OCCURRENCE OF CANKER AND WOOD ROT PATHOGENS IN YOUNG APPLE TREES AND POSSIBLE SOURCES OF INOCULUM

by

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SUMMARY

Apples are one of the most important deciduous fruit crops cultivated in South Africa. The deciduous fruit industry identified a higher occurrence of young apple trees that died due to canker or wood rot diseases. The infected plant part starts to die back, rapidly killing the young tree shortly after establishment. Knowledge regarding the occurrence of canker or wood rot pathogens in healthy nursery trees in South African is lacking. Therefore, the aim of this study was to investigate the occurrence of stem canker and wood rot pathogens in young apple trees, as well as to identify possible inoculum sources which include mother material used for propagation.

The sampling strategy was divided up into three phases namely diseased trees collected from 1-year-old commercial orchards, certified nursery apple trees and propagation material including scion mother block trees and rootstock layer blocks. Thirteen 1-year-old orchards, which exhibited dieback symptoms shortly after establishment were sampled (ten trees per orchard). One soil sample per orchard to a depth of 30 cm was also collected. The soil sample was collected near the roots of one of the diseased trees. A total of 480 certified nursery apple trees were collected from four nurseries to determine if seemingly healthy trees are infected with dieback pathogens. These certified trees adhere to the standards set out by the Scheme.

Cankers, pruning wounds and green shoots from 310 trees in scion mother blocks were collected as well as asymptomatic 405 green shoots from rootstock layer blocks. Plant material was surface sterilised, cut open, and isolations were made from the internal vascular discolouration. Fungal cultures obtained during the study were identified to species level by DNA sequencing and phylogenetic analyses of either the ITS, β-tubulin or EF1α gene regions, within each specific taxonomic group.

A total of 45 fungal species belonging to the taxonomic groups: Basidiomycetes, Botryosphaeriaceae, Diaporthales, Diatrypaceae, Dothideomycetes, as well as, *Phaeoacremonium* species and *Truncatella angustata,* were identified in this study. The species identified in the current study have all been associated with canker or wood rot symptoms found on fruit trees in other fruit growing regions of the world. Thirty-one canker and/or wood rot causing fungi found in this study are firstly reported in apples in South Africa, of which 27 species are also reported for the first time in apples worldwide. A latent infection level of 65% was found in certified nursery apple trees with the four most predominantly isolated pathogens, including *Didymosphaeria rubi-ulmifolii s.l.*, *Diplodia seriata, Diaporthe eres* and *Didymella pomorum.* The majority of the pathogens were found from brown wood discoloration and white rot symptoms observed in the bud union and pruning wounds on the rootstock in nursery trees. The high infection rate in the bud union and pruning wound made

on apple trees indicated that nursery trees got infected during the propagation process via aerial inoculum, which was present at the time of budding and pruning back.

The same fungal species were found causing latent infections in nursery trees, which were later found to be diseased in newly established orchards. The soil analyses indicated that dieback of 1-year-old commercial trees was related to the stress conditions created when apple trees were established on sub-optimal soil. More soil samples should be collected in the future to confirm these findings. Green shoots of scion trees from mother blocks, from which buds are exiced for budding, presented 5% infection and the shoots from rootstock layer blocks had an infection of 21%. Thus, buds and rooted rootstock cuttings used during propagation can also contribute to infected nursery apple trees.

Basidiomycete and Ascomycete fruiting structures were found on dead trees and cankers in the scion mother blocks, 1-year-old commercial orchards as well as in 1-year-old nursery blocks. These fruiting structures can contribute to the aerial inoculum present during propagation and establishment. However, spore trapping studies should be done in the nurseries, to determine the incidence and extend of the inoculum present during the propagation process in the nursery. This study has found that mother plant material can be the source of infected nursery trees and that a high percentage of certified nursery apple trees were infected with possible canker and wood rot pathogens resulting in the distribution of seemingly healthy apple trees to farmers.

OPSOMMING

Appels is een van die belangrikste sagtevrugte gewasse wat in Suid-Afrika verbou word. Die sagtevrugtebedryf het 'n hoër voorkoms van jong appelbome geïdentifiseer wat gesterf het as gevolg van kanker- en houtverrottings siektes. Die besmette plantdeel begin terug te sterf, wat lei tot die spoedige dood kort na vestiging. Kennis oor die voorkoms van kanker en houtverrottings patogene in gesertifiseerde kwekery bome in Suid-Afrika ontbreek. Die doel van hierdie studie was dus om die voorkoms van kanker en houtverrottings patogene in jong appelbome te ondersoek, asook om moontlike inokulum bronne te identifiseer. Inokulum bronne wat ondersoek was sluit in moeder materiaal wat gebruik word tydens voortplanting.

Die monsterneming strategie was verdeel in drie fases, meer spesifek; siek bome gevind in 1-jarige kommersiële boorde, gesertifiseerde kwekery appelbome asook voortplantingsmateriaal insluitend bostok moedermateriaal en onderstok leier blokke. Dertien 1-jarige boorde met appelbome wat terugsterf simptome kort na vestiging getoon het was gemonster (tien bome per boord). Een grondmonster per boord tot 'n diepte van 30 cm is ook ingesamel. Die grondmonster was naby die wortels van een van die siek bome in elke boord geneem. 'n Totaal van 480 gesertifiseerde kwekery appelbome is ingesamel uit vier kwekerye om te bepaal of visueel gesonde bome wel besmet is met terugsterf patogene. Hierdie gesertifiseerde bome voldoen aan die voorwaardes wat uiteengesit is deur die Skema.

Kankers, snoeiwonde en groen lote van 310 bome in bostam moederblokke asook 405 asimptomatiese groen lote van onderstok leier blokke was ingesamel. Plantmateriaal is oppervlakkig gesteriliseer, oopgesny en isolasies is gemaak van die interne vaskulêre verkleuring. Swam kulture wat tydens die studie geïdentifiseer was, was tot spesie vlak geïdentifiseer deur DNS volgorde bepaling en filogenetiese analise van die ITS, β-tubulin of EF1α geen areas, binne elke spesifieke taksonomiese groep.

'n Totaal van 45 swam spesies was geïdentifiseer wat deel vorm van die taksonomiese groepe: Basidiomycetes, Botryosphaeriaceae, Diaporthales, Diatrypaceae, Dothideomycetes, asook, *Phaeoacremonium* spesies en *Truncatella angustata*. Die spesies wat in die studie geïdentifiseer was, is almal geassosieer met kanker of houtverrottings simptome wat op vrugtebome in ander vrugte produserede streke in die wêreld gevind is. Vir 31 kanker en / of houtverrottings swamme wat gevind was in hierdie studie is dit die eerste rapport op appelbome in Suid-Afrika, en daarvan 27 spesies ook gerapporteer vir die eerste keer op appels wêreldwyd. 'n Latente infeksie van 65% is in gesertifiseerde kwekery appelbome gevind. Die vier mees oorwegend geïsoleerde patogene sluit in; *Didymosphaeria rubi-ulmifolii s.l.*, *Diplodia seriata*, *Diaporthe eres* en *Didymella pomorum*. Die meerderheid van die patogene was geïsoleer van bruin hout verkleuring en wit sagteverrot simptome wat waargeneem was in die okkulasiewond asook by die snoeiwond wat gemaak was op die

onderstok van die kwekery bome. Die hoë infeksie in die okkulasiewond en snoeiwond op kwekery appelbome dui daarop aan dat kwekery bome besmet word tydens die voortplantingsproses via lug inokulum wat teenwoordig is tydens die tyd van okkuleering en terug snoei.

Soortgelyke patogene was gevind wat latente infeksies in kwekery bome veroorsaak, wat ook siekte uitdrukking veroorsaak het in nuutgevestigde boorde. Die grond ontledings het getoon dat terugsterf van 1-jarige kommersiële bome verband hou met stres toestande wat veroorsaak word deur vestiging op sub-optimale grond. Meer grondmonsters moet ingesamel word in die toekoms om hierdie bevindinge te bevestig. Daar was ook gevind dat 5% van groen lote wat versamel was van bostam moederblokke latente infeksies gehad het. Onderstam leier blokke het 'n latente infeksie van 21% gehad. Dus, ogies en gewortel onderstok plante wat gebruik word tydens voortplanting kan ook bydra tot die besmetting van kwekery appelbome.

Basidiomycete en Ascomycete vrugstrukture was gevind op dooie bome en kankers in die bostam moederblokke, 1-jarige kommersiële boorde asook in 1-jarige kwekery blokke. Hierdie vrug strukture kan bydra tot die lug inokulum wat teenwoordig is tydens voortplanting en vestiging. Daar moet egter spore vang studies gedoen word in die kwekerye, om die voorkoms van die inokulum te bepaal wat teenwoordig is tydens die voortplantingsproses in die kwekery. Hierdie studie het bevind dat moedermateriaal die bron van besmette kwekery bome kan wees en dat 'n hoë persentasie van gesertifiseerde kwekery appelbome besmet is met moontlike kanker en houtverottings patogene wat lei tot die verspreiding van visueel gesonde appelbome aan boere.

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CONTENTS

CHAPTER 1

A review of canker and wood rot pathogens causing decline on apple trees

INTRODUCTION

Dieback is a term used when describing a condition where apple trees die prematurely due to the infection of opportunistic canker and wood rot pathogens (Menapace *et al.*, 2015). Canker and wood rot pathogens can infect trunks, branches, and shoots of apple trees and give rise to symptoms such as cankers, twig blight, and wood rot (Brown-Rytlewski and McManus, 2000; Borovinova *et al.*, 2012). The pathogens infect their hosts through wounds and colonise the vascular tissue resulting in blockage of the vascular system (Cloete *et al.*, 2011). The infected plant part will start to die back, eventually leading to the death of the entire tree or even failure of the orchard (Zhang *et al.*, 2014). The farmer can face major financial damages when the infections are severe enough for the orchards to become uneconomical, since dieback limits the longevity and reduces the yield of the host (Elfar *et al.*, 2013).

Canker and wood rot symptoms are commonly observed in older apple orchards in South Africa. However, the decline and eventual death of these trees can take an extended period of time (Smit *et al.*, 1996). In contrast to older trees, younger trees infected with canker or wood rot pathogens can be killed, rapidly especially when exposed to abiotic stress factors (Smit *et al.*, 1996; Marek *et al.*, 2013). Over the past 5 years, young apple orchards in South Africa have shown an increase in canker expression. Studies have found that nursery apple trees infected during the propagation process only exhibit dieback symptoms once established in new orchards (Fujita *et al.*, 1988; McCracken *et al.*, 2003; Marek *et al.*, 2013). Knowledge regarding latent infections of canker or wood rot pathogens in healthy nursery trees in South African is lacking.

This literature study will seek to examine the canker and wood rot pathogens associated with dieback on apple trees as well as the epidemiology, symptoms, host range and possible control methods for these pathogens. This review will focus on canker and wood rot pathogens found on apple trees both in South Africa and other countries. The chapter will also provide an overview of the South African apple industry, the propagation process and discuss the deciduous plant certification Scheme. Reviewing these aspects can contribute to a better understanding of canker and wood rot pathogens involved in the death of young apple trees in South Africa.

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GLOBAL APPLE PRODUCTION

The domesticated apple *Malus domestica* Borkh. is the most important fruit in temperate zones (Smit *et al.*, 1996; Robinsons *et al.*, 2000). Apple cultivation dates back to Greece as early as 325 BC (Turechek, 2004), however, little is known about apple domestication (Robinsons *et al.*, 2000). Borkharsen believed that the ancestors of *M. domestica's* were *M. sylvestris* Mill., *M. dasyphylla* Borkh. and *M. praecox* Borkh. (Robinsons *et al.*, 2000). Apples belong to the genus *Malus* and family Rosaceae (Turechek, 2004). Apples are the third most produced fruit crops in the world following bananas and grapes (Dobránszki and da Silva, 2010), with an estimated 77.0 million ton apples produced worldwide during 2015/2016 (USDA, 2015).

Apple cultivation can only occur in geographical regions with sufficient chilling requirements of 100 hours per annum under 5°C (Turechek, 2004). Apple trees are susceptible to winter damage if the temperature decreases below -30°C (Turechek, 2004). Currently, the top ten major apple growing regions of the world are; in order; China, United States of America, Turkey, Poland, Italy, India, France, Chile, Iran and Russian Federation (Krishna *et al.*, 2010; WAPA, 2013). China is the leading producer of apples in the world with an estimated 43 million tons production forecasted for the 2015/2016 marketing year (USDA, 2015). In the southern hemisphere, Chile is the leading producer with a forecast of 1.4 million tons followed by Argentina, Brazil and South Africa (WAPA, 2013; USDA, 2015). South Africa is currently the sixteenth largest apple producer in the world and fourth largest in the southern hemisphere (WAPA, 2013) with a production forecast of 865 000 tons for the 2015/2016 marketing year (USDA, 2015). South Africa is, however, the second biggest exporter in the southern hemisphere after Chile and sixth biggest exporter in the world, exporting approximately 52,6% (455 000 tons) of annual production (Hortgro, 2015).

SOUTH AFRICAN APPLE INDUSTRY

Production areas

In South Africa, apples are produced in several provinces such as the Western Cape, Northern Cape, Eastern Cape and Limpopo; with the Western Cape responsible for approximately 90% of the total apple production and exports (van Schoor *et al.*, 2009; Hortgro, 2015). The Western Cape has a Mediterranean climate with a winter rainfall and sufficient chilling requirements, which are perfect for apple production (van Schoor *et al.*, 2009; Grab and Craparo, 2011; Hortgro, 2015). The main production areas in the Western Cape Province include Ceres, Groenland, Langkloof East, Villiersdorp, Grabouw and Elgin (van Schoor *et al.*, 2009; Hortgro, 2015). The area which is under apple plantation has steadily increased from ≈20 700 ha in 2008 to 23 150 ha in 2016. The quantity of apples produced and exported have also increased over the past 8 years (Hortgro, 2015).

Establishment cost

Even though the area under apple plantations has increased over the past 8 years, the average tree age is high, with 34% of the trees being older than 25 years (Hortgro, 2015). In the 2013/2014 marketing year only 9% of the trees were younger than 3 years (Hortgro, 2015). This low orchard replanting and expansion can be blamed on the rising establishing costs, especially in years with a weak Rand exchange rate (Hortgro, 2015). Orchard establishment cost has increased annually since 2009. In 2009, orchard establishment costs were approximately R160 000 per ha (Hortgro, 2015). In 2016, an estimated R281 994 per ha was needed to establish an orchard (Theron and Steyn, 2015). This is an increase of R121 994 per ha in seven years. Without royalties, plant material contributes to 29% (R81 840 per ha) of the total establishment cost (Theron and Steyn, 2015). It is therefore very important to have quality, disease-free nursery trees to establish an orchard to ensure that the trees grow to their full potential from the start (Theron and Steyn, 2015).

CANKER AND WOOD ROT PATHOGENS OF APPLE TREES

Ten different canker diseases caused by Ascomycete pathogens have been identified from apple trees (Table 1) (Sutton *et al.*, 2015). In total 36 Ascomycete species have also been associated with cankers or dieback symptoms on apple trees (Table 1). The Basidiomycete pathogens have been grouped to cause wood rot or silver leaf disease (Table 2) (Sutton *et al.*, 2015). In South Africa, 16 Ascomycete fungi have been reported from apple trees from either dieback or canker symptoms and four Basidiomycete fungi from wood rot. Canker diseases not present in South Africa and of quarantine importance include, European canker (*Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman), Valsa canker (*Valsa ceratosperma* (Tode) Maire), Anthracnose canker and Perennial canker (*Neofabraea malicorticis* H.S. Jacks., N. perennans Kienholz).

Canker and wood rot pathogens found on apple trees in South Africa

The first report of canker on apple trees was in 1919 in South Africa with *Botryosphaeria dothidea* (Moug.) Ces. & De Not. identified from the diseased material (Biggs, 2004). This pathogen is more known for causing white rot on apple fruit (Biggs, 2004). The first report of canker found on young apple material was in 1990 when a apple rootstock from Simondium nursery got infected by *Diaporthe ambigua* Nitschke (Smit *et al.*, 1996). Smit *et al.* (1996) isolated *Dia. ambigua* from the margins of characteristic, sunken in lesions found on apple, pear, and plum rootstocks as well as on diseased branches of mature trees. Perithecia and pycnidia formed in the cracks of the canker.

A study was conducted by Adams *et al.* (2006) on *Cytospora* species found on native trees in South Africa and found that *Valsa malicola* Z. Urb., which was renamed to *Cytospora*

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schulzeri Sacc. & P. Syd. *V. nivea* (Hoffm.) Fr., were reported as canker causing pathogens on apple trees in South Africa. Slippers *et al.* (2007) studied the diversity of Botryosphaeriaceae species found on pome and stone fruit trees in South Africa and identified *Botryosphaeria obtusa* (Schwein.) Shoemaker which was renamed to *Diplodia seriata* De Not. and *Neofusicoccum australe* Crous, Slippers & A.J.L. Phillips as canker causing pathogens causing decline on apple trees.

Cloete *et al.*, (2011) conducted a study on the occurrence of canker and wood rot pathogens on mature pome trees in South Africa and found the following pathogens on apple trees: *D. seriata*, *Eutypa lata* (Pers.) Tul. & C. Tul., *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai which was renamed to *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous, and *Phomopsis theicola* Curzi which was renamed to *Diaporthe foeniculina* (Sacc.) D. Udayanga & L.A. Castlebury.

A few lesser known canker pathogens have also been found which include species of the Didymellaceae. Didymellaceae species have been associated with twig blight and fruit spot in South Africa (Crous *et al.*, 2000). The main species of this disease is *Phoma macrostoma* Mont. which was renamed to *Didymella macrostoma* (Mont.) Q. Chen & L. Cai (Crous *et al.*, 2000; Chen *et al.*, 2015). Wood rotting caused by numerous Basidiomycete fungi was identified by Matthee and Thomas (1977a) as a cause of heavy production losses of apple production in South Africa. They identified *Polyporus versicolor* (L.) Fr. renamed to *Trametes versicolor* (L.) Lloyd, *Polyporus adustra* (Willd.) Fr renamed to *Bjerkandera adusta* (Willd.) P. Karst, *Stereum purpureum* Pers. and *Schizophyllum commune* Fr., the wood rotting species involved in dieback of apple trees.

The relevance of the different fungal taxa isolated from diseased apple trees can be illustrated by data obtained from the Disease Clinic (Plant Pathology Department, Stellenbosch University). Nine different fungal taxa isolated from 60 young diseased apple trees over a seven-year period (2010-2016) (Table 3). These trees represent 60 commercial orchards expressing canker or wood rot symptoms shortly after establishment. Fungi of the Botryosphaeriaceae were the most predominant, followed by *Diaporthe* spp. and Basidiomycetes spp. (Table 3).

Symptoms associated with canker and wood rot

Canker symptoms can form on the trunks, branches or shoots of both young and old apple trees (Krishna *et al.*, 2010; Zang *et al.*, 2012; Wang *et al.*, 2014). When the symptoms are found on the trunk or main branches, severe economic losses can occur (McCracken *et al.*, 2003). However, when cankers are formed on shoots or minor branches, the use of sanitation practices in the spring and autumn and can remove the canker and subsequent inoculum

source, without major losses to the tree or harvest (McCracken *et al.*, 2003). When environmental conditions are favourable, dieback symptoms are expressed and will give rise to symptoms such as canker, twig blight and wood rotting which leads to the dieback of affected plant parts (Slippers *et al.*, 2007; Cloete *et al.*, 2011; Abdollahzadeh, 2015). Severe infection, coupled with unfavourable environmental conditions (Smit *et al.*, 1997), will result in the death of trunks, branches and shoots, which leads to the eventual death of the tree (Krishna *et al.*, 2010; Zang *et al.*, 2012; Wang *et al.*, 2014).

Generally canker and wood rot pathogens infect trunks and branches of mature apple trees resulting in elliptical cankers which can expand and eventually girdle the trunk or branch (Abreo *et al.*, 2012; Wang *et al.*, 2014). Girdling of plant parts will appear mostly on weak branches and small twigs (Wang *et al.*, 2014). The leaves on the infected branch beyond the girdled section will wilt and die (Smit *et al.*, 1997; Abreo *et al.*, 2012). Symptoms caused by *Diaporthe* species are characterised by depressed elliptical lesions that form longitudinal cracks on the surface of the bark (Smit *et al.*, 1996; Smit *et al.*, 1997; Abreo *et al.*, 2012). *Cytospora* species give rise to external symptoms, including water-soaked lesions, which dry up to form a sunken, swollen, and distorted canker (Fisher, 1931; Zang *et al.*, 2012; Wang *et al.*, 2014). Cracks form on the surface of the wood between the healthy and the diseased tissue (Zang *et al.*, 2012; Wang *et al.*, 2014). Cankers will expand in the direction of the long axis of the infected plant part (Zang *et al.*, 2012).

