0.04	0.09	0.23	0.05
8	0.01	0.2	0.09	0.22	0.07
9	0.29	0.01	0.06	0.24	0.02
10	0.32	0.03	0.13	0.29	0.02
Mean[2]	**0.10 ab ± 0.12[3]**	**0.10 ab ± 0.07**	**0.09 ab ± 0.07**	**0.17 a ± 0.09**	**0.04 b ± 0.03**
2014					
1	1.41	1.88	1.34	2.5	0.28
2	1.82	2.32	2.19	1.34	0.42
3	1.48	1.53	6.03	0.9	0.11
4	1.25	1.11	1.41	1.91	0.11
5	1.25	1.55	3.14	1.1	0.16
6	1.85	4.83	1.73	1.75	0.04
7	3.74	1.77	1.92	1.01	0.14
8	2.76	6	9.15	0.25	0.08
9	0.55	7.04	0.59	0.07	0.19
10	0.79	1.34	1.06	0.06	0.36
Mean[4]	**1.69 ab ± 0.94**	**2.94 a ± 2.17**	**2.86 a ± 2.69**	**1.09 b ± 0.82**	**0.19 c ± 0.13**
2015					
1	11.02	2.38	6.1	2.91	2.63
2	17.87	2.95	16.6	3.58	2.83
3	24.75	8.42	5.83	2.27	1.59
4	9.85	3.11	10.17	2.04	3.49
5	20.34	2.38	6.18	1.51	0.57
6	6.27	3.66	9.15	4.29	3.81
7	5.15	5.26	5.14	3.73	1.52
8	12.02	2.75	3.1	6.5	12.56
9	33.48	12.37	4.83	5.82	0.83
10	18.58	14.97	3.35	3.3	1.11
Mean[5]	**15.93 a ± 8.83**	**5.83 bc ± 4.56**	**7.05 b ± 4.03**	**3.60 cd ± 1.60**	**3.09 d ± 3.51**

[1]Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.0647
[3] Standard deviation calculated from means of the 10 sites
[4] LSD = 0.3725
[5] LSD = 0.467

Table 8. *Phytophthora* mean DNA concentrations in grapevine soil at 30-60 cm depth for the years 2013 (grapevines), 2014 (rotation crop and weeds) and 2015 (grapevines with the exception of Nursery E with weeds).

Mean DNA concentration (pg.ul^{-1})					
Site	**Nursery A**	**Nursery B**	**Nursery C**	**Nursery D**	**Nursery E**
2013					
1	0.07[1]	0.09	0.31	0.09	0.02
2	0.01	0.25	0.16	0.09	0.04
3	0.07	0.72	0.14	0.16	0.02
4	0.09	0.3	0.15	0.22	0.09
5	0.22	0.03	0.11	0.11	0.07
6	0	0.29	0.12	0.14	0.01
7	0.16	0.27	0.23	0.26	0.03
8	0.1	0.27	0.29	0.07	0.02
9	0.38	0.07	0.09	0.05	0.03
10	0.11	0.16	0.19	0.41	0
Mean[2]	**0.12 bc ± 0.11[3]**	**0.25 a ± 0.19**	**0.18 ab ± 0.08**	**0.16 ab ± 0.11**	**0.03 c ± 0.03**
2014					
1	1.09	1.19	0.99	1.74	0.08
2	1.99	3.29	1.13	0.91	0.46
3	1.58	1.64	5.46	2.93	0.09
4	1.82	3.88	2.19	0.64	0.12
5	1.43	5.07	3.44	0.39	0.31
6	1.1	1.02	3.29	3.25	0.1
7	1.84	3.12	1.75	1.36	0.05
8	1.84	1.89	1.34	0.11	0.1
9	0.82	2.91	2.88	0.11	0.07
10	0.48	1.99	0.46	0.06	0.22
Mean[4]	**1.40 bc ± 0.51**	**2.60 a ± 1.29**	**2.29 ab ± 1.50**	**1.15 c ± 1.17**	**0.16 d ± 0.13**
2015					
1	5.88	3.54	7.3	2.67	1.18
2	6.1	1.94	7.57	1.4	1.54
3	17.97	11.49	18.13	1.45	0.97
4	11.54	2.18	8.86	2.3	3.47
5	6.95	3.03	5.76	2.2	24.2
6	0.01	2.54	15.9	3.8	5.07
7	7.3	3.17	17.19	3.03	0.92
8	5.15	4.78	10.59	2.25	0.4
9	19.33	16.81	3.54	3.5	1.99
10	29.53	23.13	9.36	4.97	0.67
Mean[5]	**10.98 a ± 8.80**	**7.26 ab ± 7.39**	**10.42 a ± 5.01**	**2.76 bc ± 1.10**	**4.04 c ± 7.23**

[1]Mean of two biological repeat soil samples and two technical qPCR repeats.
[2] Least Significant difference (LSD) = 0.0647
[3] Standard deviation calculated from means of the 10 sites.
[4] LSD = 0.3271
[5] LSD = 0.6349

CHAPTER 3

Phytopythium paucipapillatum sp. nov. isolated from a nursery grapevine in South Africa

ABSTRACT

Phytopythium species (Peronosporales) are known to occur in various soil and aquatic habitats. Certain species are also known to occur in agricultural systems as plant pathogens. In 2013, six *Phytopythium* isolates were isolated from asymptomatic nursery grapevine roots and were characterised phylogenetically and morphologically. Phylogenetic analysis based on the internal transcribed spacer-ribosomal DNA (ITS-rDNA) and cytochrome oxidase subunit 1 (COI) gene regions clearly distinguished these isolates, together with a Japanese isolate, as a new species. *Phytopythium paucipapillatum* displayed occasionally papillate, direct sporangial germination as well as sporangia which proliferates internally. The sporangia were globose, subglobose, ovoid, obovoid, limoniform to ellipsoid or distorted. The oogonia were small, globose, with some oogonia also displaying short papillae protruding from the surface. This species is also insensistive to the fungicide hymexazol, which is used in selective media to suppress the growth of *Pythium* species. A pathogenicity trial was conducted on the grapevine rootstock 110-Richter. The trial results revealed that this species is non-pathogenic towards rootstock 110-Richter. This species may therefore be a saprophytic soil inhabitant.

INTRODUCTION

The genus *Phytopythium* Abad, de Cock, Bala, Robideau, Lodhi and Lévesque (Pythiaceae), was first described in 2010, with *P. sindhum* A.M. Lodhi, Shahzad and Lévesque as the type species (Bala *et al.*, 2010b). This genus comprises 20 species, of which 16 were previously assigned to the genus *Pythium* Pringsheim (de Cock *et al.*, 2015), one species to the genus *Phytophthora* De Bary (Baten *et al.*, 2014) and another to the marine genus, *Halophytophthora* H.H. Ho and S.C. Jong (Marano *et al.*, 2014; Thines, 2014). *Phytopythium* has been described as an intermediate genus between *Pythium* and *Phytophthora*, having morphological traits similar to both (de Cock *et al.*, 2015).

The morphological characteristics of the *Phytopythium* genus include papillate and internally proliferating sporangia and zoospore discharge by vesicle formation. Species in this genus typically also have globose to ovoid sporangia as well as laterally attached or broadly apical antheridia. Unlike *Phytophthora* species, internal proliferation and sporangial papillation does not occur on the same sporangium in *Phytopythium* species (de Cock *et al.*, 2015). The

internally proliferating sporangia and vesicular zoospore formation are traits shared with *Phytophthora* and *Pythium*, respectively.

Most species in the genus *Phytopythium* were formerly placed in the genus *Pythium* clade K (Lévesque and de Cock, 2004). Several phylogenetic studies have suggested that this clade be segregated to a new genus. Based on a study using ribosomal large subunit (LSU), Briard *et al.* (1995) determined that *Phytopythium vexans* (de Bary) Abad, de Cock, Bala, Robideau, Lodhi and Lévesque was distinct from both *Pythium* and *Phytophthora*. Similarly, a phylogenetic study based on the internal transcribed spacer (ITS) rDNA region of several oomycete genera, conducted by Cooke *et al.* (2000), demonstrated that *P. vexans* formed a separate clade. Villa *et al.* (2006) also showed that the clade K species were closely related to, but distinct from *Phytophthora*, based on ITS, mitochondrial cytochrome oxidase subunit 2 (COII) and β-tubulin gene phylogenies. Several more studies have recognised the need to separate clade K from the genus *Pythium* (Panabières *et al.*, 1997; Dick, 2001; Lévesque and de Cock, 2004; Bedard *et al.*, 2006; Belbahri *et al.*, 2008; McLeod *et al.*, 2009; Hyde *et al.*, 2014). The circumscription of the genus *Phytopythium* was completed using phylogenies based on the ITS, cytochrome oxidase subunit 1 (COI), and nuclear ribosomal large and small subunit (LSU and SSU) (de Cock *et al.*, 2015). An extensive study by Uzuhashi *et al.* (2010) attempted to reclassify *Pythium* species according to sporangial morphology and phylogenies based on the LSU and COII gene regions. This study placed the Pythium clade K species into the genus *Ovatisporangium*, however, due to the earlier generic description by Bala *et al.* (2010b), *Phytopythium* receives nomenclatural precedence over *Ovatisporangium*, which is regarded as a synonym (de Cock *et al.*, 2015).