External symptoms produced by wood rot pathogens cause irregular, dark-brown lesions on the bark which usually changes into elongated, cankerous lesions (Matthee and Thomas, 1977a). Progression of pathogen infection can give rise to two different wood decay symptoms, white rot and brown rot, of which white rot is more common (Table 2) (Sutton *et al.*, 2015). Basidiomycetes causing white rot degrade polysaccharide and lignin, which result in a soft, spongy and bleached appearance (Sutton *et al.*, 2015). Brown rot symptoms are characterised by dark brown dry wood, which is the result of the degradation of polysaccharide (Sutton *et al.*, 2015). *Trametes versicolor* infection can progress to such a state that the bark will start to flake off giving rise to the characteristic papery bark symptom (Matthee and Thomas, 1977a). The foliar of the infected *Chondrostereum purpureum* (Pers.) Pouzar branches will become silver, sunken, curled and distorted, resulting in the characteristic silver leaf symptoms (Matthee and Thomas, 1977a; Miyairi *et al.*, 1977; Spiers and Brewster, 1997; Sutton *et al.*, 2015).

When the bark of the diseased wood is removed, discolouration of the tissue beneath can be observed (Matthee and Thomas, 1977a; Wang *et al.*, 2014). Internal symptoms include brown vascular discolouration usually in wedge- or irregular-shaped sectors (Matthee and Thomas, 1977a; Spiers and Brewster, 1997). Cloete *et al.* (2011) found brown vascular streaking, black vascular streaking, wedge-shaped necrosis, watery necrosis, brown internal

necrosis, as well as soft rot which was isolated from diseased mature apple trees. These symptoms are similar to the symptom types described by van Niekerk *et al.* (2011) expressed in grapevine due to trunk diseases. The majority of the species isolated by Cloete *et al.* (2011) occured too infrequently to be correlated to a specific symptom type. However, a large incidence of wedge-shaped necrosis was associated with infection from either Botryosphaeriaceae or *E. lata* pathogens.

Dieback of plant parts are gradual and it can take years for the infection to be severe enough to kill the entire plant (Cloete *et al.*, 2011). The rate of decline and eventual death of the branches or an entire tree does, however, depends on the health of the tree. The progression of the disease is slower in mature apple trees growing vigorously in comparison to trees experiencing stress (Wang *et al.*, 2014). Thus, young trees are killed more rapidly when dieback symptoms are expressed, whereas mature apple trees are killed over an extended period of time (Smit *et al.*, 1996).

Epidemiology and ecology of dieback pathogens

Canker and wood rot pathogens infect apple trees through injury on the bark (Deflorio *et al.*, 2008; Ke *et al.*, 2014). The primary infection sites are pruning wounds, however, wounds made by machinery, hail, frostbite, and insects are also important sites for infection (Borovinova *et al.*, 2012; Zang *et al.*, 2012). A study done by Borovinova *et al.* (2012) found two bark beetles, namely *Scolytus rugulosus* Mueller and *S. mali* Bechstein, living between the bark and the surface of the wood, damaging trunks and branches of pome and stone fruit trees in Bulgaria. The insect damage on the branches and trunks served as infection sites for trunk disease pathogens (Borovinova *et al.*, 2012). Certain species belonging to the family Botryophaeriaeceae can infect apple trees through lenticels and cracks on the epidermis of the wood (Liu *et al.*, 2011). Resistance to *Botryosphaeria* canker is positively correlated with lenticel development (Liu *et al.*, 2011). Dormant buds can also have latent infections of dieback pathogens, and only express disease when the plant experiences stress (Zang *et al.*, 2012; Zhang *et al.*, 2014).

After infection, pathogens such as *Valsa ceratosperma* (Tode) Maire penetrates and colonise the host xylem and phloem (Valiuškaitė and Raudonis, 2008; Wang *et al.*, 2011; Wang *et al.*, 2014). The pathogen can move systemically through the vascular tissue, inducing tissue maceration and cell death (Cloete *et al.*, 2011). This leads to the eventual blockage of xylem tissue and necrotic dieback of the host (McCracken *et al.*, 2003; Cloete *et al.*, 2011; Ke *et al.*, 2014). *Valsa ceratosperma,* the causal organism of Valsa canker, invades the host by secreting toxins and cellwall-degrading enzymes to collapse and kill the host cells (Natsume *et al.*, 1982; Ke *et al.*, 2014; Zhang *et al.*, 2014). *Valsa ceratosperma* can also invade and reside in the xylem vessels without inducing symptoms (Zhang *et al.*, 2014).

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Basidiomycete fungi are associated with wood rot and can only infect their host through a wound (Spiers and Brewster, 1997). Wood rotting fungi excrete enzymes which can deplete the wood cell wall components of the host which include hemicelluloses, celluloses, and lignin (Deflorio *et al.*, 2008). The carbohydrates excreted are metabolized by the wood rotting fungi, resulting in severe structural damage to the tree (Deflorio *et al.*, 2008). Numerous fruiting bodies of the causal organism will form on dead infected plant material (Spiers and Brewster, 1997; Mehrabi *et al.*, 2011).

Cankers and dead material found in the orchards and surrounding orchards serve as an important source of inoculum for infection (Mehrabi *et al.*, 2011; Arzanlou *et al.*, 2014). Ascomycete fungi such as *Cytospora* species and Botryosphaeriaceae form pycnidia on the infected and cankerous wood on apple trees (Copes and Hendrix, 2004; Adams *et al.*, 2006; Zang *et al.*, 2012; Arzanlou *et al.*, 2014). Airborne conidia will exude from the fruiting bodies present in the orchards (Adams *et al.*, 2006; Arzanlou *et al.*, 2014). Basidiomycete fungi form basidiocarps which release airborne basidiospores (Spiers and Brewster, 1997). Favourable weather conditions such as rain prompt spore release to form airborne inoculum (Valiuškaitė and Raudonis, 2008).

Spores of canker and wood rot pathogens are mainly spread via the wind (Matthee and Thomas, 1977a; Valiuškaitė and Raudonis, 2008). Pathogens can also be transmitted by other means such as rain and pruning shears (McCracken *et al.*, 2003). Localised splashing, wind-blown rain, and run-off from infected trees are some of the reasons why rain is an important carrier of dieback pathogens (Bertrand and English, 1976; McCracken *et al.*, 2003). More recently Moyo *et al.* (2014) proved that trunk disease pathogens can be spread by arthropod transmission on grapevines.

Portuguese millipedes (*Ommattoiulus moreleti*) and cocktail ants (*Crematogaster peringueyi*) are regarded as vectors for trunk diseases since they can transfer pathogen spores to pruning wounds of healthy grapevines (Moyo *et al.*, 2014). Spores of *Phaeoacremonium,* Botryosphaeriaceae, Diatrypaceae, and Diaporthales were detected on the exoskeleton of arthropods collected in vineyards with dieback symptoms (Moyo *et al.*, 2014). Kubátová *et al.* (2004) isolated *Phaeoacremonium* species from the larvae of the bark beetle *Scolytus intricatus* (Ratz.) and adults of bark beetle *Leperisinus fraxini* (Panz.). These bark beetles could possibly be vectors for *Phaeoacremonium* species.

Latent infection dynamics

Canker and wood rot pathogens can infect healthy tissue without inducing symptoms. The pathogen will stay latent until the host experiences stress caused by physiological factors (Zang *et al.*, 2012; Zhang *et al.*, 2014). The susceptibility of the infected host is directly linked to environmental conditions (Slippers *et al.*, 2007). The change in temperature and rainfall

patterns can affect the yield, directly and indirectly, due to the increased susceptibility of the host to canker pathogens (Menapace *et al.*, 2015). A study done by Brown-Rytlewski and McManus (2000) looked at canker development on 2-year-old Golden Delicious apple trees in different areas in America. They found that cankers which developed on the trees in Sturgeon Bay, Wisconsin, were greater than any other apple growing area in 1998. The low rainfall in the area which was below 3.6 cm during the months before inoculation resulted in drought stress, which predisposed the trees to cankers caused by *D. seriata* and *B. dothidea*.

Other abiotic stresses responsible for low productivity in apple orchards include fluctuation in temperature, nutrient deficiencies, low-temperature injuries such as frost, water logging, and insect damage (Slippers *et al.*, 2007; Valiuškaitė and Raudonis, 2008; Krishna *et al.*, 2010; Arzanlou and Bakhshi, 2012; Marek *et al.*, 2013). Low-temperature injury readily occurs in the Northern Hemisphere where dieback worsened in apple orchards experiencing extreme winter conditions (Brown-Rytlewski and McManus, 2000; Menapace *et al.*, 2015). A survey conducted by Menapace *et al.* (2015) showed that general dieback symptoms on apple trees in Italy increased over 3 years due to climate change.

<u>Latent infection obtained during propagation</u>

A study done by McCracken *et al.* (2003) found that, after the establishment of new apple orchards in areas isolated from obvious inoculum sources, the young apple trees developed large cankers on the main stems. The trees were infected with Nectria canker and showed that certain trees had multiple cankers while neighbouring trees exhibit no symptoms. These results suggested that the trees could have been infected during propagation (McCracken *et al.*, 2003). After establishment, the pathogen can move systemically and cause severe cankers on young trees (McCracken *et al.*, 2003). Brown *et al.* (1994) also concluded that the cankers observed in the young apple orchards originated from latent infection by *Cylindrocarpon heteronema* (Berk. & Broome) Wollenw. that occurred during the propagation process in the nursery.

Fujita *et al.* (1988) found that mature apple trees inoculated with the canker pathogen *Diaporthe tanakae* Tak. Kobay. & Sakuma only exhibited typical canker lesions after 2 years, this explains the absence of symptoms in the nursery. Abiotic stresses at the end of propagation as well as during planting contribute to symptom expression in newly established orchards (Marek *et al.*, 2013). Dehydration of trees after removal from the field and prior to storage as well as temperature fluctuation during storage are important abiotic factors in the nursery contributing to symptom expression (Marek *et al.*, 2013).

Host range

Canker and wood rot fungal species are cosmopolitan and can occur on a wide range of hosts including fruit, forest and ornamental plants as endophytes and plant pathogens (Cloete *et al.*, 2011; Úrbez-Torres, 2011; Phillips *et al.*, 2012). These fungal taxa include species in the taxonomic groups *Phaeoacremonium,* Diaporthales, Botryosphaeriaceae, Basidiomycetes, and Diatrypaceae (Cloete *et al.*, 2011). These pathogens can lead to the death of various economically important crops (Spiers and Brewster, 1997; Abreo *et al.*, 2012; Phillips *et al.*, 2012).

Fruit and nut trees affected by canker and wood rot pathogens include: kiwi fruit (*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson), apricot (*Prunus armeniaca* L.), cherry (*P. pennsylvanicum* Sarg.), peach (*P. persica* L.), plum (*Prunus salicina* L.), olive (*Olea europaea* L.), grapevine (*Vitis vinifera* L.), almond (*P. dulcis* Mill.), date palm (*Phoenix dactylifera* L.), pear (*Pyrus communis* L.), blueberry (*Vaccinium angustifolium* Aiton), pistachio, (*Pistacia vera* L.), citrus (*Citrus* L.), walnut (*Juglans regia* L.), raspberries (*Rubus idaeus* L.) and cranberry (*Vaccinium macrocarpon* Aiton) (Spiers and Brewster, 1997; Ogata *et al.*, 2000; Moleleki *et al.*, 2002; Copes and Hendrix, 2004; Catal *et al.*, 2007; Damm *et al.*, 2007; Halleen *et al.*, 2007; Slippers *et al.*, 2007; Damm *et al.*, 2008a; Cloete *et al.*, 2011; Udayanga *et al.*, 2014; Elfar *et al.*, 2013; Arzanlou *et al.*, 2014; Martín *et al.*, 2014; Gramaje *et al.*, 2015).

Ornamental and forest trees which can be affected include willow (*Salix* sp.), hop bush (*Dodonaea viscosa*), Southern live oak (*Quercus virginiana* Miller), European ash tree (*Fraxinus excelsior* Linn), pine tree (*Pinus radiate* D. Don), Swedish whitebeam (*Sorbus intermedia* (Ehrh.)), elm tree (Ulmus L.) popular (*Populus* L.), scholar (*Styphnolobium japonicum* L.) and eucalyptus (*Eucalyptus obliqua* L'Hér.) (Smit *et al.*, 1996; Smit *et al.*, 1997; Spiers and Brewster, 1997; Adams *et al.*, 2002; Adams *et al.*, 2006; Halleen *et al.*, 2007; Damm *et al.*, 2008a; Gomes *et al.*, 2013; Arzanlou *et al.*, 2014; Fan *et al.*, 2014; Arzanlou and Narmani, 2015; Fan *et al.*, 2015; Gramaje *et al.*, 2015).

In the Western Cape of South Africa a larger number of horticultural crops including grapevines, stone, pome and citrus fruit are produced (Grab and Craparo, 2011). These orchards and grapevines are in close proximity to one another as well as to forest tree species (Ridgway *et al.*, 2011; Úrbez-Torres, 2011; Arzanlou *et al.*, 2014). Therefore, orchards, forest trees, and fruit trees may serve as inoculum sources for canker and wood rot pathogens for one another (Arzanlou *et al.*, 2014).

Cloete *et al.* (2011) inoculuated possible pathogens isolated from pear and apple orchards on detached grapevine, pear, and apple shoots. Some of the isolates obtained from mature pear orchards formed significant lesions on detached Granny Smith apple shoots (Cloete *et al.*, 2011). These pathogens included *Neofusicoccum vitifusiforme* (van Niekerk &

Crous) Crous, Slippers & A.J.L. Phillips, *Phaeoacremonium iranianum* L. Mostert, Gräfenhan, W. Gams & Crous, and *Paraconiothyrium brasiliense* Verkley; which is now named *Didymosphaeria rubi-ulmifolii s.l.* (Cloete *et al.*, 2011; Ariyawansa *et al.*, 2014). *Neofusicoccum* australe and *Phaeoacremonium fraxinopennsylvanicum* (T.E. Hinds) D. Gramaje, L. Mostert & Crous were also isolated from apple*,* but has been previously reported on apple in South Africa (Table 3).

Phaeoacremonium viticola J. Dupont, *Paraconiothyrium variabile* Riccioni, Damm, Verkley & Crous which is now known as *Didymosphaeria variabile* (Riccioni, Damm, Verkley & Crous) Ariyawansa & K.D. Hyde and *Phomopsis* sp. 7 isolated from apple did not cause significant lesions on apple shoots, but did caused lesions on pear or grapevine shoots. These fungi should not presently be disregarded as possible pathogens of apple since the pathogenicity test were only incubated for 14 days (Cloete *et al.*, 2011). *Didymosphaeria variabile* was also isolated from necrotic peach and plum wood by Damm *et al.* (2008b) and also found pycnidia formed on the *P. persica* necrotic material. *Diaporthe foeniculina* did not form significant lesions on any of the shoots tested, however, it is known to cause disease on citrus, specifically lemons (Udayanga *et al.*, 2014).

MANAGEMENT STRATEGIES TO CONTROL CANKER AND WOOD ROT PATHOGENS

Canker and wood rot pathogens penetrate and colonise the host phloem and xylem, making chemical control difficult (Brown-Rytlewski and McManus, 2000; Wang *et al.*, 2011; Wang *et al.*, 2014; Zhang *et al.*, 2014). Currently, there are no reliable curative control measures for canker and wood rot diseases, making preventative control essential (Wang *et al.*, 2014). The best management strategy is to reduce the inoculum present in the orchard with an integrated disease management program (Arauz and Sutton, 1990).

Cultural practices

Cultural control practices, such as sanitation practices, should be used to reduce and prevent infection (Carter, 1983; Biggs, 2004). Fruiting bodies Ascomycetes and Basidiomycetes such as *Valsa leucostoma* (Pers.) Fr. and *C. purpureum*, respectively, are found in orchards where they serve as inoculum source (Bertrand and English, 1976; Spiers and Brewster, 1997). It is, therefore, important to remove the dead and diseased parts of the tree, as well as dead trees, contaminated with these fungi (Cooley and Autio, 2011). The entire orchard can be replanted or rebudded to decrease the incidences of canker pathogens and increase the productivity of the orchard. The goal of sanitation practices is to remove and reduce inoculum sources from the orchard, delaying and slowing epidemics as well as decreasing the incidence and severity of the disease (Cooley and Autio, 2011). Sanitation practices are most effective when done in the winter or early spring (Cooley and Autio, 2011). It is, important not to prune during wet

weather or after rain since it has been proven that spores are released after rainfall (Bertrand and English, 1976; Carter, 1983). It is most beneficial if the farmer prune at the time most unfavourable for infection for a specific canker and wood rot pathogen (Matthee and Thomas, 1977b).

Summer pruning can also be used as a sanitation practice by removing foliar, fruit and canker infected tissue (Cooley and Autio, 2011). The purpose of summer pruning is to alter the microclimate and architecture of the apple canopy to favour an increase in fruit quality and quantity. Sanitation practices can be applied during summer pruning to reduce the inoculum source, however, this is not the primary purpose since sanitation practices can alter the canopy architecture in ways that do not adhere to horticultural goals (Cooley and Autio, 2011). Sanitation practices during summer pruning are more readily used to reduce the incidence of foliar diseases but have also had an effect on *D. seriata* (Black rot) and *B. dothidea* (White rot) (Cooley and Autio, 2011).

Sanitation practices during summer pruning are not that beneficial for canker disease management since it creates wounds which can be infected during a time when trees are more prone to canker development (Brown-Rytlewski and McManus, 2000). The fresh wounds are also more susceptible to infection with pathogens such as *Valsa mali* Miyabe & G. Yamada (Ke *et al.*, 2014). Summer pruning can be beneficial for controlling pathogens which can cause disease as both cankers and fruit decay such as *B. dothidea* and *D. seriata*, the removal of affected branches will reduce the inoculum sources for fruit and canker infection (Brown-Rytlewski and McManus, 2000). Pruning cuts should be smooth and at an angle of 45° which will ensure that the excess water can easily run off (Matthee and Thomas, 1977b). When water accumulates on the surface of the wound, spores can also accumulate and infect the wound easier (Matthee and Thomas, 1977b).

Climatic change is also a big concern for the agriculture industry since the rainfall and temperature patterns affect the yield directly as well as indirectly when the trees are more susceptible to various pests and diseases (Valiuškaitė and Raudonis, 2008; Menapace *et al.*, 2015). The farmer can apply control strategies to counter the possible negative effect climate change can have on dieback. Management strategies include changing planting and harvesting times, irrigation and fertilizer applications, and cultivar selection (Menapace *et al.*, 2015).

Pruning wound protectants

Pruning wound susceptibility

Winter, spring and summer pruning leave fresh wounds on the trees which are susceptible to canker and wood rot pathogens. After an apple tree sustains a wound, a layer of cells situated between the wood and the bark becomes active and starts dividing (Matthee and Thomas,

1977b; Biggs, 1990). For the wound to heal the cells grow from the edges of the wound and form callus tissue which eventually covers the wound completely (Biggs, 1990). The duration of the healing process depends on the size of the pruning wound and the vigour of the tree and can take more than one season to complete (Matthee and Thomas, 1977b).

Studies conducted on the susceptibility of pruning wounds on grapevines found that the pruning wound susceptibility is dependent on the time of pruning and the age of the pruning wound (Halleen *et al.*, 2010). The susceptibility of the pruning wound is also influenced by the specific canker and wood rot pathogen infecting the wound. It was found that grapevine pruning wounds were susceptible to *Phomopsis* and *Botryosphaeria* species for only 3 weeks, whereas susceptibility of the pruning wounds to *P. minimum* and *D. seriata* was up to 16 weeks (Kotze *et al.*, 2011). All of these pathogens have been found to cause disease on apple trees in South Africa. However, the susceptibility of apple pruning wounds against these pathogens have not been investigated.

Wounds made on apple trees healed quicker than wounds made on sweet cherry and peach trees indicating that wounds on apple trees are more resistant to infection from pathogens such as *Cytospora* species (Biggs, 1990). Wounds made on fruit trees or grapevines are to some degree susceptible to canker and wood rot pathogens until the healing process is complete (Biggs, 1990). It is therefore very important to use a suitable pruning wound protectant during this time. The time of pruning wound application, type of wound protectant as well as the correct application of the product is important to ensure full coverage against the pathogens (Matthee and Thomas, 1977b).

Aspects of a good pruning wound protectant
A study done by Matthee and Thomas (1977b) on wound protectants recommended that the product should contain a few characteristics to be considered as a suitable product. In short, the protectant should be easy to apply and form a watertight yet elastic layer over the wound. The protectant should be resistant to melting or cracking and should not be able to wash off by rain (Matthee and Thomas, 1977b). The effectiveness of fungicides can quickly deteriorate and can easily be washed off leaving the wounds unprotected against infection (Mutawila *et al.*, 2011).

It is also important that the wound protectant contains a suitable fungicide which can inhibit the fungal growth of dieback pathogens (Matthee and Thomas, 1977b). Van Niekerk *et al.* (2011) found that the commercial wound protectant ABE Tree Seal, which is a non-fungicidal protectant, did not show any inhibition of pathogen infection on grapevine pruning wounds compared to the water control treatment. Their findings indicated that a non-fungicidal protectant was not effective in the prevention of grapevine trunk disease pathogen infection (van Niekerk *et al.*, 2011). Matthee and Thomas (1977b) also recommend that the protectant

should not be phytotoxic to the host, but rather induce callus formation to enhance the healing process. Lastly it is very important to apply the product correctly, since a good product can give poor control when applied incorrectly (Matthee and Thomas, 1977b).

Chemical control

Wounds made during pruning or sanitation practices should be protected with an effective fungicide-containing protectant (Sutton *et al.*, 2015). The protectant is usually in a paste form which can be applied with a brush. This paste provides protection against new infection and reinfection (Matthee and Thomas, 1977b; Sutton *et al.*, 2015). Currently there are no curative fungicides available to heal an infected wound. However, the protectant can control reinfection through killing mycelial growth which might still be present in the bark after infected branches were removed (Sutton *et al.*, 2015). Fungicides can be washed away when applied shortly before rainfall, resulting in a reduction in control (Kotze *et al.*, 2011). It is recommended that pruning as well as applying a pruning wound protectant should not be done if there is a chance of rain (Matthee and Thomas, 1977b). A second application can be applied if unexpected rainfall has occurred shortly after application (Kotze *et al.*, 2011).

Various fungicides are known to protect infection sites such as poluoxin, oxine-copper, and guazatine (Sutton *et al.*, 2015). A study done by Matthee and Thomas (1977b) on the effectiveness of different pruning-wound protectants containing different fungicides tested against basidiomycete wood-rotting fungi on apple trees indicated that pyracarbolid (2-methyl-5,6-dihydro-4-H-pyran-3-carboxylic acid analide) provided the best inhibition. Pyracarbolid was mixed with a range of different wound protectants and it was found that a homopolymeric polyvinyl acetate base provided adequate protection (Matthee and Thomas, 1977b). The wound protectant homopolymeric polyvinyl acetate base exhibited good characteristics such as non-phytotoxic, inexpensive, stimulated callus formation, had good tenacity ability, and ease of application as well as a good fungicidal effect. In combination with pyracarbolid, it was the most effective product in their study (Matthee and Thomas, 1977b).

A wound-protecting product, Garrison, provided significant protection of wounds made on MM106, M793 and Golden Delicious apple trees against *C. purpureum* (Spiers and Brewster, 1997). Garrison provides effective control of *C. purpureum* on apples, pears, and willows, but not on stone fruit (Spiers and Brewster, 1997). This product is, however, only registered on grapevines against *E. lata* infection and other grapevine trunk diseases in Australia (APVMA, 2007). A study done on pruning-wound protectants on apricot orchards found that treating pruning wounds individually with a wound protectant is not always practically possible in large commercial orchards (Carter, 1983). Thus, they recommended applying a spray containing benomyl applied with a high-pressure pneumatic system. This proved to control *E. lata* and *C. purpureum* in apricot orchards (Carter, 1983).

This method can be especially effective when controlling canker pathogens such as *B. dothidea* and *D. seriata* which can also infect the fruit causing post-harvest decay (Brown-Rytlewski and McManus, 2000). Several *Botryosphaeria* species are sensitive to fungicides such as benomyl and tebuconazole (Sutton *et al.*, 2014). Fungicide sprays can be applied in late autumn after harvest and in early spring after pruning (Sutton *et al.*, 2014). Currently, two products, namely Neocil-Plus and Steriseal, are registered as general pruning wound protectant products in South Africa and can be used on apple trees (van Zyl, 2015).

Biological control

The use of fungicides are becoming more restricted as a result of the heightened public concern for a safe environment, thus alternative wound protectants should be developed to replace chemical methods (Mutawila *et al.*, 2011). The use of an effective biological control agent on susceptible pruning wounds can potentially offer long-term protection (Kotze *et al.*, 2011). *Fusarium lateritium* Nees was tested as a pruning wound protectant of apricot wounds and it was found that *F. lateritium* can be used as a biological control agent to control *E. lata* and *C. purpureum* infection. This biological control agent can be used as an alternative to benomyl (Carter, 1983).

Trichoderma species have been investigated most often as biocontrol agents to control various diseases including wood rot pathogens on pruning wounds of various woody hosts (Fravel *et al.*, 2012). *Trichoderma*-based biological control agents are the only registered grapevine pruning wound protectant available in South Africa (Halleen *et al.*, 2010; Mutawila *et al.*, 2011). *Trichoderma* species have a suppressive effect on grapevine trunk disease pathogens and it can be due to the plant-fungi interaction and the antagonistic properties of *Trichoderma* to grapevine trunk disease pathogens (Kotze *et al.*, 2011; Mutawila *et al.*, 2011). A study was done by Kotze *et al.* (2011) that indicated that the *in-vitro* tests indicated different antagonistic properties including mycoparasitism, antibiosis by volatile and non-volatile compounds and competition for nutrients and space.

The ability of *Trichoderma* species to colonise pruning wounds and sustain its presence during unfavourable environmental conditions, indicates that *Trichoderma*-based protectants can provide long-term protection against dieback pathogens (Kotze *et al.*, 2011). In New Zealand, the Trichoprotection® range from Agrimm® has a variety of *Trichoderma* products containing *Trichoderma harzianum* Rifai and *Trichoderma atroviride* P. Karst. which are registered for protection against *Eutypa* infection on grapevine (Halleen *et al.*, 2010). *Trichoderma viride* Pers. is a biological control agent present in a few commercial products such as Trichoseal and Tricospray, which are registered as a pruning wound protectant in New Zealand (Spiers and Brewster, 1997). Trichoseal and Trichospray products were applied to wounds made on apple and pear rootstocks in New Zealand but it was found that the

products did not protect the wounds against *C. purpureum* infection (Spiers and Brewster, 1997). *Trichoderma viride* did, however, protect apple wood blocks from decay by the wood rotting fungus *T. versicolor* (Ogawa and English, 1991). In South Africa, *Tr. atroviride* isolate USPP-T1 proved very effective at reducing infection of grapevine pruning wounds (Kotze *et al.*, 2011). The potential of this isolate should be studied further as well as tested on apples. Currently, there are no biological control agents registered for apple trees in South Africa.

Alternative control methods

Cultural and chemical practices do not sufficiently control dieback on apple tree. It is thus important to investigate the potential of alternative control methods (Adams *et al.*, 2002).

Breeding for resistance and induced resistance

Development of resistant cultivars can be achieved by breeding with high-resistance germplasm (Valiuškaitė and Raudonis, 2008; Liu *et al.*, 2011). In one example an apple cultivar with less lenticels showed resistance against Botryosphaeria canker (Liu *et al.*, 2011). It is known that *Botryosphaeria* species can infect peach trees through pruning wounds, damaged tissue as well as directly through lenticels (Weaver 1979; Damm *et al.*, 2007). When breeding for resistance to Botryosphaeria canker, lenticel abundance is a key factor to evaluate.

A study conducted on 4600 Golden Delicious x Jonathan hybrid seedlings by Liu *et al.* (2011) indicated that high-resistance was found towards certain isolates of *B. dothidea* but not to others. This shows that the resistant seedling is not resistant towards all *B. dothidea* isolates and it implies a complicated gene-for-gene interaction (Liu *et al.*, 2011). Apple cultivars vary in susceptibility to *Dia. tanakae* causal organism of *Diaporthe* canker (Sutton *et al.*, 2014). Cultivars Jonagold and Jonathan are more susceptible than Starking Delicious, Tsugara, and Indo (Sutton *et al.*, 2014). These cultivars are not commonly planted in South Africa

Apple trees can activate an efficient and active defence system when shoots, branches or trees are inoculated with an avirulent pathogen (Zhang *et al.*, 2014). This will induce resistance against further infection by a more virulent pathogen (Zhang *et al.*, 2014). Zhang *et al.*, (2014) inoculated 5-year-old Fuji and Gala apple trees with a less virulent isolate namely *Valsa mali* var. *mali* LXS081501, and found that apple cultivars inoculated with the avirulent isolate successfully induced resistance and displayed resistance against the pathogenic *V. mali* var. *mali* pathogen. This shows that successful control can be obtained through induced resistance. However, it is unknown what the resistance reaction would be if more than one canker and wood rot pathogen are involved. The use of resistant cultivars seems like the ideal control method. However, the use of resistant cultivars is not an immediate solution at this

moment since the development of resistant cultivars takes a long time and economically more important diseases such as apple scab take priority in breeding programs (Krishna *et al.*, 2010; Borovinovo *et al.*, 2012). Breeding for resistance is currently very limited, but will become more and more important in the oncoming decades in controlling economically important diseases that occur on fruit trees (Liu *et al.*, 2011).

Micropropagation

Micropropagation or *in-vitro* tissue culture is defined as the propagation of plants through *in-vitro* cloning of different somatic cells, tissue or organs of plants under controlled conditions to regenerate new plants (Dobránszki *et al.*, 2010). The aim of this method is to produce large numbers of progeny plants, which are disease-free and genetically identical to the mother plant (Dobránszki *et al.*, 2010). Micropropagation has numerous advantages over conventional propagation, such as that micropropagation can be completed in a relatively short time when compared to conventional methods (Abbott and Whiteley, 1975; Dobránszki *et al.*, 2010). The main advantages of micropropagation are the capability to produce large numbers of trees with desired horticultural qualities; the ability to produce progeny plants year round under controlled *in-vitro* conditions; production of disease-free plants; possibility of reproducing genotypes which are difficult to produce under normal nursery practices; as well as a high reproduction rate (Dobránszki *et al.*, 2010; Laubcher, 2016).

Currently, the possible use of micropropagation to combat canker and wood rot pathogens which infect apple and stone fruit trees during propagation is being investigated (Laubcher, 2016). The first results for the cherry tissue cultured trees are promising as only one tree died compared to the death of numerous trees planted in nearby locations propagated with conventional rootstocks. This research is still in the laboratory research phase but can become a big scale possibility in the near future (Laubcher, 2016).

Plant health

Reduced stress

Apple trees are more susceptible to canker pathogens when exposed to abiotic stresses (Abdollahzadeh, 2015). These stresses are responsible for low productivity in apple orchards and contribute to disease development and should be kept to the minimum (Krishna *et al.*, 2010; Marek *et al.*, 2013). An important preventative practice for dieback management is to maintain tree health, vigour and reduce stress (Krishna *et al.*, 2010).

Abiotic stresses experienced by the young nursery tree during propagation, during storage, as well as during planting play a major role in disease expression during the establishment of an orchard (Marek *et al.*, 2013). When removing the trees from the nursery soil it is important that the nursery trees have defoliated completely (Theron and Steyn, 2016).

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The application of chemical or manual defoliation practices to the nursery trees should not be done too early in autumn since this practice will ensure untimely bud break (Theron and Steyn, 2016). Symptom expression of pathogens *Diaporthe* specie and *Didymella* species has been observed when rainfall occurred during the time of lifting (Marek *et al.*, 2013). It is thus important to time the lifting of trees correctly.

Several studies have found that drought stress is the main physiological stress factor influencing symptom expression (Brown-Rytlewski, and McManus 2000). Dehydration of nursery trees during storage and unfavourable storage conditions, especially temperature fluctuation during storage, will contribute to disease expression (Marek *et al.*, 2013). Stress factors should be minimized as far as possible by providing an optimum environment during storage by keeping the trees well hydrated and between 1°C and 4°C before transporting them to the farmer (Marek *et al.*, 2013).

Drought conditions during planting and during the growing season should be avoided. Apple trees infected with *C. purpureum* can recover from infection when fertilizers are applied to increase the vigour of the tree. An increase in vigour will result in a stronger tree (Sutton *et al.*, 2015). When establishing an orchard it is important to start with quality, registered disease-free large nursery trees to ensure that the trees grow to their full potential from the start (Theron and Steyn, 2015). Larger trees have a higher vigour compared to weaker smaller nursery trees which lead to a higher survival rate of larger trees after establishment (Theron and Steyn, 2015).

Application of arbuscular mycorrhizal fungi (AMF) to the soil or to the tree roots can ensure a mutualistic relationship. When plants have a mutualistic relationship with AMF, the trees can grow more vigorously and subsequently be more tolerant to pathogen attack (Krishna *et al.*, 2010). Arbuscular mycorrhizal fungi is a symbiont. This mutualistic relationship will ensure improved plant health by increasing plant nutrient uptake, disease resistance, water uptake, ecosystems establishment, as well as an increased productivity of the plant (Krishna *et al.*, 2010).

THE DECIDUOUS PLANT CERTIFICATION SCHEME

Background and goal of the Scheme

A scheme was established in 1960 by the deciduous fruit industry to determine if the plant material used for propagation are of quality (van Rensburg, 1997). The Scheme, namely the Deciduous Fruit Plant Certification Scheme, was published by the Government Notice no. R. 1971 on 15 October 1993 (PlantSA, Paarl, South Africa). This provided inspection of visually clean plant material (van Rensburg, 1997; Mostert *et al.*, 2016). The last revised version of the Scheme is dated 28 January 2011 (PlantSA, Paarl, South Africa).

The Scheme provides certified plant material which prescribes to a minimum requirement based on the instruction of the Plant Improvement Act (Act 53 of 1976) and the Agricultural Pest Act (Act 36 of 1983) (van Rensburg, 1997). The Scheme ensures that the nursery trees supplied to the farmers are disease and pest free. Over time, a decline in the quality of plant material could be observed and that is largely due to the accumulation of harmful pathogens and genetic degeneration (van Rensburg, 1997). It is thus of the utmost importance that the plant material used for propagation should meet the requirement of the Scheme and should be evaluated. Good quality plant material plays a major role in the continuous production of apples in the long term.

In 1954, the Department of Agriculture of South Africa recognised that the physical, phytosanitary and genetic status of the propagation material were the main factors contributing to the growth in agricultural production (van Rensburg, 1997). In 1974, the South African Plant Improvement Organisation (SAPO) was established to ensure that the physical, phytosanitary and genetic requirements set out by the Scheme for the production of apple trees are met (Mostert *et al.*, 2016). After the establishment of SAPO, two private organisations, specifically Topfruit and Stargrow, were established (van Rensburg, 1997; Mostert *et al.*, 2016). SAPO, Stargrow, and Topfruit are the three plant improvement organisations (PIO) of South Africa (Mostert *et al.*, 2016). The PIO's maintain a regulated system of plant improvement and it is their duty to ensure disease-free registered nucleus, foundation and mother blocks which comply with the theoretical and practical requirements set out by the Scheme (Mostert *et al.*, 2016).

Inspectors

Internal inspectors appointed within the PIO's inspect the nucleus, foundation and mother blocks. These inspectors will search for virus free material with favourable characteristics, such as the colour of the fruit. They will also continuously search for unusual characteristics in the plant material and take precautionary measures to prevent the collection of deviant plant material for further propagation (Mostert *et al.*, 2016). During the nursery process, contractual inspectors visit the nurseries three times a year to ensure that the trees sold to the farmers are certified as disease-free, true to type and assigned to specific size class. The inspectors are contracted by the Deciduous Fruit Plant Improvement Association (DPA) and their role is to ensure that the nursery trees meet the physical requirement set out by the Scheme (van Rensburg, 1997; Mostert *et al.*, 2016). The DPA was founded in 1990 by the industry and the goal of the association is to supply improved plant material. The inspectors visit the nurseries during the summer, autumn and winter, and mark all plant material with disease or unusual symptoms which should be removed and destroyed (Mostert *et al.*, 2016). The DPA implement traceability checks on all the nursery trees to ensure that the plant material comes from a

registered and certified mother block (Mostert *et al.*, 2016). The nurseries are also obligated to submit a nursery-return to the DPA which includes information regarding the origin and certification of the scion and rootstock material used for each combination planted (Mostert *et al.*, 2016).

Plant material must be clean from pest and diseases

According to the Scheme, plant material in registered blocks belonging to the PIO's must be visually clean from pathogens, insects as well as viral diseases (van Rensburg, 1997). The rootstock plant material must be free from 22 viral diseases whereas the scion plant material must be clean from only four viral diseases. Nucleus, foundation, and mother blocks are tested for specific viruses via ELISA or PCR on a regular basis. Both the scion and rootstock plant material must be visually clean of various bacterial, fungal and Oomycete pathogens. Of the different canker and wood rot diseases only, *C. purpureum*, are listed in the Scheme. Steps should be taken to ensure that the plant material in the established nucleus, foundation and mother blocks are clean from harmful diseases (Mostert *et al.*, 2016). Phytosanitary processes are used to eliminate viruses, viroids, mycoplasmas and bacteria from the plant material. Tissue culture or meristem culture, heat treatment at 40°C, hot water treatment and embryogenesis are used to ensure disease-free nucleus material (van Rensburg, 1997; Dobránszki and da Silva, 2010).

Nursery process

Budding

Propagation of apples are primarily done through vegetative methods, specifically budding (Dobránszki and da Silva, 2010). Budding can be defined as the transplantation of a scion bud onto a rootstock seedling in the nursery (Turechek, 2004; Dobránszki and da Silva, 2010). The plant material received by the nurseries for the propagation of new apple trees is controlled by the three PIO's and comply with the Scheme (Mostert *et al.*, 2016). The propagation of new apple trees is demonstrated in Figure 1. During the propagation of new apple trees, rooted rootstock plants are planted out in fumigated nursery soil in July of year one (Fig 1B, C) (Marek *et al.*, 2013). The Western Cape has a winter rainfall (van Schoor *et al.*, 2009) which allows the rootstock plant to establish a root system in the soil during the winter rain period (Marek *et al.*, 2013).

The shoot and the roots of the rootstock plant will grow for a year in the nursery soil (Fig. 1D) before a scion bud (Fig. 1E) is budded onto the sapling in February of year two. If budding was successful, the scion bud will start to bud in the spring of year two. The rootstock shoot will be cut back when the budding was successful (Fig. 1F) (Marek *et al.*, 2013). The scion shoot starts to develop and will grow until the end of summer (Theron and Steyn, 2016).

All the lateral shoots are removed from the main shoot to ensure a nursery tree with only one shoot (Fig. 1I). The first inspection from the contractual inspectors takes place when the budded trees are approximately one meter tall (Mostert *et al.*, 2016). The second inspection occurs in the autumn of year three. During the first and second inspection, the inspectors will look for unusual characteristics and mark the trees to be destroyed when lifted from the soil (Mostert *et al.*, 2016).

Paradormancy

During the autumn of year three, when the environmental temperature is moderate and irradiation is high, the tree will enter paradormancy, or otherwise known as correlation inhibition (Theron and Steyn, 2015; Theron and Steyn, 2016). This is temporary dormancy which is controlled within the tree; the extent of paradormancy determines the final physical quality of the tree (Dennis, 1994; Theron and Steyn, 2016). During paradormancy, the tree hardens off in preparation for endodormancy which occurs during the winter (Dennis, 1994). The tree will develop lateral and terminal buds during paradormancy (Theron and Steyn, 2016). The extent to which these buds develop during this phase of dormancy will play a determining role in how vigorous the trees will grow once planted out into a new orchard (Theron and Steyn, 2015). The leaf primordia will develop at a rate of one per week, thus, the development of a good terminal bud takes 6 to 7 weeks to develop (Theron and Steyn, 2015). During paradormancy, the tree stores reserves, specifically nitrogen and carbohydrates and will also develop a more comprehensive root system (Theron and Steyn, 2016). A well-formed root system, as well as sufficient reserves, are needed in the spring during transplantation of trees into new orchards (Theron and Steyn, 2016).

Final inspection and classification

In early winter, before winter inspection, when the trees have entered endodormancy the trees are lifted from the nursery soils (Marek *et al.*, 2013). After the trees are removed they are graded into different size classes according to the diameter of the base of the scion shoot and height (Fig. 1J) (Marek *et al.*, 2013; Mostert *et al.*, 2016). The size classes include Tall Large, which is 15 mm or larger in diameter and taller than 1.8 m; Large, which is 15 mm or larger in diameter and shorter than 1.8 m; First, 12-15 mm in diameter; Medium, 10-12 mm in diameter; Standard, 8-10 mm in diameter and Small, 7 mm in diameter (Theron and Steyn, 2015). There are advantages and disadvantages to both smaller and larger trees. For smaller trees, a higher mortality rate has been found in smaller trees since it is a weaker tree in general and cannot fill its allocated space in the orchard as quickly as a bigger tree (Theron and Steyn, 2015). The larger trees are more desired by farmers and thus to increase the amount of larger trees produced, the producers often force nursery growth until late in autumn. This can lead to a

weak physiological quality in the larger trees (Theron and Steyn, 2015). Larger trees also have a higher shoot to root ratio and the risk of high root loss during lifting are higher thus a larger tree can suffer more transplant shock (Theron and Steyn, 2015).