Most *Phytopythium* species survive as saprophytes in water and soil environments (Marano *et al.*, 2016). Several species are known to be phytopathogenic, with species such as *P. oedochilum* (Drechsler) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque, *P. helicoides* (Drechsler) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque and *P. vexans* having broad plant host ranges (Van der Plaats-Niterink, 1981). Together with *Pythium* and *Phytophthora* species, certain *Phytopythium* species have been associated with grapevine decline (Spies *et al.*, 2011). This soilborne disease of grapevines is characterised by root and crown rot lesions, reduced root biomass and leads to plant stress, which predisposes the plant to infection by other soilborne and grapevine trunk fungal pathogens (Spies *et al.*, 2011). *Phytopythium vexans*, *P. helicoides*, *P. litorale* (Nechw.) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque, *P. chamaehyphon* (Sideris) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque and *P. mercuriale* (Belbahri, B. Paul and Lefort) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque have previously been isolated from diseased nursery and vineyard grapevines, with *P. vexans* confirmed as a pathogen when inoculated onto healthy, potted

vines (Spies *et al.*, 2011). In addition to the aforementioned species, *P. oedochilum* and the undescribed clade K species *Pythium* sp. WJB-3 has also been reported on *Cynara cardunculus and Persea americana* in South Africa (McLeod *et al.*, 2009).

During a grapevine nursery survey in 2013, six isolates of an unknown *Phytopythium* species were isolated from roots of visually healthy nursery vines. The aim of this study was to describe this putative new *Phytopythium* species and to determine whether it is pathogenic towards grapevine rootstock 110-Richter.

MATERIALS AND METHODS

Sampling

The sampling was conducted in 2013, in a grapevine nursery in the Wellington area (Western Cape Province, South Africa). Rooted grapevine cuttings were sampled in a zig-zag ('W') pattern across an open field nursery. Isolations were made from symptomless roots of 1-year-old cuttings and plated onto PARP medium amended with 0.4 $g.L^{-1}$ Switch fungicide (cyprodinil 375 $g.kg^{-1}$ and Fludioxonil 250 $g.kg^{-1}$, Syngenta, South Africa) and PARPH (Jeffers and Martin, 1986). These plates were incubated at 25°C for 3 days. Any growth was transferred to potato dextrose agar (PDA) and incubated at 25°C for one week. The isolates were hyphal tipped twice to obtain axenic cultures. Isolates were stored in the Stellenbosch University culture collection (STE-U) on corn meal agar (CMA) slants and bottles containing sterile water and citrus leaf pieces.

Morphological observation

The *Phytopythium* isolates (STE-U 7843-7848 and MAFF241149) were cultured on corn meal agar (CMA) containing ß-sitosterol (30 $mg.L^{-1}$) and incubated for one week at 25°C. In addition, the isolates were grown in sterile soil extract (SSE) (Jeffers and Aldwinckle, 1987) containing grass blades (*Pennisetum clandestinum* Hochst. ex Chiov) and hemp seeds (*Cannabis sativa* L.) (de Cock and Lévesque, 2004), and incubated for three days at room temperature. Observations and measurements of morphological structures were made using differential interference contrast (DIC) microscopy (Nikon Eclipse Ni, Nikon South Africa) at 400X and 1000X magnification. The biometric indices were calculated according to Shahzad *et al.* (1992). The monograph by Van der Plaats-Niterink (1981) and the keys of Dick (1990) were consulted for morphological descriptions.

Growth studies were conducted on all isolates including an isolate of *Ovatisporangium* sp. 5 (MAFF241149) from Uzuhashi *et al.* (2010) that had high internal transcribed spacer (ITS) sequence similarity to the South African *Phytopythium* isolates (see Results). The

isolates were sub-cultured onto potato carrot agar (PCA) and CMA plates and incubated at temperatures ranging from 5°C to 35°C at 5° intervals. Colony radii were measured, using a digital caliper (Mitutoyo corp., Japan), at 24 hour intervals for three consecutive days.

Genomic DNA extraction, PCR and sequencing

DNA extraction

DNA was extracted from fungal mycelia in pure culture using a modified CTAB DNA extraction protocol by Lee *et al.* (1990). The modifications were as follows: harvested mycelia were disrupted by adding 0.5 g of glass beads and shaking at 30 Hz in a Retsch MM400 mixer/miller (Retsch, GmbH and Co., Haan, Germany) for 5 minutes. Two chloroform-isoamyl alcohol steps were done instead of one to enhance the purification of the DNA. The DNA concentrations were determined using a NanoDrop UV spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

PCR and Sequencing

Polymerase chain reaction (PCR) amplification of the ITS-rDNA (ITS1-5.8S-ITS2) and COI gene regions were conducted. For the ITS PCR, the primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990) and ITS6 (5'-GAA GGT GAA GTC GTA ACA AGG-3') (Cooke and Duncan, 1997) were used. Each reaction (40 µL) of the ITS PCR consisted of 1x NH_4 buffer (Bioline, USA Inc., Taunton, MA), 2.5 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche, Switzerland), 0.2 mM of each dNTP, 0.2 µM of each primer, 0.5 U of BIOTAQ (Bioline) and 50 ng of DNA. The PCR was conducted in an Applied Biosystems 2720 thermal cycler with an initial denaturation temperature of 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 50°C for 1 min, 72°C for 90 s and a final extension step at 72°C for 5 min. The COI PCR was conducted using the primer pair OomCOILevup (5'-TCA WCW MGA TGG CTT TTT TCA AC-3' and FM85mod (5'- RRH WAC KTG ACT DAT RAT ACC AAA-3') (Bala *et al.*, 2010a). Each 40 µL reaction consisted 1x NH_4 buffer (Bioline), 3.5 mM $MgCl_2$ (Bioline), 0.2 mg BSA Fraction V (Roche), 0.1 mM of each dNTP, 0.1 µM of each primer, 0.5 U of BIOTAQ (Bioline) and 50 ng of DNA . PCR cycling consisted of 94°C for 5 min followed by 32 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s and a final extension step at 72°C for 7 min. The PCR products were run on a 1.5% agarose gel stained with ethidium bromide followed by a post-PCR clean-up using the MSB Spin PCRapace kit (STRATEC Molecular GmbH, Berlin, Germany). For both gene regions the PCR product was sequenced in the forward and reverse directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

All the samples were sequenced on an ABI 3130XL Genetic Analyser by the Central Analytical Facilities at Stellenbosch University. The resulting ITS and COI sequences were aligned and consensus sequences were generated using Geneious 9.1.4 (http://www.geneious.com) (Kearse *et al.*, 2012). The consensus sequences were then compared to sequences on GenBank using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were deposited in GenBank with accession numbers KX372742-KX372748 for the COI sequences, and KX372749-KX372754 for the ITS sequences (Table 1).

Phylogenetic analysis

Phytopythium sequences were downloaded from GenBank for use in phylogenetic analysis (Table 1). No COI sequences were available for *P. palingenes* ((Drechsler) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque) and *P. polytylum* (Drechsler) Abad, De Cock, Bala, Robideau, Lodhi and Lévesque, as well as no ITS sequences for *P. polytylum,* and these were thus excluded from phylogenetic analyses. Also, the invalid taxa *P. megacarpum*, *P. sterile*, and *P. cucurbitacearum*, as well as *P. indigoferae* were excluded from our analyses (de Cock *et al.*, 2015).