After classification, the trees are presented for final inspection and certification (van Rensburg, 1997; Mostert *et al.*, 2016). During final or winter inspection the trees are evaluated to determine if they adhere to the physical requirements as well as if they developed a well-formed bud union and acceptable shoot and root growth (Mostert *et al.*, 2016). If the trees do not adhere to the physical requirements, or any insects or diseases are visible, the inspector will reject the tree (van Rensburg, 1997). The nurseries have 14 days to re-select trees for final inspection (Mostert *et al.*, 2016). Only trees that meet all the requirements will be authorised with a blue certification label. The blue certification labelling system guarantees the farmers that the nursery trees met the minimum requirement as set out by the Scheme (Mostert *et al.*, 2016).

Storing of nursery trees

Trees can be stored in cold store rooms or outside in sawdust beds before being sold to the farmers (Marek *et al.*, 2013). In cold storage, the trees are exposed to temperatures around 4°C which can be beneficial to the trees before planting when the outside temperatures in the nurseries are not cold enough for required winter chilling (Theron and Steyn, 2016). When a lack of winter chilling is observed in milder winter climates, the growth of the buds can be affected. This can result in an expression of basal dominance in the tree once planted out in the new orchards (Theron and Steyn, 2016). Planting trees in sawdust beds in the nursery are labour intensive and time-consuming (Marek *et al.*, 2013). Cold storage also provides better temperature and humidity control to ensure that the trees stay endodormant (Marek *et al.*, 2013).

Cold store rooms can, however, have a progressive effect on canker development when temperatures are not between 1°C and 4°C or the store rooms are not properly disinfected. Marek *et al. (*2013) found that a latent infection in the apple nursery trees was more likely to be expressed in cold storage when abiotic stresses were present in the nursery, during lifting, grading as well as during storage. Apple trees are more susceptible to canker pathogens when exposed to biotic and abiotic stresses (Abdollahzadeh, 2015) since these trees are sensitive to various factors such as fluctuation of temperature (Valiuškaitė and Raudonis, 2008). These stresses are responsible for low productivity in apple orchards and contribute to disease development (Krishna *et al.*, 2010; Marek *et al.*, 2013). Stress factors should be minimized as far as possible by providing an optimum environment during storage by keeping the trees well hydrated and between 1°C and 4°C before transporting them to the farmer (Marek *et al.*, 2013).

Farmers establish new orchards in spring using the certified plant material obtained from the nurseries. However, recently canker development has been observed on the 1-year-old trees shortly after establishment. Hortgro Science was assigned by the Rootstock Evaluation Committee (REC) to investigate the high incidence of young Geneva® rootstocks deaths due to wood rot pathogens. The committee concluded that a more scientific study is needed to determine the prevalence of canker and wood rot pathogens in both apple orchards and nurseries. Thus this thesis will investigate the occurrence of canker and wood rot pathogens in young apple trees and possible inoculum sources.

CONCLUSION

Apples are one of the most important deciduous fruit crops being produced in South Africa, contributing to the revenue produced from exporting deciduous fruit, mainly to African countries (Hortgro, 2015). An increase in apple plantation have been observed in the Western Cape over the past 8 years, however, the average age of the orchards are old and re-establishment of orchards will be inevitable in the near future (Theron and Steyn, 2015). With the increased establishment costs, it is vital for farmers to start new orchards with disease free, quality plant material to ensure that the orchards can reach its full producing potential as soon as possible (Hortgro, 2015; Theron and Steyn, 2015).

Canker and wood rot pathogen species can infect and colonise its host causing decline on apple and other woody host trees worldwide (Zhang *et al.*, 2014; Menapace *et al.*, 2015). Even though canker and wood rot symptoms are commonly seen in older orchards in South Africa, the incidence and severity of the decline and death of young apple trees are still largely unknown. Various species of canker and wood rot pathogens have been identified as causal organisms for the decline of mature apple trees in South Africa (Cloete *et al.*, 2011). Recently canker development has been observed on 1-year-old trees shortly after establishment. Little information is available on the species involved in the decline of young apple trees shortly after planting.

AIM AND OBJECTIVES

The aim of this study was to identify inoculum sources for canker pathogens of young apple orchards including propagation material, nursery trees and newly planted apple orchards. The objectives were:

1. To determine the incidence of 1-year-old orchards showing decline symptoms shortly after establishment in three apple producing areas in the Western Cape and to determine the canker and wood rot pathogens involved in the decline.
2. To evaluate the incidence of latent infections in seemingly healthy certified nursery trees.

3. To evaluate mother plant material used during the propagation of apple trees.

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TABLE AND FIGURES

Table 1. Ascomycete pathogens associated with canker on apple trees.

Taxonomic group	Casual organism [a]	Country	Reference
Amphisphaeriaceae	*Seiridium unicorne* (Cooke & Ellis) B. Sutton (Monochaetia twig canker)	USA	Sutton *et al.*, 2015
Botryosphaeriaceae	*Botryosphaeria dothidea* (Moug.) Ces. & De Not.	Australia, Argentina, Brazil, China, Japan, South Africa, USA	Brown-Rytlewski and McManus, 2000; Crous *et al.*, 2000; Biggs, 2004; Liu *et al.*, 2011
	Botryosphaeria ribis Grossenb. & Duggar	India, South Africa	Crous *et al.*, 2000; Krishna *et al.*, 2010
	Botryosphaeria stevensii Shoemaker (*Diplodia* canker)	Worldwide (Including South Africa)	Sutton *et al.*, 2015
	Diplodia bulgarica A.J.L. Phillips, J. Lopes & Bobev 2012	Bulgaria, Iran	Phillips *et al.*, 2012
	Diplodia mutila (Fr.) Mont.	New Zealand, USA	Slippers *et al.*, 2007
	Diplodia seriata De Not.	Bulgaria, Japan, South Africa, USA	Brown-Rytlewski and McManus, 2000; Slippers *et al.*, 2007; Cloete *et al.*, 2011; Borovinova *et al.*, 2012;
	Neofusicoccum australe Crous, Slippers & A.J.L. Phillips	South Africa	Slippers *et al.*, 2007
	Neofusicoccum ribis (Slippers, Crous & M.J. Wingf.) Crous, Slippers and A.J.L. Phillips	USA	Slippers *et al.*, 2007
	Neofusicoccum parvum (Pennycook & Samuels) Crous, Slippers and A.J.L. Phillips 2006	New Zealand	Slippers *et al.*, 2007
Cadophora	*Cadophora luteo-olivacea* (J.F.H. Beyma) T.C. Harr. & McNew	Italy	Spadaro *et al.*, 2011
Dermateaceae	*Cryptosporiopsis corticola* (Edgerton) Nannf.	South Africa	Crous *et al.*, 2000
	Neofabraea malicorticis H.S. Jacks.*, N. perennans* Kienholz (Anthracnose canker and Perennial canker)	Canada, Europe, USA	de Jong *et al.*, 2001; Gariépy *et al.*, 2003; Sutton *et al.*, 2015
Diaporthales	*Cytospora chrysosperma* (Pers.) Fr.	Iran	Mehrabi *et al.*, 2011
	Cytospora schulzeri Sacc. & P. Syd.	China, Iran, Japan, South Korea, South Africa, USA	Cloete *et al.*, 2011; Mehrabi *et al.*, 2011; Wang *et al.*, 2011; Wang *et al.*, 2014
	Diaporthe ambigua Nitschke	South Africa	Smit *et al.*, 1996; Smit *et al.*, 1997; Crous *et al.*, 2000; Moleleki *et al.*, 2002
	Diaporthe eres Nitschke	Europe, Japan, South Africa, Uruguay, USA	Smit *et al.*, 1996; Smit *et al.*, 1997; Crous *et al.*, 2000; Abreo *et al.*, 2012; Sutton *et al.*, 2015

Table 1. Continue.

Taxonomic group	Casual organism [a]	Country	Reference
Diaporthales	*Diaporthe foeniculina* (Sacc.) D. Udayanga & L.A. Castlebury	South Africa	Cloete *et al.*, 2011
	Diaporthe perniciosa Marchal & É.J. Marchal	New Zealand, South Africa	Smit *et al.*, 1996; Crous *et al.*, 2000; Gomes *et al.*, 2013
	Diaporthe tanakae Tak. Kobay. & Sakuma (Diaporthe canker)	Japan	Sutton *et al.*, 2015
	Leucostoma cinctum (Fr.) Höhn. (Leucostoma canker)	Bulgaria, Iran, USA	Mehrabi *et al.*, 2011; Borovinova *et al.*, 2012; Wang *et al.*, 2014; Sutton *et al.*, 2015
	Leucostoma persoonii (Nitschke) Höhn.	China, Japan, South Korea, South Africa	Miyairi *et al.*, 1977; Crous *et* al., 2000; Cloete *et al.*, 2011; Wang *et al.*, 2011
	Phomopsis cotoneastri Punith.	Uruguay	Abreo *et al.*, 2012
	Phomopsis spp.	Bulgaria, USA	Borovinova *et al.*, 2012; Marek *et al.*, 2013
	Valsa ceratosperma (Tode) Maire (Valsa canker)	China, Japan, South Korea	Fisher, 1931; Ke *et al.*, 2014; Sutton *et al.*, 2015
	Valsa nivea (Hoffm.) Fr.	South Africa	Adams *et al.*, 2006
	Valsella melastoma (Fr.) Sacc.	USA	Wang *et al.*, 2014
Diatrypaceae	*Eutypa lata* (Pers.) Tul. & C. Tul.	South Africa	Cloete *et al.*, 2011
Dothideomycetes	*Didymellaceae* spp.	South Africa	Crous *et al.*, 2000
Nectriaceae	*Nectria cinnabarina* (Tode) Fr. (Nectria twig blight)	Europe, Malawi, New Zealand, Germany, USA	Sutton *et al.*, 2015
	Neonectria ditissima (Tul. & C. Tul.) Samuels & Rossman (Nectria canker)	Worldwide except Australia and South Africa	McCracken *et al.*, 2003; Valiuškaitė and Raudonis, 2008; Sutton *et al.*, 2015
Phaeoacremonium	*Phaeoacremonium angustius* W. Gams, Crous & M.J. Wingf. 1996	USA	Rooney-Latham *et al.*, 2006
	Phaeoacremonium fraxinopennsylvanicum (T.E. Hinds) D. Gramaje, L. Mostert & Crous	South Africa, USA	Rooney-Latham *et al.*, 2006; Cloete *et al.*, 2011
	Phaeoacremonium iranianum L. Mostert, Gräfenhan, W. Gams & Crous 2006	Iran	Arzanlou *et al.*, 2013
	Phaeoacremonium minimum (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous	Iran, South Africa	Cloete *et al.*, 2011; Arzanlou *et al.*, 2013
Xylariaceae	*Biscogniauxia marginata* (Fr.) Pouzar (Nailhead canker)	USA	Sutton *et al.*, 2015

[a] If known disease name it is included after the causal organism.

Table 2. Basidiomycete pathogens found worldwide causing wood rot on apple trees.

Casual organism[a]	Country	Decay type	Reference
Abortiporus biennis (Bull.) Singer	New Zealand	White	Sutton *et al.*, 2015
Armilaria spp.	Australia, New Zealand	Brown	Sutton *et al.*, 2015
Bjerkandera adustra (Wild.) P. Karst.	South Africa, USA	White	Matthee and Thomas, 1977a; Crous *et al.*, 2000; Sutton *et al.*, 2015
Chondrostereum purpureum (Pers.) Pouzar (Silver leaf)	Australia; Japan; New Zealand, South Africa	White	Miyairi *et al.*, 1977; Bishop, 1978; Spiers and Brewster, 1996; Crous *et al.*, 2000; Sutton *et al.*, 2015
Coriolopsis gallica (Fr.) Ryvarden	USA	White	Sutton *et al.*, 2015
Flammulina velutipes (Curtis) Singer *sensu lato*	USA	White	Sutton *et al.*, 2015
Fomitopsis pinicola (Sw.) P. Karst.	USA	Brown	Sutton *et al.*, 2015
Ganoderma applanatum (Pers.) Pat.	USA	White	Sutton *et al.*, 2015
Phellinus ferruginosus (Schrad.) Pat.	USA	White	Sutton *et al.*, 2015
Phellinus igniarius (L.) Quél.	USA	White	Sutton *et al.*, 2015
Phlebia radiata Fr.	USA	White	Sutton *et al.*, 2015
Pholiota adiposa (Batsch) P. Kumm.	USA	White	Sutton *et al.*, 2015
Pholiota squarrosa (Vahl) P. Kumm.	USA	White	Sutton *et al.*, 2015
Pleurotus sp.	Brazil, New Zealand	White	Sutton *et al.*, 2015
Schizophyllum commune Fr.	Brazil, Bulgaria, South Africa	White	Crous *et al.*, 2000; Borovinova *et al.*, 2012; Sutton *et al.*, 2015
Stereum spp.	Brazil	White	Sutton *et al.*, 2015
Trametes pubescens (Schumach.) Pilát	USA	White	Sutton *et al.*, 2015
Trametes versicolor (L.) Lloyd	Australia, Brazil, New Zealand, South Africa, USA	White	Matthee and Thomas, 1977a; Sutton *et al.*, 2015
Tyromyces fissilis (Berk. & M.A. Curtis) Donk	Japan	White	Sutton *et al.*, 2015

[a] If known disease name it is included after the causal organism.

Table 3. Incidence of pathogens isolated from 60 young diseased apple trees by the Disease Clinic of Stellenbosch University from 2010 to2016.

Pathogens[a]	Incidence (Trees infected)
Botryosphaeriaceae spp.	31
Diaporthe spp.	22
Basidiomycetes spp.	16
Leucostoma spp.	8
Cytospora spp.	4
Phaeoacremonium spp.	2
Collophora spp.	1
Coniothyrium spp.	1
Eutypella spp.	1

[a] Fungal isolates were identified to genus level.
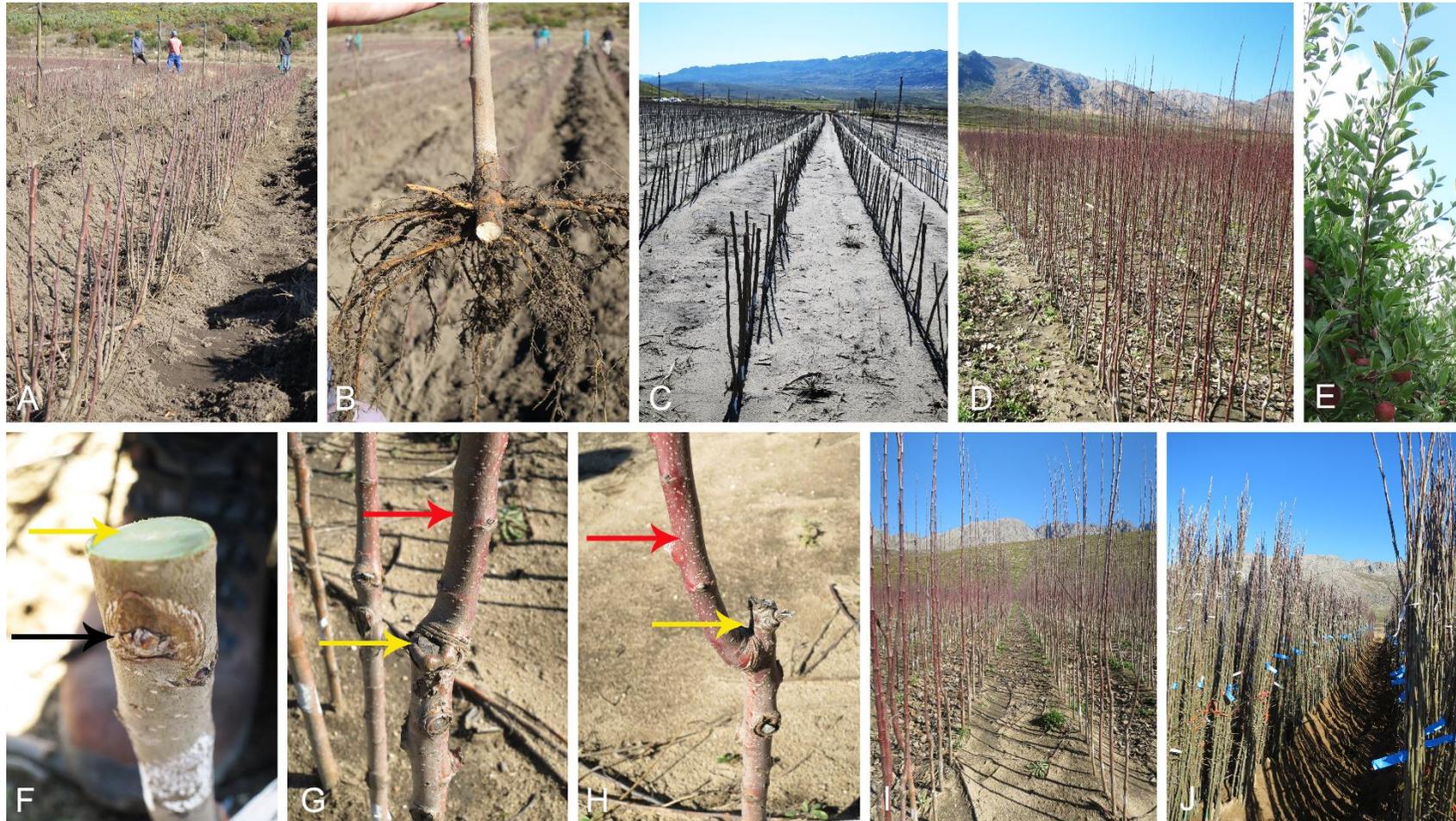
Figure 1. The propagation process of an apple tree via budding. A rootstock layer block (A). A rooted rootstock cutting (B). Rooted rootstock cuttings established in the nursery (C). Rootstock plant in 1-year-old nursery soil (D). Scion shoot with buds (E). Established bud union and new pruning wound made on the rootstock shoot (F). Scion bud developed into a scion shoot with visible pruning wounds (G, H). Nursery trees just before lifting (I). Certified and graded nursery trees ready to be sold (J). Black arrow indicate bud union, yellow arrow indicates pruning wound made on the rootstock shoot and red arrow scion bud which developed into a scion shoot.

CHAPTER 2

Occurrence of canker and wood rot pathogens in young apple trees and propagation material in the Western Cape of South Africa

ABSTRACT

Canker and wood rot pathogens can infect wounds and colonise the vascular tissue of young apple trees causing rapid decline and eventual death when exposed to abiotic stress factors. Recently, a higher occurrence of canker development on 1-year-old apple trees shortly after establishment was observed in apple orchards in the Western Cape Province of South Africa. The source of infection of these trees is unknown and could be latent infections of nursery trees. Knowledge regarding latent infections of canker or wood rot pathogens in certified nursery trees in South Africa is lacking. This study aimed to assess the phytosanitary status of nursery trees in relation to canker and/or wood rot pathogens and to investigate propagation material as possible inoculum sources for stem canker pathogens. Thirteen 1-year-old apple orchards showing canker or dieback symptoms were sampled from three apple producing areas in the Western Cape. Certified nursery apple trees were collected from four nurseries as well as scion and rootstock mother plant material, which are used during propagation of apple trees. The propagation material sampled include cankers and pruning wounds from apple scion mother blocks, 1-year-old scion shoots, rootstock layer blocks, and diseased 1-year-old rootstock shoots. Isolations were made from the discolouration observed in the vascular tissue of the plant parts and from asymptomatic material. Causal organisms involved in the infection of the apple plant material were determined with PCR and sequence comparisons of the relevant genes and phylogenetic analyses. Similar canker and wood rot fungi were isolated from 1-year-old diseased apple trees, nursery apple trees and from the propagation material. A total of 45 fungal species associated with canker or wood rot symptoms reported on fruit trees or other woody hosts were identified in this study. *Didymosphaeria rubi-ulmifolii s.l.* was the dominant fungal specie causing latent infection in the certified nursery apple trees and was also found causing dieback in 1-year-old apple trees. Other canker and wood rot pathogens isolated belonged to the Basidiomycetes, Botryosphaeriaceae and Diatrypaceae. It also included species in the genera *Didymella*, *Didymosphaeria*, *Phaeoacremonium*, *Diaporthe*, *Cadophora*, *Coniochaeta*, *Cytospora* and *Truncatella angustata*. Sixty-five percent of certified nursery apple trees were infected with canker and wood rot pathogens, although, apple producers do not find a similarly high disease incidence in newly established apple orchards. The soils of the 1-year-old apple orchards were analysed, and in most of the cases, were found to be sub-optimal soils, which resulted in

abiotic stress experienced by the young apple trees. The bud unions and pruning wounds on the rootstock were most infected in the nursery trees, suggesting that aerial spores are also sources of inoculum. Basidiomycete and Ascomycete fruiting structures were found on the scion mother block trees, 1-year-old apple trees, and the 1-year-old nursery rootstock shoots which can contribute to aerial inoculum. Propagation material is also a source of inoculum with scion shoots used for budding (5%) and rooted rootstock cuttings from layer blocks (21%) having latent infections of canker and wood rot pathogens. This study found thatcertified nursery apple trees can have latent infections of canker and wood rot pathogens, resulting in the distribution of seemingly healthy trees to producers.

INTRODUCTION

Dieback is a condition where apple trees die prematurely due to the infection of both true and opportunistic canker and wood rot pathogens giving rise to general symptoms such as cankers, twig blight, and wood rotting (Brown-Rytlewski and McManus, 2000; Borovinova *et al.*, 2012; Menapace *et al.*, 2015). The pathogens responsible for canker and wood rot symptoms infect their hosts through wounds and colonise the vascular tissue resulting in blockage of the vascular system (Cloete *et al.*, 2011). Infected trunks, branches, and shoots start dying back, eventually leading to the death of the entire tree or even failure of the orchard (Zhang *et al.*, 2014). The farmer can face major financial damages when the infections are severe enough for the orchards to become uneconomical since dieback limits the longevity and reduce the yield of the host (Elfar *et al.*, 2013).

Seventeen canker pathogens have been reported to cause dieback or canker symptoms on apple trees in South Africa. These include *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Diplodia mutila* (Fr. : Fr.) Mont., *Diplodia seriata* De Not*., Neofusicoccum australe* Crous, Slippers & A.J.L. Phillips*, Cryptosporiopsis corticola* (Edgerton) Nannf*, Cytospora schulzeri* Sacc. & P. Syd*., Diaporthe ambigua* Nitschke*, Diaporthe eres* Nitschke*, Diaporthe foeniculina* (Sacc.) D. Udayanga & L.A. Castlebury*, Diaporthe perniciosa* Marchal & É.J. Marchal*, Leucostoma persoonii* (Nitschke) Höhn*, Valsa nivea* (Hoffm.) Fr*., Eutypa lata* (Pers.) Tul. & C. Tul*., Didymelaceae* species, *Phaeoacremonium fraxinopennsylvanicum* (T.E. Hinds) D. Gramaje, L. Mostert & Crous and *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (Smit *et al.*, 1996; Smit *et al.*, 1997; Crous *et al.*, 2000; Adams *et al.*, 2006; Cloete *et al.*, 2011; Sutton *et al.*, 2015). Four Basidiomycetes species have also been reported to be associated with wood rot symptoms on apple trees in South Africa. These include *Bjerkandera adustra* (Wild.) P. Karst*., Chondrostereum purpureum* (Pers.) Pouzar*, Schizophyllum commune* Fr. and *Trametes versicolor* (L.) Lloyd (Matthee and Thomas, 1977; Crous *et al.*, 2000; Sutton *et al.*, 2015).

Canker and wood rot symptoms are commonly observed in older apple orchards in South Africa. The decline and eventual death of these trees can take an extended period of time (Smit *et al.*, 1996). In contrast, younger trees infected with canker or wood rot pathogens can be killed more rapidly, especially when exposed to abiotic stress factors (Smit *et al.*, 1996; Marek *et al.*, 2013). Studies conducted in Europe on the death of young newly established apple trees found that young trees developed large cankers on the main stems shortly after establishment due to *Cylindrocarpon heteronema* (Berk. & Broome) Wollenw. (Brown *et al.*, 1994) and *Neonectria ditissima* Tul. & C. Tul. Samuels & Rossman (McCracken *et al.*, 2003). Both of these studies concluded that the cankers observed in the young apple orchards originated from latent infections that occurred during the propagation process in the nursery.

Marek *et al.* (2013) investigated the causal agents of cold storage canker of young apple trees. The study found that latent infections in nursery trees caused disease symptoms during cold storage or shortly after planting (Marek *et al.*, 2013). A study done by Fujita *et al.* (1988) found that mature apple trees artificially inoculated with the canker pathogen *Diaporthe tanakae* Tak. Kobay. & Sakuma only exhibited typical canker lesions after 2 years. The time required for symptom development of canker and wood rot together with stress due to cold storage used at producer level explains the absence of symptoms in the nursery.

Recently, a high occurrence of canker development was observed on 1-year-old trees shortly after establishment in the Western Cape Province of South Africa. Serious losses have incurred with two instances of 30% and 70% of newly planted apple trees in orchards that have died due to stem cankers (Disease Clinic, Department of Plant Pathology, Stellenbosch University). Over the past 6 years, 60 trees between the ages of 1 and 3 years, were sent to the Disease Clinic of Stellenbosch University for analysis. These 60 trees represented 60 commercial orchards in the Western Cape with canker or wood rot symptoms. The deciduous fruit industry of South Africa also observed a high incidence of young apple trees dying due to dieback, which launched an investigation into the contamination of specific rootstocks.

Knowledge regarding latent infections of canker or wood rot pathogens in certified nursery trees in South Africa is lacking. Thus the aim of this study was to assess the phytosanitary status of nursery trees and to identify inoculum sources for stem canker pathogens of diseased 1-year-old apple trees. This was determined by assessing the occurrence of canker and wood rot pathogens in 1) 1-year-old apple trees showing cankers or dieback; 2) certified nursery apple trees, and 3) plant material used during the propagation of apple trees.

MATERIALS AND METHODS

Sampling of planting material

1-year-old orchards

Thirteen 1-year-old commercial orchards in the Kouebokkeveld, Hermanus, and Grabouw areas were surveyed in March 2015 and March 2016. These orchards exhibited dieback symptoms within 1 year of establishment. Ten trees with canker symptoms per orchard were sampled. The samples was stored at 4°C until isolations were conducted.

Certified nursery apple trees

Nursery plants with scion Golden Delicious and rootstocks MM109, M793 and Geneva® (G222) were collected from four fruit tree nurseries in South Africa. In total, 480 trees (160 trees per rootstock) were collected in August – September 2015. Due to the unavailability of some scion-rootstock combinations, other scion cultivar options were also accepted when Golden Delicious was not available. Forty trees with the rootstock CG4202 was also collected instead of G222 rootstock, forming part of the Geneva® rootstock sampling. Trees beloying to different size classes were also requested from these nurseries (Table 1). Trees were stored at 4°C for no longer than one week until isolations were conducted.

Scion mother block orchards

Scion mother block trees were sampled in February and March of 2015 and 2016. Three scion cultivars were used, including Rosy Glow, Golden Delicious and Early Red One. Six scion mother blocks per scion cultivar were sampled, which resulted in 18 mother blocks in total. Fifteen (2016) or 20 (2015) trees were sampled per block. First year's results indicated 15 trees would be adequate, and the number was therefore reduced for the following year's sampling. From each tree, a typical canker symptom, pruning wound as well as a 1-year-old shoot were sampled. If there were no cankers present on the tree, two pruning wounds were sampled, and if there were more than one canker present on the tree, two cankers were sampled (Table 2). The plant material was stored at 4°C for no longer than 2 weeks.

Rootstock mother blocks

Rootstock plant material was collected in June 2016 in collaboration with the three PIOs of South Africa. Three rootstock cultivars were selected namely G222, M793, and MM109. Three layer blocks per rootstock were investigated, and 45 asymptomatic shoots randomly removed from each block. In total 405 asymptomatic rooted rootstocks were collected. In one of the

nurseries, dieback symptoms were found in the MM109 and M793 1-year-old nursery blocks. These are the rootstock cuttings which were removed from the layer blocks and established in the nursery soil in August 2015. Dieback occurred on the rootstock shoots shortly after establishment. Forty-five M793 and 42 MM109 symptomatic shoots were sampled from the corresponding 1-year-old nursery blocks.

Isolation of planting material

Canker lesions were excised from the 1-year-old trees and triple surface sterilized by soaking in 70% ethanol solution for 30 s, then 1% NaOCl solution for 60 s, and finally in 70% ethanol solution for 30 s. The cankered wood pieces were left to air-dry on sterile tissue paper in the laminar flow cabinet. A pruning shear (with blades flame sterilised) was used to cut the wood pieces in half, exposing the vascular tissue. Twelve wood pieces measuring approximately 2 x 2 mm were removed from the section between the diseased dark brown vascular discolouration and the healthy tissue, and placed onto 2% potato dextrose agar (Biolab, Midrand) amended with streptomycin sulphate (40 mg/L, Calbiochem, Merck) (PDA+s). Four wood pieces were placed per PDA+s Petri dish, thus three Petri dishes per symptom. Plates were incubated at 23°C under natural light for 2 to 3 weeks, or until substantial fungal growth were observed. Representative subcultures were made, using the hyphal tip method, from each primary isolation plate and incubated under the same conditions.

Preparation of plant material and isolations were done thoughout the study as previously described in the 1-year-old sampling. For the nursery trees isolations were made from four parts found on the nursery trees namely: wounds made on the scion shoot, the bud union, the pruning wound made when the rootstock was cut back and additional wounds found on the rootstock. If no wounds were found on the scion or rootstock shoot, asymptomatic pieces were used. For the scion mother block samples, isolations were made from the vascular discolouration found in the cankers and pruning wounds. Furthermore, eight buds were removed from each scion shoot and placed onto 2 PDA+s dishes. Four disks were also cut through the internodes of the scion shoot and placed onto one PDA+s dish.

For the rootstock mother block, isolations were made from asymptomatic tissue. A sterile pruning shear was used to cut disks though the buds on the rootstock shoot of the rooted cutting. Disks were cut into quarters and placed onto PDA+s. Three disks were removed from each rooted cutting and placed onto three PDA+s dishes (four wood pieces per Petri dish). For the 1-year-old nursery material, isolations were made from the internal vascular discoloutation. Fruiting bodies which formed on the surface of the canker were scraped off and placed onto PDA+s.

Soil sampling from 1-year-old orchards

A soil sample was taken with an auger from each orchard next to the root system of one of the affected trees to a depth of 30 cm. The soil samples were sent to Bemlab (Somerset West, South Africa) for analysis.

Identification of fungal species

Fungal isolates were arranged into different taxonomic groups based on its cultural and morphological characteristics. Cultural characteristics included colony size, colour, texture and shape. Microscopic slides were made from unknown cultures to observe conidia shape and colour. Examination of microscopic slides was done using Bright-field microscopy. The following fungal groups were identified: species of the Botryosphaeriaceae, Diaporthales, *Phaeoacremonium* and in some cases Basidiomycetes where fruiting bodies formed on the Petri dish. Species within the Botryosphaeriaceae were identified by using the descriptions of Phillips *et al.* (2012) and van Niekerk *et al.* (2004), for *Phaeoacremonium* species Gramaje *et al.* (2015) and Damm *et al.* (2008) were used; for species in the order Diaporthales the description by Mostert *et al.* (2001) was used. Unknown cultures were also grouped and selections were made for further molecular identification. All known saprophytes were discarded. One isolate per taxonomic group for each plant part was stored and used for further identification.

Molecular identification

DNA extraction

Ten isolates from each taxonomic group were selected for molecular identification. DNA was extracted from 3 week-old fungal cultures on PDA+s. The DNA isolation protocol of Damm *et al.* (2008) was used with some modificatios. Fungal mycelium was placed into 2-mL Eppendorf tubes with 0.5 mg glass beads and 600 µl CTAB (2% CTAB, 1 M Tris, pH 7.5; 5 M NaCl; 0.5 M EDTA, pH 8.0). The Eppendorf tubes were shaken for 5 min at 30 s after which the tubes were incubated at 65°C for 15 min. After the incubation period, 400 µl chloroform:isoamylalcohol (24:1) was added instead of chloroform:phenol and centrifuged for 15 min at 13 500 rpm. The supernatant was extracted and added to a new 2-mL Eppendorf tube containing 200 µl of ammonium acetate (7.5 M) and 600 µl isopropanol. Tubes were centrifuged for 15 min at 13 500 rpm. The supernatant was discarded and 70% ethanol was added and centrifuged for a further 5 min. The supernatant was discarded and the tubes were centrifuged for 1 min. The supernatant was removed and pellets dissolved in 200 µl double distilled water (ddH$_2$O).

Polymerase chain reaction (PCR) and electrophoresis

Primers for amplification were selected according to taxonomic groups. For Diatrypaceae, Diapothales, Basidiomycetes, and unidentified fungal cultures the internal transcribe spacers 1 and 2 and the 5.8S rDNA gene area was amplified with ITS-5F and ITS-4R (White *et al.*, 1990). In a total reaction volume of 20 µl, the PCR reaction contained 1 µl of DNA, 10 µl 2x KAPA Taq ready mix (KAPABiosystems, Massachusetts, United States), 0.8 µl ITS-5 (0.4 pmol/µl), 0.8 µl ITS-4 (0.4 pmol/µl), and 7.4 µl ddH$_2$O. Reaction conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, and a final extension step at 72°C for 7 min.

For species in the Botryosphaeriaceae, the elongation factor 1-alpha gene (EF-1 α) was amplified with primers EF-1 728F and EF-1 968R (Carbone and Kohn, 1999) following the previously described PCR reaction volumes. PCR conditions consisted of an initial denaturation step at 94°C for 5 min followed by 30 cycles of 45 s at 94°C, 45 s at 53°C and 90 s at 72°C with a final extension step at 72°C for 7 min. Fungal cultures with *D. seriata*-like morphological characteristics were identified using the species-specific primers DS3.8 S3 and DS3.8 R6 (Martín *et al.*, 2014). In a total reaction volume of 20 µl, the PCR reaction contained 1 µl of genomic DNA, 8 µl 2x KAPA Taq ready mix, 0.8 µl ITS-5 (0.4 pmol/µl), 0.8 µl ITS-4 (0.4 pmol/µl) and 8.4 µl ddH$_2$O. Reaction conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 s at 94°C, 45 s at 57°C and 45 s at 72°C with a final extension step at 72°C for 7 min.

Species of *Phaeoacremonium* were identified with the partial β-tubulin (β-tubulin) gene amplified using the primers T1 (O'Donnell and Cigelnik, 1997) and Bt2B (Glass and Donaldson, 1995). The same PCR reaction volumes and concentrations were used as described earlier. Reaction conditions consisted of an initial denaturation step at 94°C for 5 min followed by 36 cycles of 45 s at 94°C, 45 s at 55°C and 90 s at 72°C with a final extension step at 72°C for 6 min. All PCR reactions were performed in an Applied Biosystems 2700 PCR machine (Carlsbad, California, USA). A non-template control was also included in each PCR run.

PCR products were separated by electrophoresis on a 1% (w/v) agarose gel in TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5) after ethidium bromide staining. The GeneGenius Gel Documentation and Analysis System (Syngene, UK) were used to visualize the gel under ultraviolet (UV) light alongside a 100-bp DNA ladder (GeneRuler, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Sequencing of PCR products

PCR products were purified using the MSB Spin PCRapase kit (Invitek, Berlin, Germany) and prepared for forward and reverse sequencing. Thermocycler conditions were 1 min at 95˚C,

30 cycles of 10 s at 95˚C, 5 s at 50˚C and 4 min at 60˚C, with a final extension of 30 s at 60˚C. The gene areas were sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California, United States) with the primers used in the initial PCR reactions. The nucleotide order of samples was read in an ABI 3130xl DNA sequencer (Perkin-Elmer, Norwalk, California, United States) at the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University.

Phylogeny

Forward and revers sequences for each isolate were aligned in Geneious R 9.1.7 and a consensus sequence was extracted. Consensus sequences were run through the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information's (NCBI) nucleotide database. Isolates obtained from this study were aligned with representative sequences from each taxonomic group obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) with the program Geneious R 9.1.7 (Biomatters Ltd., Auckland, New Zealand).

Individual taxonomic group sequences' data sets were aligned with L-INS-I method using the program MAFFT v7.222 (Katoh *et al.*, 2002) in Geneious R 9.1.7. Maximum likelihood (ML) analyses were performed using PHyML (Guindon and Gascuael, 2003) in Geneious R 9.1.7 under the general time reversible (GTR) model. Both the gamma distribution parameter and proportion of invariable sites were estimated. Bootstrap support values were calculated from 100 replicates. Clades with bootstrap support ≥ 70% were considered significant and highly supported (Hillis and Bull, 1993).

Statistical analysis

Chi-square tests were used to test for differences in the frequencies of the different factors using Frequency Procedure (PROC FREQ) of the SAS software version 9.2 (SAS Institute Inc, Cary, USA). The percentage infected trees were calculated. Factor (treatment) means (ie. PIO, cultivars, rootstocks etc.) were tested using PROC TTEST with SAS software. The number of infections caused by the different pathogens was also expressed as a percentage of the total number of infected trees for the different factors (treatments). Normality of standardized residuals was confirmed by Shapiro-Wilk test (Shapiro and Wilk, 1965). Levene's test was used to verify the homogeneity of factor (treatment) variances (Levene, 1960). The data was subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software. Fisher's least significant difference (LSD) was calculated at the 5% level to compare factor (treatment) means (Ott and Longnecker, 2001).

RESULTS

Morphological and cultural groups were identified and representatives were selected for which the relevant genes were sequenced. Species identification was based on phylogenetic analyses.

Phylogenetic analyses

Separate phylogenetic analyses were conducted according to the taxonomic groups identified. Table 3 lists the representative isolates that were used in the phylogenetic analyses. *Truncatella angustata* (Pers.) S. Hughes (bootstrap support of 97%) was the only species identified in the Amphisphaeriaceae (Fig. 1).

Seven Basidiomycete species were identified (Figs. 2-4). The majority of the isolates (50.7%) belonged to *T. versicolor* (bootstrap support of 82%) (Table 4) (Fig. 2), followed by *S. commune* (bootstrap support of 100%) (Fig. 3) and *Bj. adustra* (bootstrap support of 94%) (Fig. 3). *Chondrostereum purpureum* (100% bootstrap support) and *Stereum hirsutum* (Willd.) Pers. (62% bootstrap) were only found once in the study (Fig. 3). The *Peniophora* isolates formed two separate clades, but did not group with any known species and was therefore named *Peniophora* sp. 1 (100% bootstrap support) and *Peniophora* sp. 2 (99% bootstrap support) (Fig. 4). *Peniophora* sp. 2 grouped with a *Peniophora lycii* Höhn. & Litsch sequence with 74% bootstrap support. The identity of *Peniophora lycii* is uncertain since sequences identified as this species does not form a monophyletic clade.

Isolates of the Botryosphaeriaceae were identified as species of *Botryosphaeria, Diplodia,* and *Neofusicoccum* (Figs. 5 – 7). The majority of the Botryosphaeriaceae isolates (78.8%) were identified as *D. seriata* (74% bootstrap support) (Table 4) (Fig. 5). *Botryosphaeria dothidea* (72% bootstrap support) (Fig. 6) and three species of *Neofusicoccum* were identified, namely *N. australe* (bootstrap support of 85%), *Neofusicoccum viticlavatum* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips (bootstrap support of 89%) and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (bootstrap support of 64%) (Fig. 7).

Cadophora luteo-olivacea (J.F.H. Beyma) T.C. Harr. & McNew (65% bootstrap value) and an unknown *Cadophora* sp. (Bootstrap value of 71%) were identified. *Cadophora* sp. did not form a monophyletic clade with any other known species (Fig. 8). *Coniochaeta fasciculata* (J.F.H. Beyma) Z.U. Khan, Gené & Guarro (100% bootstrap value) and *Coniochaeta velutina* (Fuckel) Cooke (62% bootstrap value) (Fig. 9) were identified. One of the isolates obtained in the study was identified as a new *Coniochaeta* species, not forming a monophyletic clade with any other known species (Fig. 9).

One *Cytospora* specie was found namely *Cy. schulzeri* (bootstrap value of 52%) which formed a clade with an isolate found on apple trees in Michigan (JX438604.1). The isolates

found in this study did not form a clade with the other *Cy. schulzeri* found on apple trees in South Africa (DQ243792.1). Adams *et al.* (2006) used both isolates in his study and concluded that *Cy. schulzeri* and *Cytospora germanica* Sacc. is part of the *Valsa malicola/Valsa germanica* species complex. Rossman *et al.* (2015) found that *Cytospora* is the correct name for this genus instead of *Valsa.*

Seven *Diaporthe* species were identified in this study. The majority of the isolates (55.9%) were identified as *Diapothe eres* (bootstrap value of 88%) (Table 4), followed by *Diaporthe* sp. (100% bootstrap value) (Fig. 11). *Diaporthe* sp. grouped with *Diaporthe arecae* (H.C. Srivast., Zakia & Govindar.) R.R. Gomes, C. Glienke & Crous and *Diaporthe pseudophoenicicola* R.R. Gomes, Glienke & Crous, but with a low bootstrap support (Fig. 11). *Diaporthe foeniculina* (67% bootstrap value), *Diaporthe cynaroidis* Marinc., M.J. Wingf. & Crous (99% bootstrap value), *Dia. ambigua* (100% bootstrap value), *Phomopsis* sp. 5 (88% bootstrap value) and *Phomopsis* sp. 4 (59% bootstrap support) were also identified (Fig 11).

Three species were identified in the Diatrypaceae. The majority of the isolates (82.8%) were identified as *Eutypa lata* (84% bootstrap value) (Table 4), followed by *Eutypella citricola* Speg. (98% bootstrap value) and lastly *Eutypa* specie (79% bootstrap value) (Fig. 12). *Eutypa* specie grouped with *Eutypa tetragona* (Duby) Sacc. & *Eutypa leptoplaca* (Durieu & Mont.) Rappaz (Fig. 12).