The ITS and COI consensus sequences of the putative new *Phytopythium* species were aligned with *Phytopythium* reference sequences obtained from GenBank using the L-INS-i alignment method of the MAFFT (v7.299) online alignment tool (Katoh and Toh, 2008). These sequences were subjected to maximum parsimony analysis using PAUP* (v4.0b10) (Swofford, 2003) as well as Bayesian analysis. *Phytophthora batemanensis* Gerr.-Corn. and J.A. Simpson and *Phytophthora boehmeriae* Sawada served as outgroups. The parsimony analysis was conducted by using 100 random replicate additions. Tree bisection and reconstruction (TBR) was used as the branch swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5. All characters were of equal weight and of no specific order. Bootstrap values were determined from 1000 heuristic search replicates and 10 random taxon additions. Additional parsimony measures were calculated which include the retention index (RI), the rescaled consistency index (RC) values, consistency index (CI) and tree length (TL).

Bayesian analysis was performed in PhyloBayes version 4.1 (Lartillot *et al.*, 2009). Two independent chains were run under the non-parametric, CAT-GTR mixture model (Lartillot and Phillipe, 2004) for 5000 cycles. Convergence was assessed using the tracecomp and bpcomp commands, and found to be acceptable (maxdiff < 0.3, effective size > 50) when discarding the first 1000 cycles as burn-in.

Pathogenicity trial

Five isolates of the putative new *Phytopythium* sp. (STE-U 7843– 7847) were grown on PDA plates for two weeks at 25°C. Pearled wheat (*Triticum aestivum* L.) (100 g) was soaked in 100 mL deionised water overnight, after which it was autoclaved on two consecutive days at 121kPa for 20 minutes. The autoclaved pearled wheat was then inoculated with agar blocks cut from the two-week old cultures of the putative new species. The inoculated pearled wheat was incubated at 25°C for three weeks and shaken intermittently to ensure even colonisation of the wheat grains.

Rooted 110-Richter rootstocks were subjected to hot water treatment (50°C for 30 min) and planted in a pasteurised potting soil and perlite mixture (3:1) (Master Organics, Cape Town) in 4.5 L planting bags. The plants were kept in a greenhouse at 26°C and were watered twice daily by drip irrigation. After the first shoots emerged, one shoot was retained per plant and its length was measured. The plants were then inoculated by adding the colonised pearled wheat to the soil (2% w/v). The control plants were inoculated with sterile pearled wheat only. The plants were left to grow for eight months. Plant fertilisers Nutrifeed (Starke Ayres, Johannesburg) and Seagro (Seagro, Cape Town) were administered alternatively on a weekly basis. The trial was conducted using a randomised block design and seven replicates were included for each isolate including the controls.

After eight months, the trial was evaluated by measuring all the shoot lengths for each treatment. The roots were inspected for the presence of any necrotic lesions. Re-isolations were made from the roots of all the plants to fulfil Koch's postulates. The pathogenicity results were analysed using a one-way analysis of variance (ANOVA) (Statistica v13, Dell Software, Texas, USA) at a 95% confidence interval. The results ere analysed for significant differences using Student's L.S.D. T-test.

RESULTS

Isolates

In total, six *Phytopythium* isolates displaying rosaceous growth on PDA were obtained from asymptomatic roots of a single plant in the open field nursery. Several isolates of *P. litorale* and *P. helicoides* were also obtained from the same plant. The type strain (STE-U 7843) of the new *Phytopythium* species was also deposited in the culture collection of the Centraal Bureau vir Schimmelcultures (CBS accession number pending) in the Netherlands. An isolate (MAFF241149, *Ovatisporangium* sp. 5) which, upon BLAST analysis, displayed high sequence homology to the six grapevine isolates was obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank, Ibaraki, Japan.

Taxonomy

Phytopythium paucipapillatum S.D. Langenhoven, W.J. Botha, and L. Mostert, sp. nov.

MycoBank MB819417, Fig 1., Table 2.

Etymology

Latin. The specific epithet refers to the sparsely papillated sporangia and oogonia

Main hyphae up to 5 µm wide. Sporangia mostly apapillate germinating directly, some papillate, internally proliferating with extended proliferation. Sporangia globose, subglobose, ovoid, obovoid, limoniform to ellipsoid or distorted shapes 15–34 µm diam, most 19–25 µm diam. Position of papilla apical or subapical close to sporangiophore, 4-5 µm. Sporangia apical, unilaterally intercalary or perpendicular on sporangiophore, some sporangia clustered in groups of 3–5 at apex of sporangiophore, connected by short hyphal segments. Discharge tubes 3.7–5 µm wide, 7.5–11 µm long. Zoospores differentiated extrasporangially in an ephemeral vesicle. Zoospores biflagellate. Zoospore cysts spherical 9–11 µm diam. Hyphal swellings not observed. Oogonia small globose terminal, intercalary, some unilaterally intercalary, (18–)20–23(–26) (av. 22) µm diam, some oogonia ornamented with one to three short, blunt papillae. Antheridia up to three per oogonium, mostly monoclinous, or occasionally diclinous at a distance. Antheridia applied lengthwise to oogonium wall with a central fertilisation tube, antheridial cell 4-5 x 11-20 µm with an undulating contour and one to several constrictions; some antheridia applied broadly apical to oogonium. Oospores plerotic or nearly so, (14–)18–20(–23) (av. 20) µm diam. Occasionally two oospores per oogonium. Oospore wall thickness 0.9-1.9 µm. Ooplast 7-13 µm diam. Aplerotic index 76.9%, ooplast index 54.4%, oospore wall index 45.2%

Colony characteristics – colony growth pattern on PDA and PCA rosaceous, CMA slight aerial mycelium with coarsely radiate pattern and numerous micro tufts of aerial mycelium (Fig. 2). Grows on PARP and PARPH selective media. Cardinal temperatures - minimum 10°C, optimum 25°C, maximum 30°C on PCA. Average growth rate at the optimum temperature was 8.55 mm/day for the STE-U isolates and 7.44 mm/day for MAFF241149 on PCA (Fig. 3). Growth study on CMA, minimum 10°C, maximum 30°C and 32°C for STE-U isolates and MAFF isolate, respectively. The optimum growth temperature was 25°C for STE-U 7843, 7844, 7847 and MAFF. The optimum temperature for STE-U 7845, 7846 and 7848 was 30°C (Fig. 4). The average growth rate for the STE-U isolates with optimum growth temperatures at 25 °C and 30 °C were 9.77 mm/day and 10.63 mm/day, respectively. The daily growth rate for The MAFF isolate was 8.79 mm/day on CMA.

Type. South Africa, Wellington, *Vitis vinifera* roots, May 2013, *S.D. Langenhoven.* Culture ex-type deposited as STE-U 7843 at the Stellenbosch University culture collection.

Additional cultures examined. South Africa, Wellington, grapevine roots (STE-U 7844, STE-U 7845, STE-U 7846, STE-U 7847, STE-U 7848). Japan, Ibaraki, uncultivated soil (*Ovatisporangium* sp. 5 MAFF241149).

Notes. Three differences were observed between the *Phytopythium paucipapillatum* STE-U and MAFF isolates. STE-U displayed mainly plerotic oospores whereas MAFF had mainly aplerotic oospores. There were also differences in colony growth and optimum growth temperatures between STE-U and MAFF on CMA, but not on PCA. *Phytopythium paucipapillatum* STE-U displayed rosaceous growth on CMA and coarsely radiate growth on PDA, whereas MAFF displayed a rosette growth pattern on both CMA and PDA (Fig. 2).

Regarding internal proliferation, no nested proliferation has been observed in *P. paucipapillatum*, as compared to *P. chamaehyphon*, *P, fagopyri* and *P. helicoides* – all of which display internal, nested proliferation. *Phytopythium chamaehyphon*, *P. fagopyri*, *P. helicoides*, *P. palingenes*, *P. polytylum* and *P*. sp. WJB-3 are exclusively aplerotic whereas *P. paucipapillatum* isolates were both plerotic and aplerotic. In addition, *P. paucipapillatum* is the only species with oogonial ornamentation as compared to its closest taxa (Table 2).

Phytopythium chamaehyphon differs from *P. paucipapillatum* by having mainly globose and sub-globose, non-papillated sporangia. Also, *P. chamaehyphon* had larger (av. 26.5 µm diam.), smooth and globose oogonia. Oospores were also larger and only aplerotic. (Van der Plaat-Niterink, 1981) (Table 2).

In comparison to *P. paucipapillatum, P. fagopyri* had larger globose, subglobose to pyriform, terminal sporangia (28.5–32 µm diam.). Oogonia were larger (av. 29 µm diam.), smooth and globose. Antheridia were monoclinous and were attached to coiled stalks. Oospores were also larger (24.3 µm diam.) and aplerotic (Baten *et al.*, 2015) (Table 2).