Isolates of *Didymella* and *Didymosphaeria* belonging to the class Dothideomycetes were identified. Two species of *Didymella* were found, including *Didymella pomorum* (Thüm.) Q. Chen & L. Cai (bootstrap value of 55%) and *Didymella* specie (Fig. 13). Two species of *Didymosphaeria* were identified, specifically *Didymosphaeria rubi-ulmifolii s.l.* H.A. Ariyawansa, E. Camporesi & K.D. Hyde (72% bootstrap value) and *Didymosphaeria variabile* (Riccioni, Damm, Verkley & Crous) Ariyawansa & K.D. Hyde (89% bootstrap value) (Fig. 14).

The highest diversity of species found for a genus was for *Phaeoacremonium*. Twelve *Phaeoacremonium* species were identified namely *P. inflatipes* W. Gams, Crous & M.J. Wingf. (99% bootstrap support), *P. fraxinopennsylvanicum* (100% bootstrap support), *P. australiense* L. Mostert, Summerb. & Crous (94% bootstrap support), *P. subulatum* L. Mostert, Summerb. & Crous (100% bootstrap support), *P. scolyti* L. Mostert, Summerb. & Crous (72% bootstrap support), *P. prunicola* L. Mostert, Damm & Crous (73% bootstrap support), *P. iranianum* L. Mostert, Gräfenhan, W. Gams & Crous (100% bootstrap support), *P. minimum* (100% bootstrap support), *P. austroafricanum* L. Mostert, W. Gams & Crous (100% bootstrap support), *P. viticola* J. Dupont (90% bootstrap support), and two new *Phaeoacremonium* spp. (Fig. 15). Isolate STEU 8402 (*Phaeoacremonium* sp. 1) did not form a clade with any known *Phaeoacremonium* species, whereas two other isolates (STEU 8401 and 8400) (*Phaeoacremonium* sp. 2) grouped with a new species identified from a canker sampled from an apricot tree (100% bootstrap support) (Fig. 15).

Diversity of fungal taxa

A total of 45 fungal species belonging to the Basidiomycetes, Botryosphaeriaceae, Diatrypaceae, also the genera *Didymella*, *Didymosphaeria*, *Phaeoacremonium*, *Diaporthe*, *Cadophora*, *Coniochaeta*, *Cytospora* and *Tr. angustata* were identified in this study (Table 4). The species identified have all been associated with canker or wood rot symptoms on fruit trees or other woody hosts in South Africa or somewhere else. The majority of the species (38) were identified as Ascomycetes that could form cankers and seven species in the Basidiomycetes that could form wood rot (Table 4). *Didymosphaeria rubi-ulmifolii s.l.* was the predominant fungal specie isolated from 276 plant parts throughout the study and was the predominant pathogen found in the 1-year-old trees (present in 11 of the orchards investigated) as well as the nursery material (241 plant parts). Five pathogens were found in all four sampling strategies namely *S. commune*, *D. seriata*, *Dia. foeniculina*, *Did. variabile* and *Did. rubi-ulmifolii s.l.* Apart from the possible plant pathogens, a large variety of fungal taxa were isolated such as *Trichoderma, Epicoccum, Alternaria, Aureobasidium* and *Penicillium,* which are known to be saprophytic fungi and were not included in the results.

1-year-old commercial apple orchards

From the 130 diseased 1-year-old trees that were investigated, possible canker and wood rotting pathogens were found in 55 trees (42.3%). Twenty-one different fungal taxa were isolated from dieback symptoms (Table 4). The most predominant pathogen was *Did. rubi-ulmifolii s.l.* which was isolated from 15 trees, followed by *D. seriata* (isolated from 11 trees) and *Eu. citricola* (isolated from 5 trees) (Table 5). The *Didymosphaeria* species were found most often, then species within Botryosphaeriaceae and then wood rotting fungi of the Basidiomycetes. The occurrence of the *Didymosphaeria* species was significantly higher than the Diaporthales, Coniochaetales, Diatrypaceae, Didymellaceae, *Phaeoacremonium* spp. and *Tr. angustata* (Table 5). The three geographical areas Hermanus, Grabouw, and Kouebokkeveld had similar levels of infection in the sampled trees (P = 0.734), with mean percentage infection of 55%, 44%, and 36.7%, respectively (Table 6). The same pathogens were isolated from all the geographical areas except for *Diaporthe* species which were found more often in the diseased trees collected from Hermanus with a mean percentage infection of 15%, statistically higher (P = 0.039) than trees from Grabouw (4%). No *Diaporthe* species were isolated from the Koubokkeveld trees.

Typical symptoms that were observed on the 1-year-old trees were constricting cankers (Figs. 16A, B). A cross section through the cankers exposed dark brown vascular discoloration, streaking down into the live tissue of the tree (Fig. 16C). The majority of trees that was sampled had well-developed root systems (Fig. 16D) indicating that the above-

ground symptoms were not due to soil borne diseases. Cankers were found on different parts of the trees. From 55 trees, the majority of the pathogens were isolated from the scion shoot, followed by the pruning wound on the rootstock, the rootstock and lastly from the bud union (Table 7).

Internal and external symptoms associated with canker on the scion shoot of the tree are illustrated in Figure 17. Dieback symptoms caused by Ascomycete and Basidiomycete pathogens infecting the pruning wound made on the rootstock during propagation are presented in Figure 18. In some of the tree samples more than one pathogen was isolated and more importantly on some trees, canker and wood rot symptoms occurred on more than one plant part (Fig. 19). Multiple infections were often on the scion shoot and from the pruning wound on the rootstock. Fruiting bodies (pycnidia) of Ascomycete fungi specifically *Coniochaeta* sp., *Did. rubi-ulmifolii s.l.* and *Di. pomorum* were found on the cankers that formed on the scion shoot. The fruiting structures of *S. commune* were found on one of the Royal Beaut trees that have died (Fig. 20).

The soil analyses indicated that the eight orchards experiencing dieback and cankers in the trees showed a pH (KCl) below of 4.6 and phosphorus levels (Bray II) higher than 60 mg/kg (Table 8). The majority of the orchards had loam or sandy soil types.

Certified nursery apple trees

From the 480 certified healthy nursery trees collected, 312 harboured possible canker or wood rot pathogens, resulting in a 65% latent infection. The nursery trees did not exhibit any external symptoms; however, a cross section through the bud union and pruning wound did often show vascular discoloration (Fig. 21). Thirty-two different fungal taxa were isolated from these nursery trees (Table 4). In Table 9 the occurrence of different taxonomic groups found causing latent infections in the nursery trees are presented. The same taxonomic groups found in the diseased 1-year-old trees were found causing latent infections in the nursery trees with the most prominent group being species in *Didymosphaeria,* followed by wood rotting fungi of the Basidiomycetes and species in the Botryosphaeriaceae (Table 9).

The bud union had significantly higher infection levels compared to the rootstocks, but was not different from infection levels found in the pruning wounds and scion (Table 10). Similar pathogens were isolated from all four plant parts with an exception for the Basidiomycetes and *Cadophora* species (Table 11; Table 12). Basidiomycetes were isolated more often from the bud union than from the rootstock and the scion shoot (Table 11), whereas *Cadophora* species were isolated more from the bud union and pruning wound than from the rootstock (Table 12).

Didymosphaeria rubi-ulmifolii s.l. was isolated from 274 plant parts and was the predominant fungus isolated from the nursery material (Fig. 22). *Didymosphaeria rubi-ulmifolii s.l.* was also the dominant pathogen isolated from all four plant parts. *Diplodia seriata, Dia. eres, Di. pomorum, T. versicolor, Did. variabile, C. luteo-olivacea, P. austroafricanum, Co. velutina, S. commune, Co. fasciculata*, and *Diaporthe* sp. were isolated from more than ten plant parts (Table 4). Figure 23 represents internal vascular discolouration and soft rot associated with Basidiomycetes found in these nursery trees.

High infection was found in all four nurseries with percentage infections ranging from 51.3% to 71.3% (Table 13). The infection levels found in the rootstock part of the tree (including pruning wound, bud union and the rootstock) for the three rootstock cultivars M793 (61.9%), MM109 (50%) and G222 (58.8%) was not significantly different (Table 14)

Scion mother block orhards

Of the 310 scion mother block trees that were investigated, 146 trees (47%) were infected with possible canker or wood rotting pathogens. A total of 25 fungal species were identified (Table 4), belonging to seven taxonomic groups (Table 15). The Diatrypaceae was the predominant taxonomic group isolated followed by species within the genus *Phaeoacremonium* (Table 15). Significantly less infection was caused by the Botryosphaeriaceae, Basidiomycetes, *Didymosphaeria* spp., *Diaporthe* spp. and the *Coniochaeta* spp. *Eutypa lata* was isolated from 44 pruning wounds and 26 cankers and was the predominant pathogen isolated in the scion mother block trees. Fungal pathogens that were isolated from more than 10 pruning wounds or cankers include *T. versicolor*, *D. seriata*, *P. fraxinopennsylvanicum*, *P. viticola*, *Did. rubi-ulmifolii s.l.* and *P. minimum*.

The incidence of infected cankers and pruning wounds were similar with 27.6% and 27.4% respectively ($P = 0.992$). The same pathogens were isolated from both the pruning wounds and cankers with exception of *Didymosphaeria* species and *Diaporthe* species, which were found more often in pruning wounds (Table 16). *Didymosphaeria* species were isolated from 16 pruning wounds and two cankers found on the scion mother block apple trees.

Typical symptoms associated with pruning wounds and cankers found on the scion mother block trees are shown in Figures 24 and 25. It was observed that especially cankers found on older trees were more difficult to isolate from. The scion mother blocks were divided into three age groups. The 1:1:1 ratio obtained through the Chi-square analysis indicated that infection in the different age groups differed from one another. Orchards of 10-20 years had a significantly higher infection (66%) than orchards older than 20 years (41.4%). Orchards younger than 10 years old were significantly less infected (25.2%) than older trees (Table 17).

The three plant improvement organisations had infection levels of 60%, 37.3%, and 34.7%, which did not statistically differ from one another. Golden Delicious was the most

infected cultivar (58.9%) followed by Early Red One (44.4%) and lastly Rosy Glow with an infection of 25.3%. Infection levels of cultivar Early Red One did not differ from Golden Delicious or Rosy Glow, however, the infection in Golden Delicious trees was higher than on Rosy Glow trees (*P* = 0.007). The diversity of fungal taxa isolated from the three different cultivars were similar. *Eutypa lata* was the predominant fungus isolated from the three cultivars; Golden Delicious (41 times), Early Red One (30 times) and Rosy Glow (6 times).

Fruiting bodies of Basidiomycete wood rotting fungi were found on dead wood in several of the older mother block orchards (Fig. 26). *Trametes versicolor* and other wood rot pathogens were isolated from pruning wounds and cankers that were collected from these orchards (Fig. 27). Figure 27 illustrates the brown and white wood rotting caused by the wood rotting fungi found in the cankers sampled in this study.

Sixteen of the 310 (5%) new shoots that were investigated had canker and wood rot pathogens present either in the shoot or the buds. The pathogens found in the green shoots were *Co. velutina, E. lata, Eutypa* sp., *Did. rubi-ulmifolii s.l.*, *D. seriata,* and *T. versicolor* (Table 18). These pathogens were also isolated from the cankers and pruning wounds

Rootstock mother blocks

Layer blocks

A total of seven fungal species were isolated from asymptomatic layer block shoots (Table 4). All of the pathogens isolated from the asymptomatic layer block were found in the nursery trees. The predominant pathogens belong to the taxonomic group Didymellaceae (Table 19) specifically, *Di. pomorum* and *Didymella* sp. followed by *Tr. angustata*. These three pathogens have been isolated from both the 1-year-old trees and the nursery trees.

The mean percentage infection of the three rootstock cultivars M793 (23.8%), MM109 (23%), and G222 (20.7%) was not statistically different (Table 20). The PIO's had latent infections in the layer block shoots of 12%, 33% and 24% (Table 21). The level of infection for the PIO's was not statistically different (Table 22).

1-year-old nursery trees

From the 87 symptomatic shoots that were sampled, 36 (41%) shoots harboured possible canker causing pathogens. For M793, 21 (47%) shoots harboured possible canker pathogens and for MM109, 15 (36%) shoots harboured possible canker pathogens (data not shown). The predominant pathogen was *D. seriata*, followed by *Cy. schulzeri* (Table 4). Both fungal taxa are known to cause dieback on apple trees. *Phaeoacremonium viticola*, *B. dothidea,* and *S. commune* were also isolated and are known dieback pathogens which have all been isolated from the nursery material. *Didymella* sp., which was the predominant species in the asymptomatic material, was reported once from the symptomatic material. Typical symptoms

that were observed on the affected 1-year-old rootstock shoots were constricting cankers (Figs 28A, B). A cross section through the cankers exposed dark brown vascular discoloration, streaking down into the live tissue of the non-budded tree (Fig. 28C). Fruiting structures which formed on the cankerous part of the shoots belonged to *D. seriata, Cy. schulzeri,* and *Didymella* sp. (Table 23).

DISCUSSION

This study has found that 65% of certified nursery apple trees investigated yielded fungi associated with canker and wood rot symptoms resulting in the distribution of seemingly healthy trees to farmers. A study done by McCracken *et al.* (2003) in England also found that the death of young newly established apple trees originated from latent infections of *Neonectria ditissima* that occurred during the propagation process in the nursery. All the nurseries and rootstocks that were evaluated had similarly infection rates, for which can be concluded that the problem is not restricted to a specific nursery or rootstock. Canker and wood rot species that caused disease in the 1-year-old diseased commercial orchards were also found in the nursery trees as latent infections.

Even though infection levels in the nursery trees were high, apple producers do not find a similarly high disease incidence of cankers or wood rot in newly established commercial orchards. Canker and wood rot pathogens are opportunistic and can induce symptoms when the host experiences stress (Menapace *et al.*, 2015). Symptoms of the canker pathogen *V. mali* var. *mali* was observed due to stress that the apple trees experienced (Zang *et al.*, 2012). In the present study, the high phosphorus level in the soils indicated that the plant had difficulty taking up water and the low, acidic pH resulted in an Aluminium toxicity (Marschner *et al.*, 1987; IPNI, 2010). These factors indicate that the trees were planted in sub-optimal soil, which will either place toxicity or mineral deficiency stress on the trees (Marschner *et al.*, 1987; IPNI, 2010). Stress conditions definitely contribute to making the tree more vulnerable to dieback development. Only one soil sample per orchard was collected and to confirm these findings more soil samples per orchard should be taken.

The fruiting bodies of Ascomycete and Basidiomycete fungi were found on the diseased 1-year-old trees. These fruiting bodies produce spores contributing to the aerial inoculum present in orchards. The spores can spread by means of the wind and infect new wounds (Matthee and Thomas, 1977; Valiuškaitė and Raudonis, 2008). It is very important to remove the dead trees from the young orchards. The presence of the wood rot pathogens in both the young trees and the nursery trees were surprising since these pathogens usually occur on older trees and dead wood (Matthee and Thomas, 1977).

Most of the cankers formed on the scion shoot of the young tree. McCracken *et al.* (2003) also found that cankers caused by *Ne. ditissima* mostly developed on the scion shoots

after establishing apple trees. Infection of the scion shoot can occur during the final stages of propagation when the lateral shoots are removed from the main stem.Infection also occured through wounds made on the scion shoot in newly established orchards.

The pruning wound on the rootstock and the bud union had the highest infection in the nursery trees. Canker and wood rot symptoms that developed from these sites were found in 19% of the infected 1-year-old apple trees. These two wound sites are important sites for infection. Infection would be via aerial inoculum present in the nursery when the wounds are made or via infected bud material. It is important to determine the possible inoculum sources during the propagation of the nursery trees to reduce the infection levels.

Possible inoculum sources are scion mother blocks, as well as older established orchards and other fruit trees, which were planted in close proximity to nurseries (McCracken *et al.*, 2003). The same canker and wood rot pathogens found causing disease on apple trees have been reported on other fruit hosts (Halleen *et al.*, 2007; Slippers *et al.*, 2007; Damm *et al.*, 2008). Basidiomycete fruiting structures were found on diseased and dead trees, able to produce basidiospores which can contribute to aerial inoculum in the surrounding area. The presence of the wood rot pathogens in both the young trees and the nursery trees can be explained by the aerial inoculum produced in the surrounding orchards as well as the infected scion buds used during propagation.

Similar canker and wood rot fungi were isolated from pruning wounds and cankers of scion mother block trees as found in 1-year-old and nursery trees. The one pathogen isolated frequently from all three scion mother block cultivars was *E. lata*, a well-known dieback pathogen. This pathogen often occurs on grapevines causing *Eutypa* dieback (Trouillas and Gubler, 2010) and is associated with decline on older plant material (White *et al.*, 2011; Moyo *et al.*, 2016). This explains why only a few isolates were found in the nursery trees. *Eutypa lata* was reported as a canker pathogen on apple in South Africa by Cloete *et al.* (2011).

Golden Delicious trees were more infected than the Rosy Glow trees. This is due to the average ages of the orchards. Rosy Glow is a newer cultivar with an average age of 8 years, compared to Golden Delicious orchards which had an average age of 20 years. The younger orchards, which were, in this case, all Rosy Glow trees had few to no cankers on the trees. It is expected that older orchards would harbour more canker pathogens since they have been pruned for longer, accumulating more wounds on the trees which have been exposed to aerial inoculum for a longer period of time. High infection in the pruning wounds indicates that apple wounds are susceptible to canker and wood rot pathogens and can develop into cankers. In some of the older, severely diseased trees and larger cankers, the causal organisms were not isolated easily resulting in a lower infection percentage obtained for trees older than 20 years versus between 10 and 20 years. This can be due to the

hardiness of the wood after several years or the pathogen may only be present as fruiting structures on the surface of the canker.

A low infection rate (5%) was found in the new green shoots, however, this study shows that infection of newly grafted plants can occur directly through infected scion buds used during propagation. The fungal pathogens, *Did. rubi-ulmifolii s.l.*, *E. lata* and *T. versicolor,* were isolated from infected scion buds and were also found in the nursery trees and 1-year-old diseased orchards.

The rooted rootstock cuttings also proved to be a direct inoculum source for infection of the nursery trees, with an overall infection of 21%. Species in *Didymella* were the predominant pathogens found and have been reported as canker causing pathogens of apples in South Africa by Crous *et al.* (2000). This was followed by *Tr. angustata*, which is found as a weaker pathogen causing decline on grapevines in Iran (Arzanlou *et al.*, 2013), Texas (USA) (Úrbez-Torres *et al.*, 2009) and Australia (Sergeeva *et al.*, 2005). Latent infections in the rootstock layer blocks of *Didymella* species, *Tr. angustata, B. dothidea* and *Dia. foeniculina* were also found in the nursery trees.

This latent infection in the rootstock layer blocks can be expressed when the shoot experiences stress such as what was found in the 1-year-old nursery blocks. All of the canker pathogens except for *P. viticola* found on the 1-year-old nursery shoots are associated with canker on apple trees in South Africa (Crous *et al.*, 2000; Cloete *et al.*, 2011). Fruiting structures were found on the cankers of the 1-year-old nursery shoots which can provide aerial inoculum during budding.

Didymosphaeria rubi-ulmifolii s.l., which belong to the class Dothideomycetes was the most dominant pathogen causing canker symptoms in commercial orchards and causing latent infections in the nursery trees. The class Dothideomycetes is lesser known as canker causing pathogens. However, a pathogenicity study conducted by Cloete *et al.* (2011) found that *Did. rubi-ulmifolii s.l.*, previously known as *Paraconiothyrium brasiliense* Verkley., formed significant lesions on apple shoots. Cloete *et al.* (2011) only isolated the pathogen from canker symptoms collected from diseased pear trees, this is thus the first report of *Did. rubi-ulmifolii s.l.* from apple trees in South Africa. Recently *Did. rubi-ulmifolii s.l.* were found to be the causal agent of cankers found near the base of young apple trees in an African country (unpublished). Personal communication with the orchard manager revealed that, from 2012 until 2016, 9 000 trees out of 25 000 were lost due to stem cankers caused mainly by this fungus (a first report is pending, therefore no further information can be provided). Little is known about the biology of this fungus and requires further investigation.

Fifteen of the 45 canker or wood rot species have been reported on apple trees in South Africa. The other 31 species were first reports on apple trees in South Africa, which included *Tr. angustata, Peniophora* sp. 1, *Peniophora* sp. 2, *St. hirsutum, N. parvum,*

Neofusicoccum sp., *C. luteo-olivacea, Cadophora* sp., *Co. fasciculate, Co. velutina,* *Coniochaeta* sp., *Dia. cynaroidis, Phomopsis* sp. 4, *Diaporthe* sp., *Phomopsis* sp. 5, *Eutypa* sp., *Eu. citricola*, *Didymella* sp., *Did. variabile, Did. rubi-ulmifolii s.l.*, *P. australiense, P. austroafricanum P. inflatipes, P. iranianum, P. prunicola, P. scolyti, P. subulatum, P. viticola, Phaeoacremonium* sp. 1, and *Phaeoacremonium* sp. 2. From the above-mentioned list only three canker and one wood rot species have been reported as pathogen on apple trees in other countries. *Neofusicoccum parvum* was reported as a canker causing pathogen in New Zealand (Slippers *et al.*, 2007), *P. iranianum* from Iran (Arzanlou *et al.*, 2013), *C. luteo-olivacea* from Italy (Spadaro *et al.*, 2011) and wood rot pathogen *St. hirsutum* from Brazil (Sutton *et al.*, 2015).