Phytopythium helicoides had larger, mostly obovoid to ovoid, papillated sporangia (av. 21–31 µm) as compared to *P. paucipapillatum*. Oogonia were larger, smooth and globose (av. 33.5 µm diam.). Antheridia were diclinous, with 1–4 antheridia per oogonium and had coiled stalks. Oospores of *P. helicoides* were larger (av. 30.5 µm diam.) and aplerotic (Baten *et al.*, 2015) (Table 2).

Phytopythium palingenes had larger (29 x 33 µm), globose, sub-globose to ovoid sporangia as compared to *P. paucipapillatum*. The sporangia of *P. palingenes* were papillated and proliferating. Secondary sporangia often formed at the base of the primary sporangia. Oogonia were larger (av. 34 µm diam.) and sub-globose. Antheridia were diclinous with 1-4 per

oogonium. Antheridial stalks and vegetative hyphae often coiled around the oogonial stalks. Oospores were large (31.3 µm diam.), sub-globose, thick walled (av. 2.6 µm), and aplerotic (Van der Plaats-Niterink, 1981).

As no DNA sequences were available for *P. polytylum*, its morphological description was compared to *P. paucipapillatum* as to avoid a possible re-description of the former species. *Phytopythium polytylum* differs from *P. paucipapillatum* by forming secondary sporangia on short branches close to the base of the primary sporangia. Oogonia were larger (av. 32.5 µm diam.) and there were 1-4 antheridia per oogonium. Oospores were larger, subglobose (av. 28.8 µm diam.), aplerotic and had a thicker wall (av. 2.5 µm) (Van der Plaats-Niterink, 1981).

Phytopythium sp. WJB3 is an undescribed species isolated from *Persea americana* in South Africa. In addition to (sub-) globose sporangia, P. sp. WJB-3 also had ellipsoid, reniform and lobed sporangia as compared to *P. paucipapillatum*. No internal proliferation was observed. Oogonia developed on short side branches and had attached 2-6 diclinous antheridia. Oospores were aplerotic and thick-walled (2-4 µm) (McLeod *et al.*, 2009).

Phylogenetic analysis

During BLAST analysis, the ITS sequences of the putative new *Phytopythium* sp. STE-U 7843-7848 displayed a 99% sequence identity over a length of 710 nucleotides (GenBank AB468814; Identities = 704/710 (99%); Gaps = 3/710 (0%)) to the undescribed Japanese *Phytopythium* species *Ovatisporangium* sp. 5 (isolate MAFF241149 = UZ612, GenBank accession AB468814). Similarly, the COI sequences of *Phytopythium* STE-U showed 98% sequence identity over a length of 750 nucleotides to the COI sequence of this isolate that was generated *de novo* during this study (Identities = 736/750 (98%); Gaps = 4/750 (0%)). The ITS and COI sequences of *Ovatisporangium* sp. 5 was subsequently included in the phylogenetic analyses. The ITS sequences of the South African isolates were 89% and 88% similar to *Phytopythium chamaehyphon* CBS 259.30 and *Phytopythium helicoides* CBS 286.31, respectively. Also, the COI sequences of the South African isolates were 96% similar to *Phytopythium chamaehyphon* PPRI8625 and 95% similar to *Phytopythium helicoides* CBS 286.31. Also, the *Phytopythium* sp. STE-U COI sequences were 96% similar to *Phytopythium chamaehyphon* PPRI8625 and 95% similar to *Phytopythium helicoides* CBS 286.31.

Maximum parsimony analysis of the ITS rDNA region yielded a total of three equally parsimonious trees of length 2146 (TL = 2146, CI = 0.565, RI = 0.78, RC = 0.441, HI = 0.435). A total of 505 characters were constant, with 158 variable characters which were parsimony uninformative. There were 537 parsimony informative characters in total. The maximum parsimony analysis of the COI gene region resulted in 23 equally parsimonious trees with a length of 488 (TL = 488, CI = 0.520, RI = 0.728, RC = 0.379, HI = 0.480). Constant characters

numbered 477, with 41 parsimony uninformative variable characters and 154 parsimony informative characters.

Phylogenetic analysis of the ITS rDNA and COI gene regions revealed the presence of four well supported main clades. In both the ITS and COI phylogenies, the six South African isolates formed a single monophyletic grouping with good bootstrap support that also included *Ovatisporangium* sp. 5 isolate MAFF241149 (Figs. 5 and 6). This clade was distinct within the larger *Phytopythium* clade that also contained *P. chamaehyphon*, *P. helicoides*, *P. fagopyri*, *P.* sp. WJB-3 and *P. palingenes*.

Pathogenicity

The pathogenicity trial yielded re-isolation percentages of 33.6%, 4.3%, 30.7%, 23.6% and 7.1% for isolates STE-U 7843, 7844, 7845, 7846, and 7847, respectively. Most of the re-isolations were obtained from thicker roots. No root lesions were observed. The control plants yielded no positive re-isolations. The average shoot lengths of the inoculated plants ranged from 1010 mm to 1210 mm. The average shoot length of the uninoculated control plants was 986.1 mm Statistical analysis of the pathogenicity trials showed no significant differences ($F(5, 35) = 0.44634$, $P = 0.81297$) in shoot lengths between all the treatments and controls (Tables 3).

DISCUSSION

Six isolates of a previously unknown *Phytopythium* species were obtained from the roots of a visually healthy nursery grapevine. Morphological and phylogenetic analyses of the South African (STE-U) isolates and one Japanese (MAFF241149) isolate revealed it to be of the same novel species, herein named *Phytopythium paucipapillatum*.

The seven *P. paucipapillatum* isolates examined in this study exhibited micro-morphological characteristics that are generally considered typical of the genus *Phytopythium* (Bala *et al.*, 2010b; Uzuhashi *et al.*, 2010; de Cock *et al.*, 2015), eg. globose, ovoid, to distorted internally proliferating sporangia with occasional papillation. Phylogenetic analysis based on the ITS-rDNA and COI gene regions revealed that *P. fagopyri*, *P. chamaehyphon*, *P. helicoides* and *P. palingenes* are closely related to *P. paucipapillatum.* A few morphological traits have been observed in *P. paucipapillatum* which distinguishes it from these closely related species. In comparison to these species, *P. paucipapillatum* shows clear differences with regard to the number of oospores per oogonium, and the sporangia with multiple germ tubes, sporangial clustering, oogonial size and cardinal temperatures (Table 2). The most distinguishing trait of *P. paucipapillatum* is the occasional sparsely papillated oogonia, a trait only observed in one other *Phytopythium* species, *P. carbonicum* Paul (Paul, 2003). The

erratic occurrence of oogonial ornamentation has also previously been noted in certain *Pythium* clades (Levesque and de Cock, 2004). Therefore, the oogonial papillation observed in *P. paucipapillatum* might not be representative of the genus *Phytopythium*, but may be used as an aid in identifying this species. Based on the description of by Van der Plaats-Niterink (1981), *P. paucipapillatum* is also morphologically distinct from *P. polytylum*. Similar to *P. vexans* (Kato *et al.*, 1990), *P. paucipapillatum* also displays an insensitivity to the isoxazole fungicide, hymexazol, as this species is able to grow on the *Phytophthora* selective medium, PARPH (Jeffers and Martin, 1986). The extent of this insensitivity is yet unknown, but may be used to our advantage when isolating and identifying *P. paucipapillatum*. The inclusion of *Ovatisporangium* sp. 5 isolate MAFF241149 in the species *P. paucipapillatum* is supported by morphological and phylogenetic data. The Japanese isolate (MAFF241149) however, differed from the South African isolates with regard to colony growth on CMA and PDA (Fig. 4), optimum growth temperature, daily growth rate and oospore pleroticity. These differences may be ascribed to natural intra-species variation, as in the genus *Pythium* (Shahzad *et al.*, 1992); or due to adaptation to local environmental conditions, as these isolates are from geographically separated locations.

In the phylogenetic analysis, MAFF241149 clustered together with *Phytopythium* sp. STE-U with high bootstrap support and Bayesian probability, and could clearly be seen as being conspecific by both ITS rDNA and COI phylogenies. The COI gene region, together with the ITS rDNA, have been selected as the oomycete barcoding region to better discriminate between different species (Robideau *et al.*, 2011; Hyde *et al.*, 2014). There has been a renewed call for the COI gene region to be substituted by the COII gene region due to certain shortcomings (Choi *et al.*, 2015). However, the ITS and COI gene regions were sufficient to resolve the species in our phylogenies of the genus *Phytopythium* and did not warrant the sequencing of additional gene regions.

The *P. paucipapillatum* isolates tested during pathogenicity trials proved to be non-pathogenic towards grapevine rootstock 110-Richter. Most species within the genus *Phytopythium* are known to be non-pathogenic (Van der Plaats-Niterink, 1981). Even though, *P. chamaehyphon, P. helicoides, P. litorale* and *P. mercuriale* (Belbahri *et al.,* 2008) have previously been isolated from diseased grapevines, the pathogenicity of these species were never determined. In contrast, *P. vexans* is a proven grapevine pathogen (Spies *et al.*, 2011). *Phytopythium paucipapillatum* was isolated from the roots of only one plant from an open field nursery, which may indicate that it is a soil inhabiting species. This argument is supported by the fact that *Ovatisporangium* sp. 5 MAFF21149 was isolated from uncultivated soil in Japan. *Phytopythium paucipapillatum* may also be saprophytic in nature, as is the case with other *Phytopythium* species (Marano *et al.*, 2016). The fact that *P. paucipapillatum* was not

pathogenic towards grapevine rootstock 110-Richter in this trial, does not necessarily exclude it from being pathogenic toward other rootstocks and crops. Further pathogenicity trials, on rootstock 110-Richter, which is moderately susceptible to *Phytophthora* (Loubser and Uys, 1997), and various other hosts would be recommended. Furthermore, instead of only using shoot length as a measure of pathogenicity, other measures such as a root rot rating and plant and root mass should be included for a more accurate determination of pathogenicity. Also, pathogenic controls should be included in the trial to assess whether the trial has been successful.

The study described a novel *Phytopythium* species associated with roots of healthy nursery grapevines. The genus *Phytopythium* is purported to contain many undescribed species (Lévesque and de Cock, 2004). Therefore, the description of *P. paucipapillatum* adds to the diversity within this genus. Most species within this genus are regarded as saprophytes within soil and water habitats. However, special attention should be paid to the species found in agricultural systems as these species are poorly studied, and to ascertain whether they can pose a threat to crop production.

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Table 1. *Phytopythium* species isolation details, and ITS-rDNA and COI sequence accessions in GenBank.

				GenBank Accessions	
Species	**Strain[a]**	**Host/Substrate**	**Location**	**ITS[b]**	**COI[c]**
Phytopythium aichiense	CBS 137195	sludge	Japan	AB948197	AB948191
P. boreale	CBS 551.88	soil under *B. caulorapa*	China	AB725879	HQ708419
P. carbonicum	CBS 292.37	coal dust	France	AB690620	-
	CBS 112544	spoil heap soil	France	AB725876	HQ708420
P. chamaehyphon	CBS 259.30	*Carica papaya*	Hawaii	AY598666	HQ708421
	PPRI8625	*Vitis vinifera* soil	South Africa	FJ415975	-
P. citrinum	CBS 119171	vineyard soil	France	HQ643375	AB690649
	ADC9442		Netherlands	HQ643380	HQ708427
P. delawarense	OH384	*Glycine max*	USA	EU339311	-
	382B	*Glycine max*	USA	AB725875	AB690642
P. fagopyri	CBS 293.35	Buckwheat seedlings	Japan	AB690617	AB690641
	HonMa	Buckwheat	Japan	AB690615	AB690639
P. helicoides	CBS 286.31	*Phaseolus vulgaris*	USA	AY598665	HQ708430
	CBS 167.68	soil	USA	HQ643382	HQ708429
P. kandeliae	CBS 113.91	*Kandelia candel* leaves	Taiwan	HQ643133	HQ708206
	CBS 111.91	*Kandelia candel* leaves	Taiwan	HQ643134	HQ708207
P. litorale	CBS122662	soil	France	HQ643385	-
	GUCC1072			AB920537	-
	CBS118360	reed rhizosphere soil	Germany	-	HQ708433
	GUCC7167			-	AB690634
P. mercuriale	CBS122443	*Macadamiae integrifoliae*	South Africa	AB690614	KF853239
	STE-U 6204	*Macadamiae integrifoliae*	South Africa	DQ916359	-

Species	Strain[a]	Host/Substrate	Location	GenBank Accessions	
				ITS[b]	COI[c]
	GUCC1132			-	AB920497
P. mirpurense	CBS124523	stagnant water	Pakistan	-	KJ831612
	CBS124524	pond water	Pakistan	KJ831614	-
P. montanum	CBS111349	spruce stand soil	Germany	AB725883	AB690637
	ADC9766			HQ643390	HQ708437
P. palingenes	CCIBt 3981	water	Brazil	KR092139	-
P. paucipapillatum	STE-U 7843	*Vitis vinifera* roots	South Africa	KX372749	KX372742
	STE-U 7844	*Vitis vinifera* roots	South Africa	KX372750	KX372743
	STE-U 7845	*Vitis vinifera* roots	South Africa	KX372751	KX372744
	STE-U 7846	*Vitis vinifera* roots	South Africa	KX372752	KX372745
	STE-U 7847	*Vitis vinifera* roots	South Africa	KX372753	KX372746
	STE-U 7848	*Vitis vinifera* roots	South Africa	KX372754	KX372747
Phytopythium sp. WJB-3	PPRI8418	*Persea Americana*	South Africa	FJ415976	-
Ovatisporangium sp.5	MAFF241149	uncultivated soil	Japan	AB468814	KX372748
Phytophthora batemanensis	CBS679.84		Australia	HQ643148	HQ708220
Phytophthora boehmeriae	CBS29129	*Boehmeria nivea* leaf	Japan	HQ643149	HQ708221

[a] ADC, Arthur de Cock culture collection; CCIBt, Institute of Botany culture collection, Brazil; CBS, Centraalbureau voor Schimmelcultures, Netherlands; GUCC, Gifu University culture collection, Gifu, Japan; MAFF ,Ministry of Agriculture, Forestry and Fisheries Genebank, National Institute of Agrobiological Resources, Ibaraki, Japan; OH, Ohio State University culture collection; PPRI, Plant Protection Research Institute culture collection, Agricultural Research Council, Stellenbosch, South Africa; STE-U, Stellenbosch University culture collection, Stellenbosch, South Africa.

[b] GeneBank accession numbers for Phytopythium sequences of the ITS rDNA (ITS1-5.8S-ITS2)

[c] GeneBank accession numbers for Phytopythium sequences of the cytochrome oxidase subunit 1 (COI)

Table 2. Morphological comparison between *Phytopythium paucipapillatum* and closely related taxa.

Morphological character	*P. paucipapillatum*	*P. chamaehyphon*[a]	*P. fagopyri*[b]	*P. helicoides*[b]
Sporangia	(sub)globose, (ob)ovoid, limoniform, distorted, occassionally papillated and clustered, terminal, unilaterally intercalary and av. 19-25 µm	Globose or (sub)globose, non-papillated, terminal or intercalary av. 18-28 µm	(Sub)globose or occasionally pyriform, papillated or non-papillated, terminal av. 28.5–32µm	(Sub)globose, mostly (ob)ovoid, papillated and terminal av. 31-21 µm
Proliferation	Internal proliferation	Internal and nested proliferation	Internal and nested proliferation	External, internal and nested proliferation
Simple sympodia	Absent	Absent	Present	Absent
Oogonia	Occassionally papillated, globose av. 21 µm	Smooth and globose av. 26.5 µm	Smooth and globose av. 29 µm	Smooth and globose av. 33.5 µm
Antheridia	Mostly monoclinous and occasionally diclinous at a distance, 1-3 per oogonium, applied lengthwise or broadly apical to oogonia	Monoclinous or diclinous, irregular, large and broadly laterally applied to the oogonium	Monoclinous, 1-3 per oogonium, sessile, clavate, or large and laterally applied to oogonium, antheridial stalk is coiled	Diclinous, 1-4 per oogonium, large and broadly laterally applied to oogonium, antheridial stalk is coiled
Oogonia with two oospores	Present	-	-	-
Oospores	Plerotic and aplerotic (MAFF241149) av. 20 µm	Aplerotic av. 24.3 µm	Aplerotic av. 24.3 µm	Aplerotic av. 30.5 µm
Cardinal temperatures	min 10°C, optimum 25°C, max 30°C	min 5°C, optimum 30°C, max 35°C	min 15°C, optimum 30°C, max 40°C	min 10°C, optimum 30-35°C, max 40°C
Daily growth rate	8.55 mm (PCA) (STE-U) 7.44 (PCA) (MAFF241149)	22 mm (PCA)	10 mm (PCA)	15 mm (PCA)

[a] Van der Plaats-Niterink, 1981
[b] Baten *et al.*, 2015

Table 3. Shoot lengths of rooted 110-Richter plants inoculated with five *Phytopythium paucipapillatum* isolates, measured 8 months after inoculation.