The other 27 species reported here for the first time on apples have been reported as canker or wood rotting pathogens on other fruit trees and woody hosts. The wood rot Basidiomycetes are known to cause heavy production losses and has been reported on many hosts including pear and stone fruit trees (Matthee and Thomas, 1977; Deflorio *et al.*, 2008). Species in the Botryosphaeriaceae can cause canker on a wide range of fruit crops, including apricot, grapevine, olives, peach, pears, and plum (Copes and Hendrix, 2004; Phillips *et al.*, 2012; Abdollahzadeh *et al.*, 2013; Úrbez-Torres *et al.*, 2013). *Cadophora* species are known to cause disease on grapevine and pear trees (Halleen *et al.*, 2007; Gramaje *et al.*, 2011; Spadaro *et al.*, 2011). Species in the genus *Coniochaeta* are known canker pathogens in South Africa on stone fruit (Damm *et al.*, 2010).

Species of *Diaporthe* are associated with canker on a wide range of fruit trees including apple, grapevines, peach, pear, plum as well as ornamental trees (Smit *et al.*, 1996; Smit *et al.*, 1997; Moleleki *et al.*, 2002; van Niekerk *et al.*, 2005; Elfar *et al.*, 2013; Gomes *et al.*, 2013; Udayanga *et al.*, 2014). Species of Diatrypaceae, especially *E. lata,* are major pathogens of grapevines worldwide (Trouillas *et al.*, 2010; Trouillas *et al.*, 2011), and also cause disease on almonds, apple, apricot, cherry, olive, peach and walnut trees (Catal *et al.*, 2007; Cloete *et al.*, 2011). *Phaeoacremonium* species can infect a wide host range of woody hosts specifically grapevine, stone fruit, and olive trees (Mostert *et al.*, 2003; Damm *et al.*, 2008; Arzanlou *et al.*, 2014; Gramaje *et al.*, 2015). Since the species reported here have been associated with canker and wood rot of different tree types, we can consider them as pathogens of apple trees. However, pathogenicity trials on apples should be conducted.

The Disease Clinic of Stellenbosch University found similar pathogens from diseased apple trees. The majority of the pathogens found by the Disease Clinic were species within the Botryosphaeriaceae, followed by *Diaporthe* species and wood rotting Basidiomycetes species. In this study, after *Didymosphaeria* species, these three taxonomic groups were predominantly found in the 1-year-old diseased trees. Other lesser known taxonomic groups, such as the *Didymella* species and *Tr. angustata* were not found by the Disease Clinic. These

species was mostly found in asymptomatic rootstock material and was found to a lesser extend, in the nursery and 1-year-old trees.

Future work should resolve the taxonomy of *Didymella, Cytospora* and *Didymosphaeria* by sequencing more than one gene area as well as describing the new species found in this study. Lesser known species found in this study, such as species in the *Didymosphaeria* and *Didymella*, were not found during the analysis of diseased trees by the Disease Clinic of Stellenbosch University. This can be due accidentally discarding unknown cultures as saprophytes based on cultural growth. Cultural growth on PDA after 1 and 2 weeks was captured for all species found in this study to aid in the identification of canker and wood rot species (Appendix A, Figs. 1-6).

The incidence of dieback in 1-year-old orchards due to canker and wood rot could be reduced by managing abiotic and biotic stress factors placed on the apple tree. This should be done throughout the propagation process, during storage and during the establishment of young trees. Abiotic stresses responsible for low productivity in apple orchards includes fluctuation in temperature, nutrients deficiencies, low-temperature injuries such as frost, water logging, insect damage, and improper horticultural practices (Slippers *et al.*, 2007; Valiuškaitė and Raudonis, 2008; Krishna *et al.*, 2010; Arzanlou and Bakhshi, 2012; Marek *et al.*, 2013).

To obtain less infected trees, aerial inoculum in the nurseries as well as in the 1-year-old commercial orchards can be reduced by applying sanitation practices on the surrounding orchards. Sanitation practices include the removal of dead and cankered material, especially when fruiting bodies are present. This is important in all phases of propagation as well as in established orchards. When the canker formed on the scion shoot of the 1-year-old tree, the diseased part of the tree can be removed. The tree will produce a new scion shoot in the next growing season, this will allow the tree to recover from the infection.

The cankered rooted rootstocks in the 1-year-old nursery blocks should be removed before budding as well as before the rootstock shoot gets pruned back. This will reduce infection during budding. To reduce infections in the nurseries a pruning wound protectant containing a fungicide should be used throughout the propagation process. Younger scion orchards were less infected than older orchards, thus PIO should renew mother block orchards at a younger age. This will reduce the infection of the scion buds used during propagation.

Certified nursery apple trees were infected with canker and wood rot pathogens, which resulted in the distribution of seemingly healthy trees to farmers. Nursery trees get infected during the propagation process via aerial inoculum, which is present at the time of budding and pruning back. The plant material used during propagation also play a role in infecting the nursery apple trees since a direct infection was found via the scion bud and rooted rootstock cutting. The young trees expressed canker and wood rot symptoms shortly after experiencing stress conditions during the establishment of the orchards. A pathogenicity test should be

conducted for all the new species found in this study. The post-nursery handling of nursery trees is very important and needs to be further investigated as to what extent certain practices or conditions would contribute to the expression of latent infections of canker and wood rot patohgens. For example, the storage conditions of trees, optimal planting and post-planting practices. Future work should aim to reduce aerial inoculum by investigating incidence of aerial inoculum present in the nurseries as well as test efficacy of different pruning wound protectants.

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TABLES AND FIGURES

Table 1. Nursery tree rootstock and scion combinations, collected from four different apple nurseries in South Africa.

Rootstock cultivar	Scion cultivar	Number of trees	Size class[a]
M793	Golden Delicious	40	Standard
	Golden Delicious	20	First
	Golden Delicious	20	Large
	Royal Beaut	20	Large
	Royal Beaut	20	Medium
	Golden Delicious	20	Large
	Golden Delicious	20	Medium
MM109	Golden Delicious	40	Large
	Golden Delicious	20	First
	Golden Delicious	20	Large
	Royal Beaut	20	Medium
	Royal Beaut	20	Large
	Royal Beaut	40	Standard
G222	Gale Gala	60	Medium
	Gale Gala	20	Large
	Granny Smith	40	Medium
CG4202	Gale Gala	40	Large

[a] Size classes of tree refer to the diameter of their base of the scion shoot: Standard: 8-10 mm; Medium: 10-12 mm; First: 12-15 mm; Large: 15+ mm.

Table 2. Information of scion mother block apple orchards, including the age and number of trees, pruning wounds and cankers sampled.

Cultivar	Age of orchard	Number sampled		
		Trees	Pruning wounds	Cankers
Golden Delicious	10 - 20 years	20	33	7
	10 - 20 years	20	29	11
	> 20 years	20	26	14
	10 - 20 years	15	10	20
	> 20 years	15	13	17
	> 20 years	15	12	18
Early Red One	>20 years	20	37	3
	10 - 20 years	20	35	5
	10 - 20 years	20	39	1
	>20 years	20	30	10
	>20 years	15	25	5
	>20 years	15	20	10
Rosy Glow	< 10 years	20	39	1
	< 10 years	15	21	9
	< 10 years	15	17	13
	< 10 years	15	28	2
	< 10 years	15	25	5
	< 10 years	15	18	12

Table 3. Species, taxonomic group, isolation details and GenBank accession numbers of representative cultures for each specie reported in the study.

Strain number	Taxonomic group	Fungal taxa	Area	Cultivar	Plant part	GenBank accession[a]
STEU 8283	Amphisphaeriaceae	*Truncatella angustata*	Grabouw	African Red	Canker on scion shoot of 1-year-old tree	KY312617
STEU 8284		*Tr. angustata*	Kouebokkeveld	Golden Delicious	Bud union of a nursery tree	KY312618
STEU 8285	Basidiomycetes	*Bjerkandera adustra*	Riviersonderend	Royal Beaut/MM109	Pruning wound on rootstock of a nursery tree	KY312619
STEU 8286		*Bj. adustra*	Riviersonderend	Royal Beaut/M793	Soft rot in pruning wound of nursery tree	KY312620
STEU 8287		*Chondrostereum purpureum*	Kouebokkeveld	Rosy Glow	Pruning wound on a mature tree	KY312621
STEU 8288		*Peniophora* sp. 2	Kouebokkeveld	Golden Delicious	Canker on scion shoot of 1-year-old tree	KY312622
STEU 8289		*Peniophora* sp. 2	Langkloof	Golden Delicious/M793	Bud union of a nursery tree	KY312623
STEU 8290		*Peniophora* sp. 1	Kouebokkeveld	Rosy Glow	Pruning wound on a mature tree	KY312624
STEU 8291		*Peniophora* sp. 1	Langkloof	Golden Delicious/M793	Bud union of a nursery tree	KY312625
STEU 8292		*Schizophyllum commune*	Langkloof	Golden Delicious/M793	Pruning wound on rootstock of nursery tree	KY312626
STEU 8293		*S. commune*	Kouebokkeveld	Royal Beaut	Canker on scion shoot of 1-year-old tree	KY312627
STEU 8294		*Stereum hirsutum*	Kouebokkeveld	Royal Beaut	Canker on scion shoot of 1-year-old tree	KY312628
STEU 8295		*Trametes versicolor*	Langkloof	Golden Delicious/MM109	Wound on rootstock of nursery tree	KY312629
STEU 8296		*T. versicolor*	Langkloof	Golden Delicious/MM110	Bud union of a nursery tree	KY312630
STEU 8297	Botryosphaeriaceae	*Botryosphaeria dothidea*	Riviersonderend	Gale Gala/G222	Bud union of a nursery tree	KY312606
STEU 8298		*B. dothidea*	Riviersonderend	Royal Beaut/M793	Bud union of a nursery tree	KY312607
STEU 8299		*Diplodia seriata*	Kouebokkeveld	Early Red One	Bud on new shoot	KY312608
STEU 8300		*D. seriata*	Riviersonderend	Royal Beaut/M793	Bud union of a nursery tree	KY312609
STEU 8301		*Neofusicoccum australe*	Riviersonderend	Royal Beaut/MM109	Bud union of a nursery tree	KY312610
STEU 8302		*N. australe*	Riviersonderend	Gale Gala/G222	Wound on scion shoot of nursery tree	KY312611
STEU 8303		*Neofusicoccum parvum*	Riviersonderend	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312612

Table 3. Continue

Strain number	Taxonomic group	Fungal taxa	Area	Cultivar	Plant part	GenBank accession[a]
STEU 8304	Botryosphaeriaceae	*Neofusicoccum viticlavatum*	Kouebokkeveld	Fuji Supreme	Canker on rootstock of 1-year-old tree	KY312613
STEU 8305		*N. viticlavatum*	Kouebokkeveld	Fuji Supreme	Canker on rootstock of 1-year-old tree	KY312614
STEU 8306		*N. australe*	Riviersonderend	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312615
STEU 8307		*N. australe*	Riviersonderend	Gale Gala/G223	Pruning wound on rootstock of a nursery tree	KY312616
STEU 8308	*Cadophora*	*Cadophora luteo-olivacea*	Kouebokkeveld	Royal Beaut/MM109	Pruning wound on rootstock of a nursery tree	KY312631
STEU 8309		*C. luteo-olivacea*	Kouebokkeveld	Golden Delicious/M793	Pruning wound on rootstock of a nursery tree	KY312632
STEU 8310		*Cadophora sp.*	Kouebokkeveld	Golden Delicious/M793	Bud union of a nursery tree	KY312633
STEU 8311		*Cadophora sp.*	Kouebokkeveld	Golden Delicious/M793	Bud union of a nursery tree	KY312634
STEU 8312	*Coniochaeta*	*Coniochaeta fasciculata*	Kouebokkeveld	Gale Gala/CG4202	Pruning wound on rootstock of a nursery tree	KY312635
STEU 8313		*Co. fasciculata*	Kouebokkeveld	Golden Delicious	Canker on scion shoot of 1-year-old tree	KY312636
STEU 8314		*Coniochaeta veluntina*	Riviersonderend	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312637
STEU 8315		*Co. veluntina*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312638
STEU 8316		*Coniochaeta sp*	Grabouw	Golden Delicious	Fruiting body on canker found on a 1-year-old tree	KY312639
STEU 8317	*Cytospora*	*Cytospora schulzeri*	Kouebokkeveld	M793	Canker on rooted rootstock cutting	KY312640
STEU 8318		*Cy. schulzeri*	Kouebokkeveld	MM109	Canker on rooted rootstock cutting	KY312641
STEU 8319	*Diaporthe*	*Diaporthe ambigua*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312642
STEU 8320		*Dia. ambigua*	Kouebokkeveld	Rosy Glow	Pruning wound on a mature tree	KY312643
STEU 8321		*Diaporthe cynaroidis*	Riviersonderend	Gale Gala/G222	Wound on rootstock of a nursery tree	KY312644
STEU 8322		*Diaporthe eres*	Riviersonderend	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312645
STEU 8323		*Dia. eres*	Grabouw	Sundowner	Canker on scion shoot of a 1-year-old tree	KY312646

Table 3. Continue

Strain number	Taxonomic group	Fungal taxa	Area	Cultivar	Plant part	GenBank accession[a]
STEU 8324	*Diaporthe*	*Diaporthe foeniculina*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312647
STEU 8325		*Dia. foeniculina*	Riviersonderend	Royal Beaut/M793	Pruning wound on rootstock of a nursery tree	KY312648
STEU 8326		*Phomopsis* sp. 4	Kouebokkeveld	Golden Delicious/MM109	Bud union of a nursery tree	KY312649
STEU 8327		*Phomopsis* sp. 4	Hermanus	Cripps Red	Canker on scion shoot of a 1-year-old tree	KY312650
STEU 8328		*Diaporthe sp.*	Kouebokkeveld	Golden Delicious/MM109	Bud union of a nursery tree	KY312651
STEU 8329		*Diaporthe sp.*	Langkloof	Golden Delicious/M793	Wound on rootstock of a nursery tree	KY312652
STEU 8330		*Phomopsis* sp. 5	Kouebokkeveld	Royal Beaut/MM109	Bud union of a nursery tree	KY312653
STEU 8331		*Phomopsis* sp. 5	Kouebokkeveld	Royal Beaut/MM110	Wound on rootstock of a nursery tree	KY312654
STEU 8333	Diatrypaceae	*Eutypa lata*	Kouebokkeveld	Golden Delicious	Pruning wound on a mature tree	KY312655
STEU 8334		*E. lata*	Kouebokkeveld	Golden Delicious/M793	Bud union of a nursery tree	KY312656
STEU 8335		*Eutypa sp.*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312657
STEU 8336		*Eutypa sp.*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312658
STEU 8337		*Eutypella citricolla*	Riviersonderend	Royal Beaut/MM109	Wound on scion shoot of a nursery tree	KY312659
STEU 8338		*Eu. citricolla*	Grabouw	Golden Delicious	Canker on scion shoot of a 1-year-old tree	KY312660
STEU 8339	Dothideomycetes	*Didymella pomorum*	Kouebokkeveld	Golden Delicious	Canker on scion shoot of a 1-year-old tree	KY312661
STEU 8340		*Di. pomorum*	Kouebokkeveld	M793	Rooted rootstock cutting	KY312662
STEU 8341		*Didymella sp*	Kouebokkeveld	Golden Delicious/M793	Bud union of a nursery tree	KY312663
STEU 8342		*Didymella sp*	Kouebokkeveld	M793	Rooted rootstock cutting	KY312664
STEU 8343		*Didymosphaeria variabile*	Hermanus	Cripps Red	Canker on scion shoot of a 1-year-old tree	KY312665
STEU 8344		*Did. variabile*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312666
STEU 8345		*Didymosphaeria rubi-ulmifolii s.l.*	Riviersonderend	Gale Gala/G222	Bud union of a nursery tree	KY312667
STEU 8346		*Did. rubi-ulmifolii s.l.*	Kouebokkeveld	Golden Delicious/M793	Wound on rootstock of a nursery tree	KY312668
STEU 8347	*Phaeoacremonium*	*Phaeoacremonium australiense*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312669

Table 3. Continue

Strain number	Taxonomic group	Fungal taxa	Area	Cultivar	Plant part	GenBank accession[a]
STEU 8348	*Phaeoacremonium*	*P. australiense*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312670
STEU 8349		*P. austroafricanum*	Langkloof	Golden Delicious/M793	Bud union of a nursery tree	KY312671
STEU 8350		*P. austroafricanum*	Langkloof	Golden Delicious/M793	Bud union of a nursery tree	KY312672
STEU 8351		*P. fraxinopennsylvanicum*	Kouebokkeveld	Golden Delicious	Pruning wound on a mature tree	KY312673
STEU 8352		*P. fraxinopennsylvanicum*	Langkloof	Golden Delicious/MM109	Bud union of a nursery tree	KY312674
STEU 8353		*P. inflatipes*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312675
STEU 8354		*P. inflatipes*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312676
STEU 8355		*P. iranianum*	Langkloof	Golden Delicious/M793	Pruning wound on a nursery tree	KY312677
STEU 8356		*P. minimum*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312678
STEU 8357		*P. minimum*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312679
STEU 8358		*P. prunicola*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312680
STEU 8359		*P. prunicola*	Grabouw	Golden Delicious	Pruning wound on a mature tree	KY312681
STEU 8360		*P. scolyti*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312682
STEU 8361		*P. subulatum*	Kouebokkeveld	Gale Gala/CG4202	Canker on scion shoot of a 1-year-old tree	KY312683
STEU 8362		*P. subulatum*	Kouebokkeveld	Gale Gala/CG4202	Pruning wound on a nursery tree	KY312684
STEU 8363		*P. viticola*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312685
STEU 8364		*P. viticola*	Kouebokkeveld	Early Red One	Pruning wound on a mature tree	KY312686
STEU 8402		*Phaeoacremonium* sp. 1	Kouebokkeveld	Royal Beaut/M793	Pruning wound on a nursery tree	KY312689
STEU 8401		*Phaeoacremonium* sp. 2	Langkloof	Golden Delicious/M793	Bud union of a nursery tree	KY312688
STEU 8400		*Phaeoacremonium* sp. 2	Kouebokkeveld	Rosy Glow	Pruning wound on a mature tree	KY312687

[a] Genbank accession numbers are ITS sequences except for taxonomic group Botryosphaeriaceae which is elongated factor 1-alpha sequences and *Phaeoacremonium* species which is β-tubulin sequences.

Table 4. Incidence of fungal taxa isolated from different plant parts in 1-year-old apple orchards, nursery apple trees, scion and rootstock mother blocks.

Taxonomic group	Casual organism	Overall incidence per plant part[b]	Incidence for specific sampling strategy			Rootstock mother blocks[f]	
			1-year-old orchards[c]	Nursery trees[d]	Scion mother blocks[e]	Layer block	1-year-old nursery block
Amphisphaeriaceae	*Truncatella angustata*	16	1	5	0	10	0
Basidiomycetes	*Bjerkandera adustra*[a]	8	0	7	1	0	0
	Chondrostereum purpureum[a]	1	0	0	1	0	0
	Peniophora sp. 2	4	1	3	0	0	0
	Peniophora sp. 1	6	1	3	2	0	0
	Schizophyllum commune[a]	16	4	10	1	0	1
	Stereum hirsutum	1	1	0	0	0	0
	Trametes versicolor[a]	37	2	18	17	0	0
Botryosphaeriaceae	*Botryosphaeria dothidea*[a]	9	0	7	0	1	1
	Diplodia seriata[a]	78	11	32	17	0	18
	Neofusicoccum australe[a]	10	2	8	0	0	0
	Neofusicoccum parvum	1	0	1	0	0	0
	Neofusicoccum viticlavatum	1	1	0	0	0	0
Cadophora spp.	*Cadophora luteo-olivacea*	17	0	17	0	0	0
	Cadophora sp.	6	0	6	0	0	0
Coniochaeta spp.	*Coniochaeta fasciculata*	18	3	15	0	0	0
	Coniochaeta veluntina	13	1	10	2	0	0
	Coniochaeta sp.	1	1	0	0	0	0
Cytospora spp.	*Cytospora schulzeri*[a]	12	0	0	0	0	12
Diaporthe spp.	*Diaporthe ambigua*[a]	3	0	0	2	1	0
	Diaporthe cynaroidis	3	0	3	0	0	0
	Diaporthe eres[a]	33	2	30	1	0	0
	Diaporthe foeniculina[a]	7	2	1	3	1	0
	Phomopsis sp. 4	2	1	1	0	0	0
	Diaporthe sp.	10	0	10	0	0	0

Table 4. Continue.