Isolate	Shoot length (mm)[a]	SD[b]	Re-isolation percentage[c]
STE-U 7843	1023.3	251.7	33.6%
STE-U 7844	1121	259.7	4.3%
STE-U 7845	1210	354.3	30.7%
STE-U 7846	1010.7	446.6	23.6%
STE-U 7847	1146	410.6	7.1%
Control	986.1	330.9	0

[a] No significant differences were observed between shoot length measurments (P = 0.81297)

[b] Standard deviation calculated for the shoot lengths of seven replicates

[c] Re-isolation percentage of Phytopythium paucipapillatum from inoculated roots

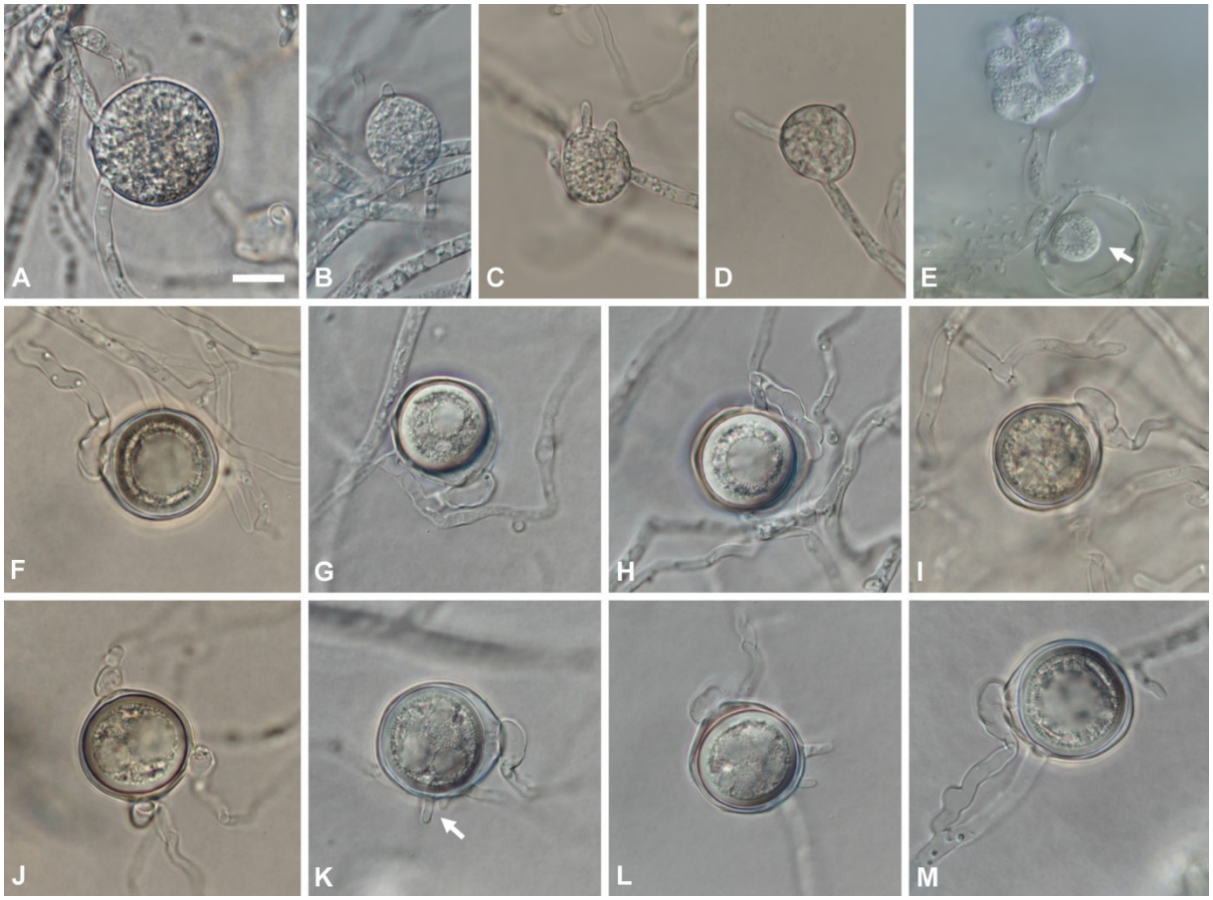

Figure 1. Morphological structures of *Phytopythium paucipapillatum*. A) a globose, intercalary sporangium without papillation. B) a sub-globose papillate sporangium. C) young, terminal mulyipapillate sporangia. D) young, terminal sporangia with papillation. E) zoospore discharge into a vesicle with a zoospore stuck in the sporangium (arrow). F-G) globose oogonia displaying broadly attached, contoured antheridia with constrictions. H) an oogonium with a monoclinous, length-wise attached antheridium. I) sub-globose oogonium displaying broadly attached, contoured antheridium with constrictions . J) an oogonium with three antheridia attached. K-L) oogonia with papillation on its surface (arrow). M) an intercalary oogonium with monoclinous antheridium. Scale bar = 10µm

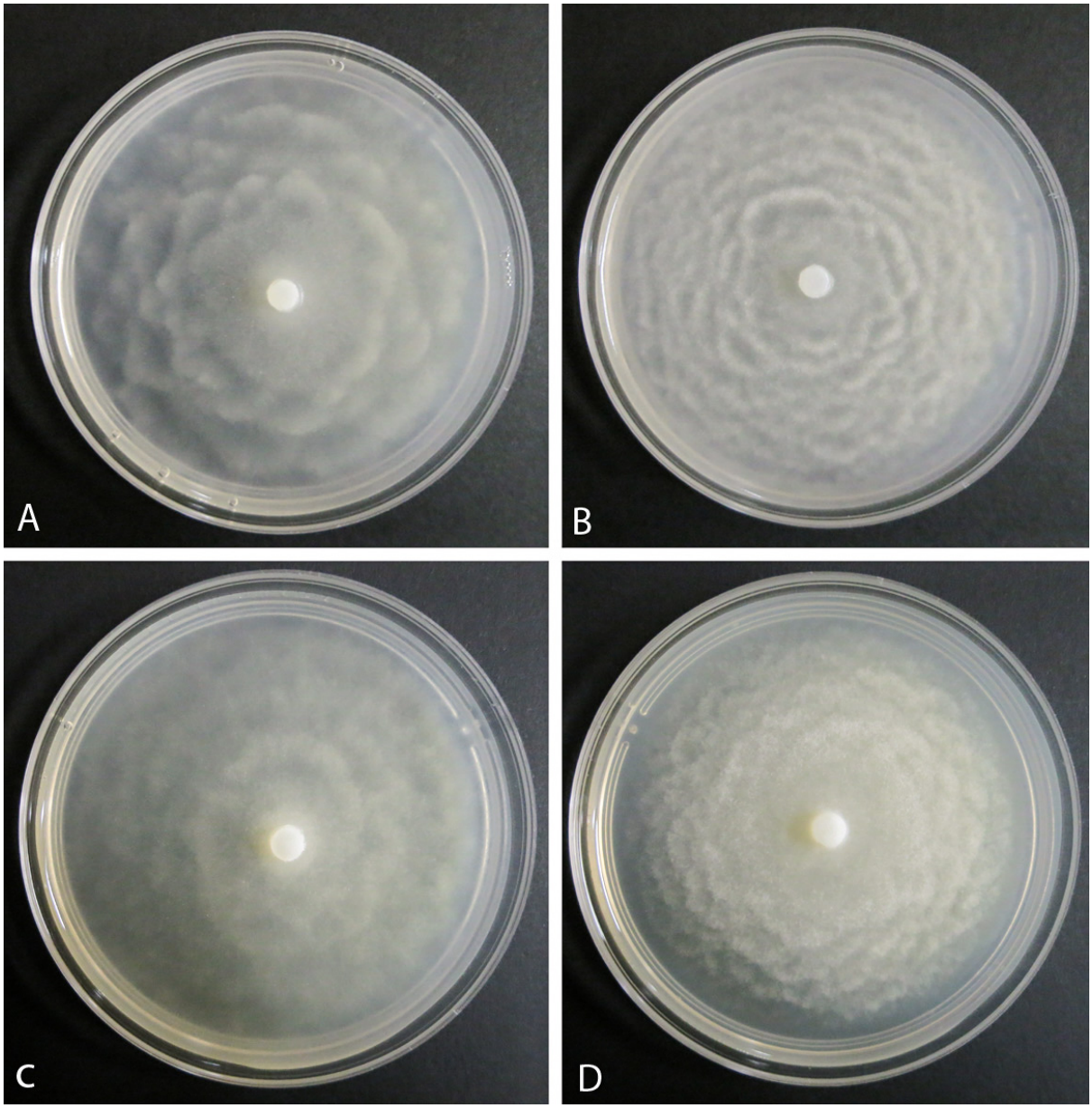

Figure 2. The colony growth patterns of *Phytopythium paucipapillatum* on corn meal agar (CMA) (A and B) and potato dextrose agar (PDA) (C and D) (1 week old cultures). A) STE-U 7843 displays rosaceous colony growth on CMA, B) MAFF241149 has a rosette growth pattern on CMA. C) STE-U 7843 grown on PDA has a coarsely radiate pattern, while, D) MAFF241149 displays a rosette pattern on PDA.

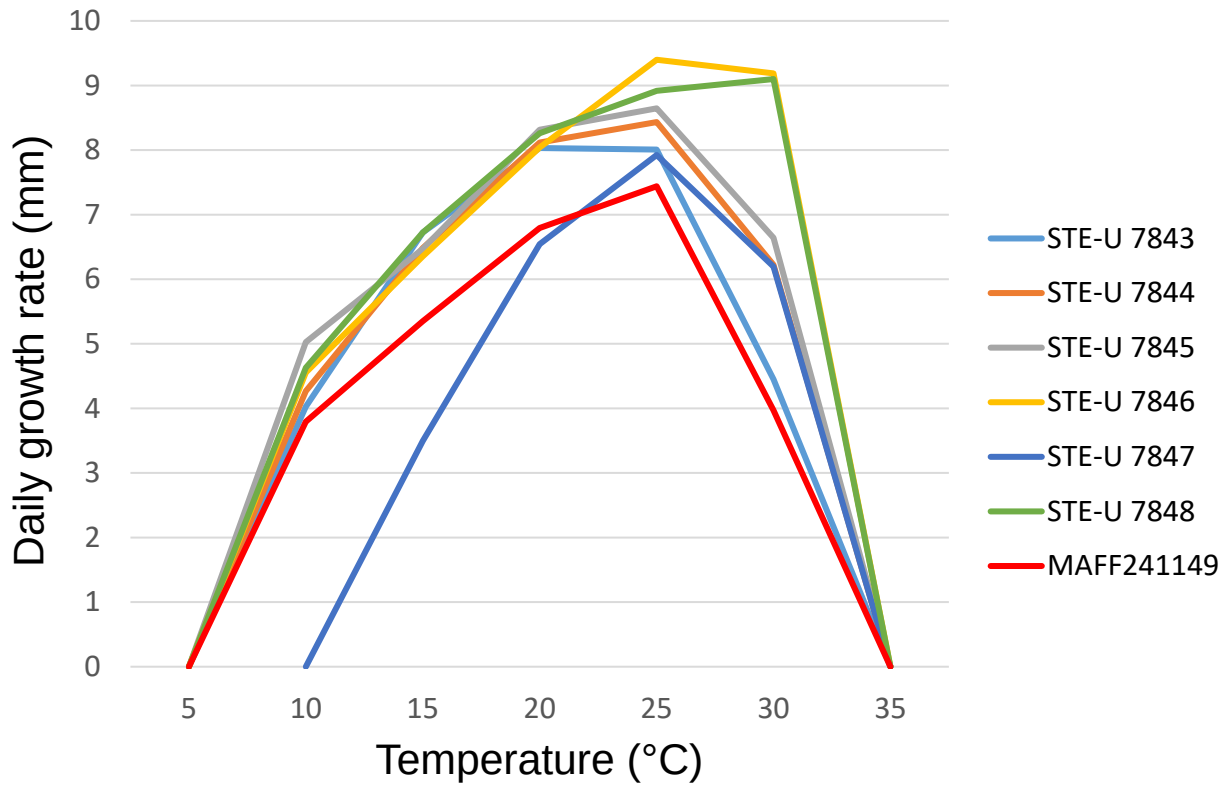

Figure 3. The average daily radial growth rate of *Phytopythium paucipapillatum* (STE-U and MAFF) on potato carrot agar. The isolates were tested at various temperatures ranging from 5°C to 35°C. *Phytopythium paucipapillatum* has an optimum growth temperature of 25°C at which it grows at 8.55 mm (STE-U) and 7.44 mm (MAFF241149) per day on potato carrot agar.

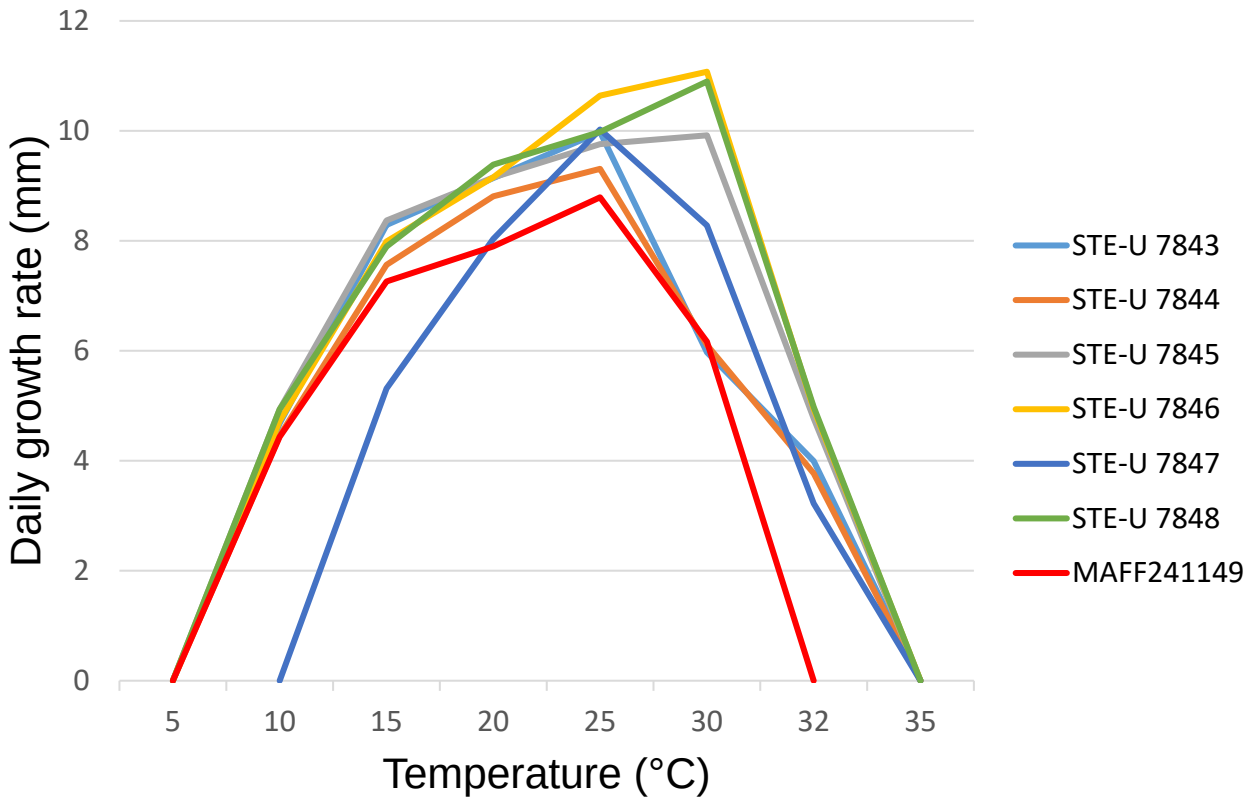

Figure 4. The average radial daily growth rate of *Phytopythium paucipapillatum* (STE-U and MAFF) on corn meal agar. The isolates were tested at various temperatures ranging from 5°C to 35°C. *Phytopythium paucipapillatum* has an optimum growth temperature of 25°C for isolates STE-U 7843, 7844, 7847 and MAFF241149; and 30°C for STE-U 7845, 7846 and 7848. The daily radial growth rate, of the STE-U isolates, at optima 25°C and 30°C, were 9.77 mm/day and 10.63 mm/day on potato carrot agar. The daily growth rate of MAFF241149 at 25°C was 8.79 mm/day

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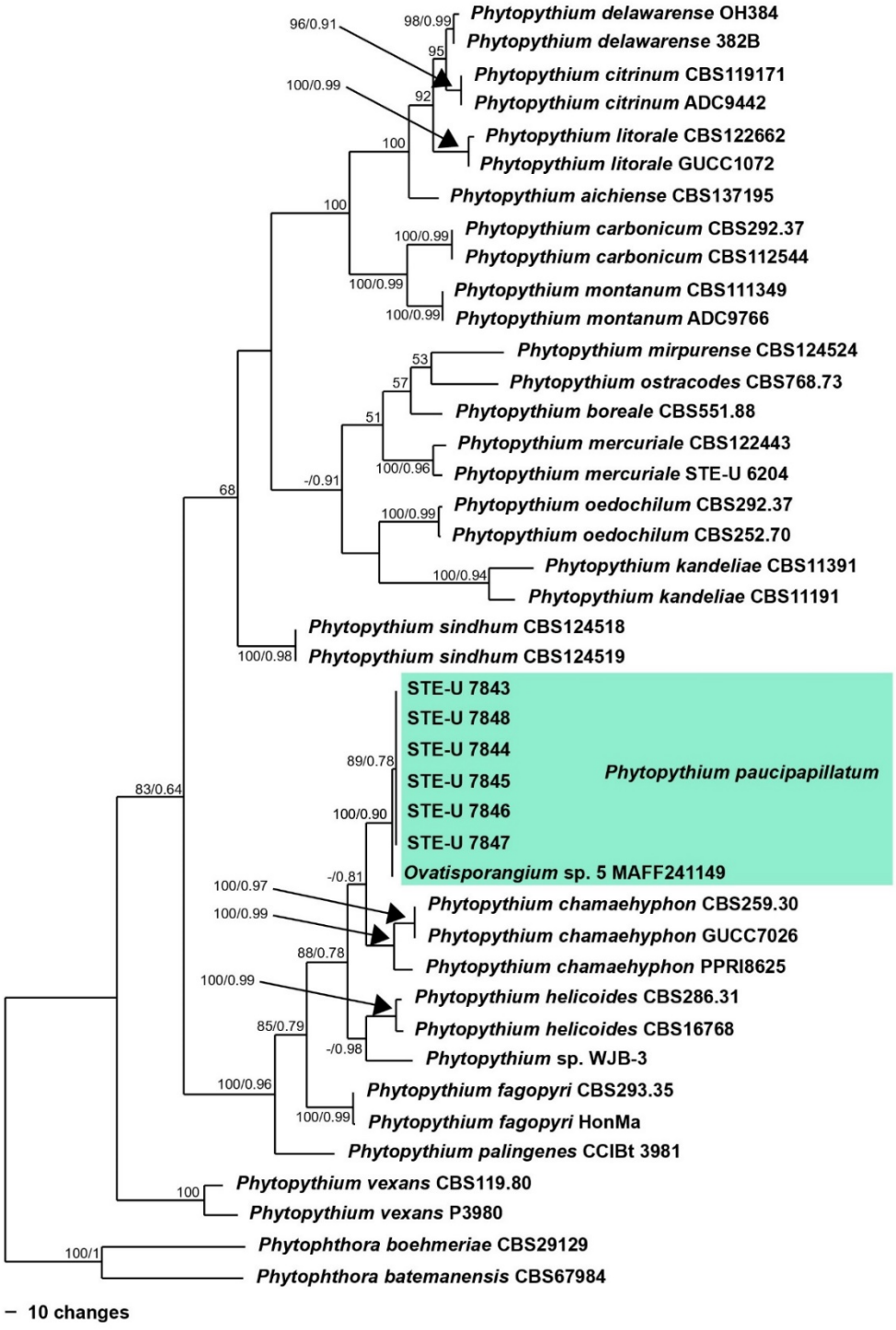

Figure 5. Maximum parsimony phylogeny (1 of 3 equally parsimonious trees) of the internal transcribed spacer-ribosomal DNA region displaying species of the *Phytopythium* genus. *Phytopythium paucipapillatum* is shown in the green shading. Bootstrap support values (parsimony analysis) and Bayesian probabilities are shown at the nodes. (TL = 2146, CI = 0.565, RI = 0.78, RC = 0.441, HI = 0.435).

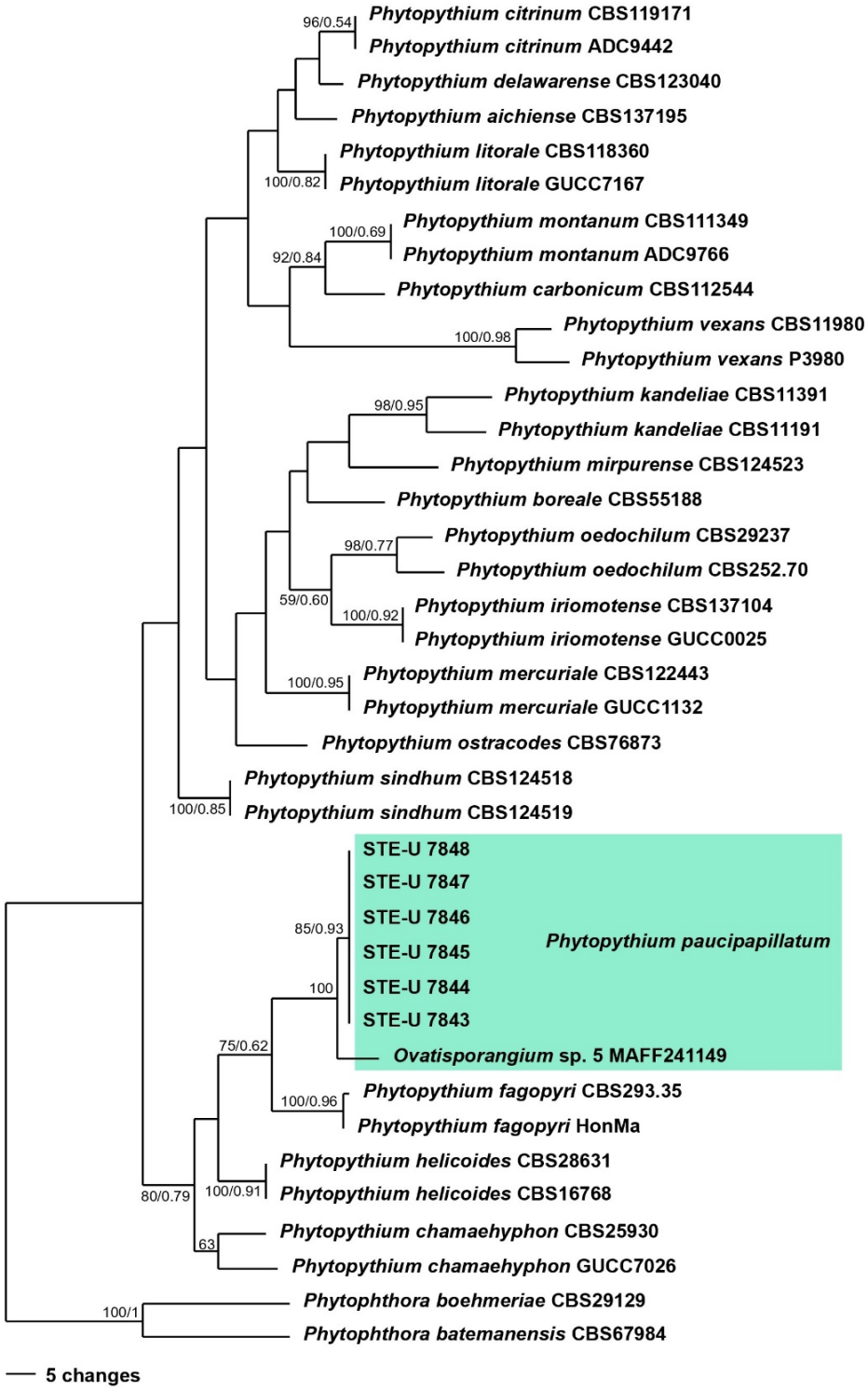

Figure 6. Maximum parsimony phylogeny (1 of 23 equally parsimonious trees) of the cytochrome oxidase subunit 1 gene region displaying species of the *Phytopythium* genus. *Phytopythium paucipapillatum* is shown in the green shading. Bootstrap support values (parsimony analysis) and Bayesian probabilities are shown at the nodes (TL = 488, CI = 0.520, RI = 0.728, RC = 0.379, HI = 0.480).