Taxonomic group	Casual organism	Overall incidence per plant part[b]	Incidence for specific sampling strategy			Rootstock mother blocks[f]	
			1-year-old orchards[c]	Nursery trees[d]	Scion mother blocks[e]	Layer block sampling	1-year-old nursery block
Diaporthe spp.	*Phomopsis* sp. 5	2	0	2	0	0	0
Diatrypaceae	*Eutypa lata*[a]	82	0	5	77	0	0
	Eutypa sp.	8	0	0	8	0	0
	Eutypella citricolla	9	5	1	3	0	0
Didymella spp.	*Didymella pomorum*[a]	58	3	25	0	30	0
	Didymella sp.	36	0	9	1	25	1
Didymosphaeria spp.	*Didymosphaeria variabile*	29	2	18	8	1	0
	Didymosphaeria rubi-ulmifolii s.l.	276	15	241	13	7	0
Phaeoacremonium spp.	*Phaeoacremonium australiense*	1	0	0	1	0	0
	P. austroafricanum	18	1	15	2	0	0
	P. fraxinopennsylvanicum[a]	19	1	3	15	0	0
	P. inflatipes	1	0	0	1	0	0
	P. iranianum	7	0	7	0	0	0
	P. minimum[a]	13	0	2	11	0	0
	P. prunicola	3	0	0	3	0	0
	P. scolyti	1	0	0	1	0	0
	P. subulatum	5	0	5	0	0	0
	P. viticola	16	0	0	15	0	1
	Phaeoacremonium sp. 1	2	0	2	0	0	0
	Phaeoacremonium sp. 2	1	0	0	1	0	0

[a] Casual organisms reported as canker or wood rot pathogen of apple trees in South Africa.
[b] Incidence is equal to number of isolates stored for each taxonomic group.
[c] Plant part included canker forming on either scion shoot, pruning wound on rootstock, bud union or rootstock of 1-year-old diseased tree.
[d] Incidence found in nursery tree on either scion shoot, pruning wound on rootstock, bud union or rootstock.
[e] Plant parts include canker, pruning wound or scion shoot.
[f] Incidence per rootstock shoot.

Table 5. Mean percentage infection of 1-year-old infected apple trees with different taxonomic groups.

Taxonomic group	Infected trees (%)[a]
Didymosphaeria spp.	11.80a
Botryosphaeriaceae	10.83ab
Basidiomycetes	6.92abc
Diaporthales	3.85bc
Coniochaetales	3.72c
Diatrypaceae	3.59c
Didymellaceae	2.50c
Phaeoacremonium spp.	1.54c
Truncatella angustata	0.77c

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 6. The number of 1-year-old apple trees infected with canker or wood rot causing pathogens in different geographical regions.

Area	Trees sampled	Infected trees	% Infection[a]
Hermanus	20	11	55.00a
Grabouw	50	22	44.00a
Kouebokkeveld	60	22	36.67a
Total	130	55	42.31

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 7. The total number of 1-year-old apple trees infected with canker or wood rot causing pathogens at a specific plant part.

Plant part	Number of trees
Scion	36
Rootstock	10
Bud Union	6
Pruning wound	5

Table 8. Soil type, pH and Phosphor content of diseased 1-year-old apple orchard soils.

Orchard	Soil type	pH (KCl)	P (mg/kg) Bray II
A[a]	Clay	5.8	394
B	Loam	4.6	380
C	Sand	4.6	133
D	Clay	4.6	100
E[a]	Loam	6.6	190
F[a]	Loam	5.1	400
G	Loam	4.5	249
H	Loam	4.2	5.4
I	Sand	4.3	222
J[a]	Sand	5.9	389
K	Sand	4.5	417
L	Clay	4.8	649

[a] Primary problem in these orchards was apple replant disease with secondary dieback on the trees.

Table 9. Mean percentage latent infection found in the nursery apple tree sampling.

Taxonomic group	Infected trees (%)[a]
Didymosphaeria spp.	12.46a
Basidiomycetes	2.46b
Botryosphaeriaceae	2.23bc
Phaeoacremonium spp.	2.07bcd
Diaporthales	1.91bcd
Didymellaceae	1.72bcd
Cadophora spp.	1.41bcd
Coniochaetales	1.31bcd
Diatrypaceae	0.67cd
Truncatella angustata	0.35d

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 10. Percentage infection in different plant parts isolated from the nursery apple trees.

Plant part	Plant parts infected (%)[a]
Bud union	38.40a
Pruning wound	30.90ab
Scion	19.70ab
Rootstocks	15.90c

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 11. Mean percentage infection by wood rotting Basidiomycete pathogens in different plant parts of the nursery apple trees.

Plant part	Plant parts infected (%)[a]
Bud union	5.00a
Pruning wound	3.44ab
Scion	1.25b
Rootstocks	0.16b

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 12. Mean percentage infection by *Cadophora* species in different plant parts of the nursery trees.

Plant part	Plant parts infected (%)[a]
Bud union	2.66a
Pruning wound	2.03a
Scion	0.94ab
Rootstocks	0.00b

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 13. Incidence of nursery apple trees infected with canker and wood rot pathogens sampled from four nurseries.

Nursery[a]	Trees sampled	Infected trees	% Infection
A	80	41	51.3
B	80	57	71.3
C	160	112	70.0
D	160	100	62.5

[a] Nursery names were kept confidential.

Table 14. Mean percentage infection and comparison of different rootstocks in the nursery apple tree sampling.

Rootstock	Infected rootstock shoots (%)	*P* value[a]	
		Rootstock	
		MM109	M793
G222	58.8	0.511	0.537
MM109	50.0	-	0.382
M793	61.9	-	-

[a] A *P* < 0.05 indicated statistical differences between the rootstocks.

Table 15. Mean percentage infected scion mother block samples with different taxonomic groups.

Taxonomic group	Infected scion mother block samples (%)
Diatrypaceae	45.71a
Phaeoacremonium spp.	26.44b
Botryosphaeriaceae	12.32c
Basidiomycetes	11.06c
Didymosphaeria spp.	7.80c
Diaporthe spp.	5.25c
Coniochaeta spp.	0.33c

[a] Different letters above bars indicate significant differences between means at $P < 0.05$.

Table 16. Mean percentage infection for pruning wounds and cankers sampled from scion mother blocks, infected with *Didymosphaeria* and *Diaporthe* species.

Taxonomic group	Infected plant parts (%)		P value[a]
	Pruning wound	Canker	
Didymosphaeria spp.	13.7	1.9	0.0253
Diaporthe spp.	10.5	0.0	0.0394

[a] A $P < 0.05$ indicated statistical differences between pruning wound and canker.

Table 17. Mean percentage infection and comparison of different age groups in the scion mother block sampling.

Orchard age	Infected trees (%)	P value[a]	
		Age group	
		Between 10 and 20 years	Older than 20 years
Younger than 10 years	25.3	0.005	0.048
Between 10 and 20 years	66.0	-	0.029
Older than 20 years	41.4	-	-

[a] A $P < 0.05$ indicated statistical differences between the age groups.

Table 18. Canker and wood rot pathogens found in the green shoots of the scion mother blocks.

Cultivar	Bud	Number of buds infected	Disk	Number of disks infected
Golden Delicious	*Trametes versicolor*	1	-	0
	Eutypa lata	1	-	0
	Didymosphaeria rubi-ulmifolii s.l.	1	-	0
Rosy Glow	-	0	*Didymosphaeria rubi-ulmifolii s.l.*	1
	-	0	*Diplodia seriata*	1
	-	0	*Eutypa sp.*	1
Early Red One	*Didymosphaeria rubi-ulmifolii s.l.*	1	*Diplodia seriata*	3
	Coniochaeta veluntina	1	*Eutypa lata*	2
	Diplodia seriata	3	-	0

Table 19. Mean percentage infected taxonomic groups found causing latent infection in rootstock layer blocks.

Taxonomic group	Infected rootstock shoots (%)[a]
Didymellaceae	54.97a
Truncatella angustata	29.63ab
Didymopshaeria spp.	5.93b
Diaporthales	1.48b
Botryosphaeriaceae	0.74b
Coniochaetales	0.59b

[a] A $P < 0.05$ indicated statistical differences between the rootstocks.

Table 20. Mean percentage latent infection in three apple rootstocks.

Rootstock	Infected rootstock shoots (%)	P value[a]	
		Rootstock	
		MM109	M793
G222	20.7	0.810	0.870
MM109	23.7	-	0.960
M793	22.9	-	-

[a] A $P < 0.05$ indicated statistical differences between the rootstocks.

Table 21. Number of asymptomatic apple shoots infected with canker and wood rot pathogens from each plant improvement organisation.

Plant improvement organisation[a]	Infected shoots	Total shoots	% Infection
A	21	180	12.0
B	22	90	24.0
C	44	135	33.0

[a] Plant improvement organisation names were kept confidential.

Table 22. Mean percentage latent infection of rootstock layer block shoots in three different plant improvement organisations.

| Plant improvement[b] organisation | Infected rootstock shoots (%) | *P* value[a] Rootstock | |
		B	**C**
A	12.00	0.161	0.388
B	24.00	-	0.530
C	33.00	-	-

[a] A *P* < 0.05 indicated statistical differences between the rootstocks.

[b] Plant improvement organisation names were kept confidential.

Table 23. Canker and wood rot pathogens that formed fruiting structures on cankers on 1-year-old nursery apple shoots.

| Taxonomic group | Number of shoots infected | |
	MM109	**M793**
Diplodia seriata	4	3
Cytospora schulzeri	4	2
Didymella sp.	0	1
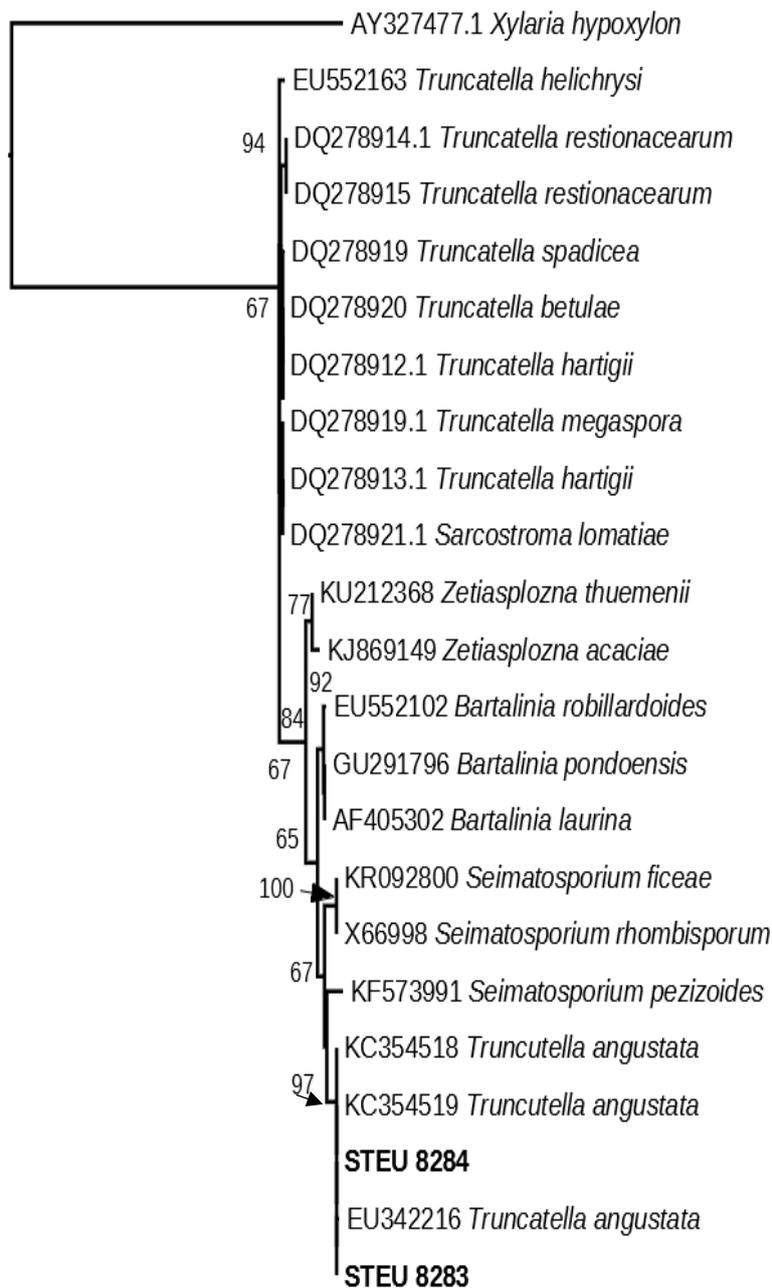
Figure 1. Maximum likelihood phylogenetic tree of *Truncatella* species which was based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Xylaria hypoxylon* was used as the outgroup. Isolates obtained in this study are indicated in bold.
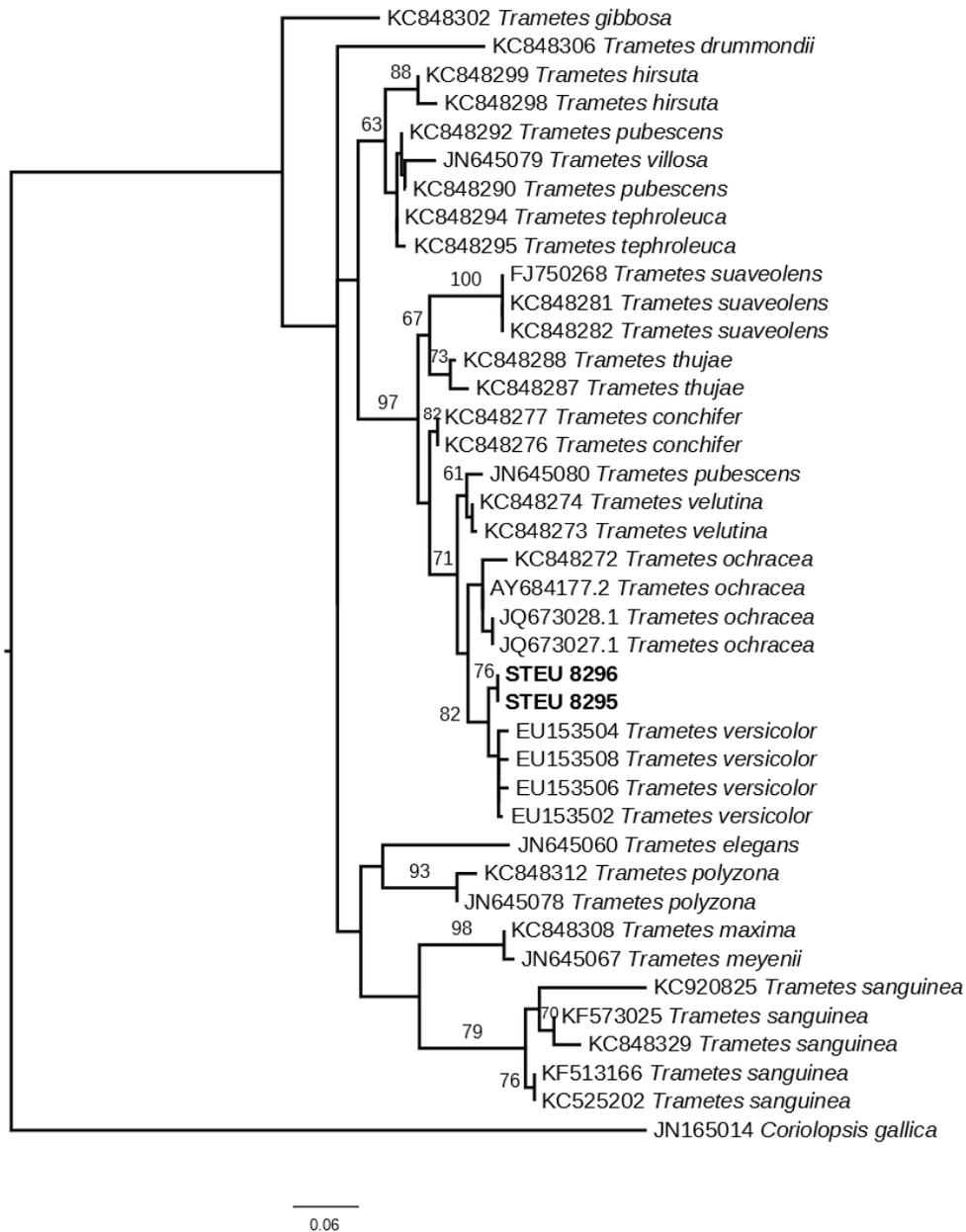
Figure 2. Maximum likelihood phylogenetic tree of wood rotting fungi of the Basidiomycetes specifically species of *Trametes* which was based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Coriolopsis gallica* was used as the outgroup. Isolates obtained in this study are indicated in bold.
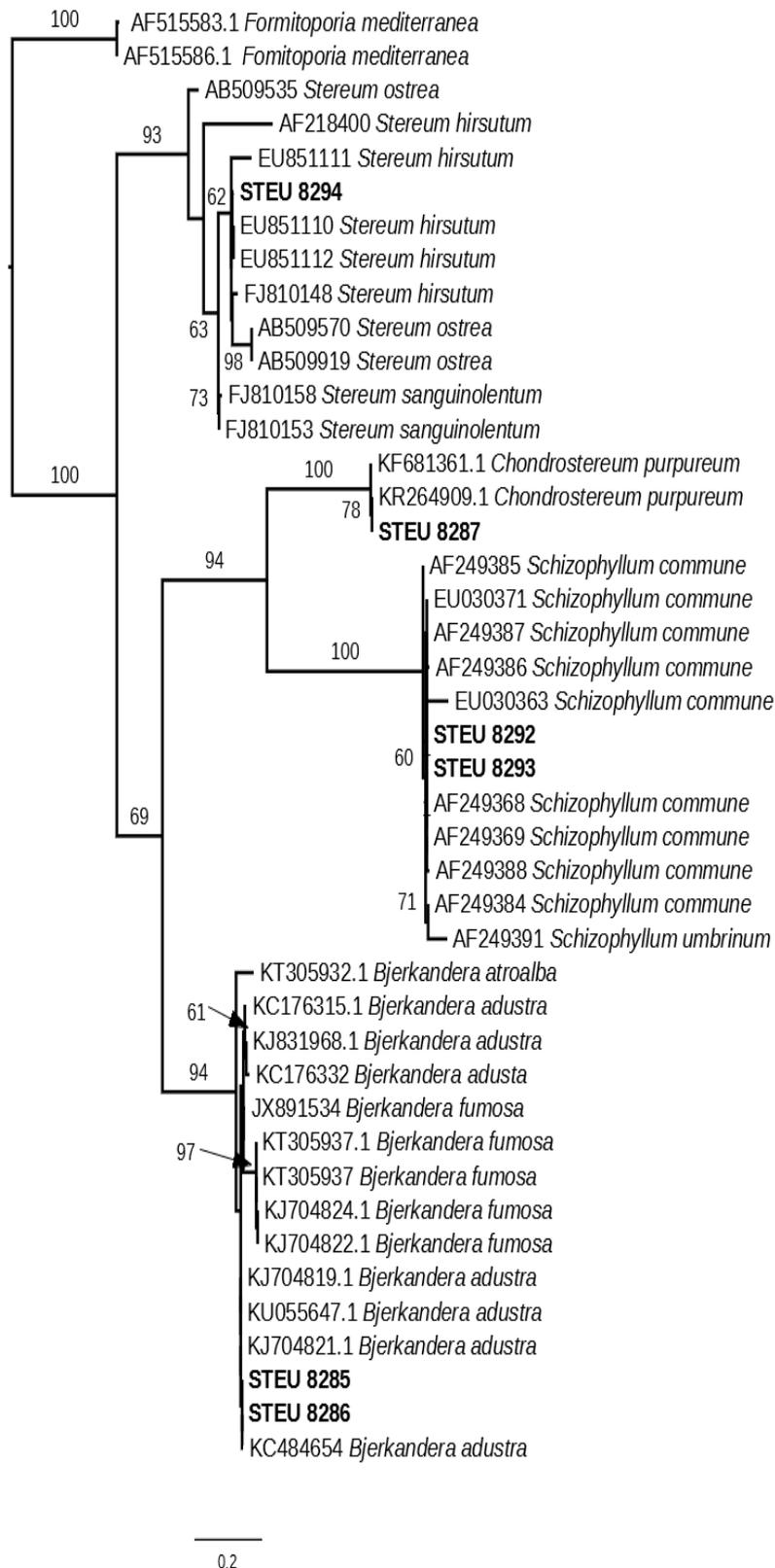
Figure 3. Maximum likelihood phylogenetic tree of wood rotting fungi of the Basidiomycetes which was based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Fomitoporia mediterranea* was used as the outgroup. Isolates obtained in this study are indicated in bold.
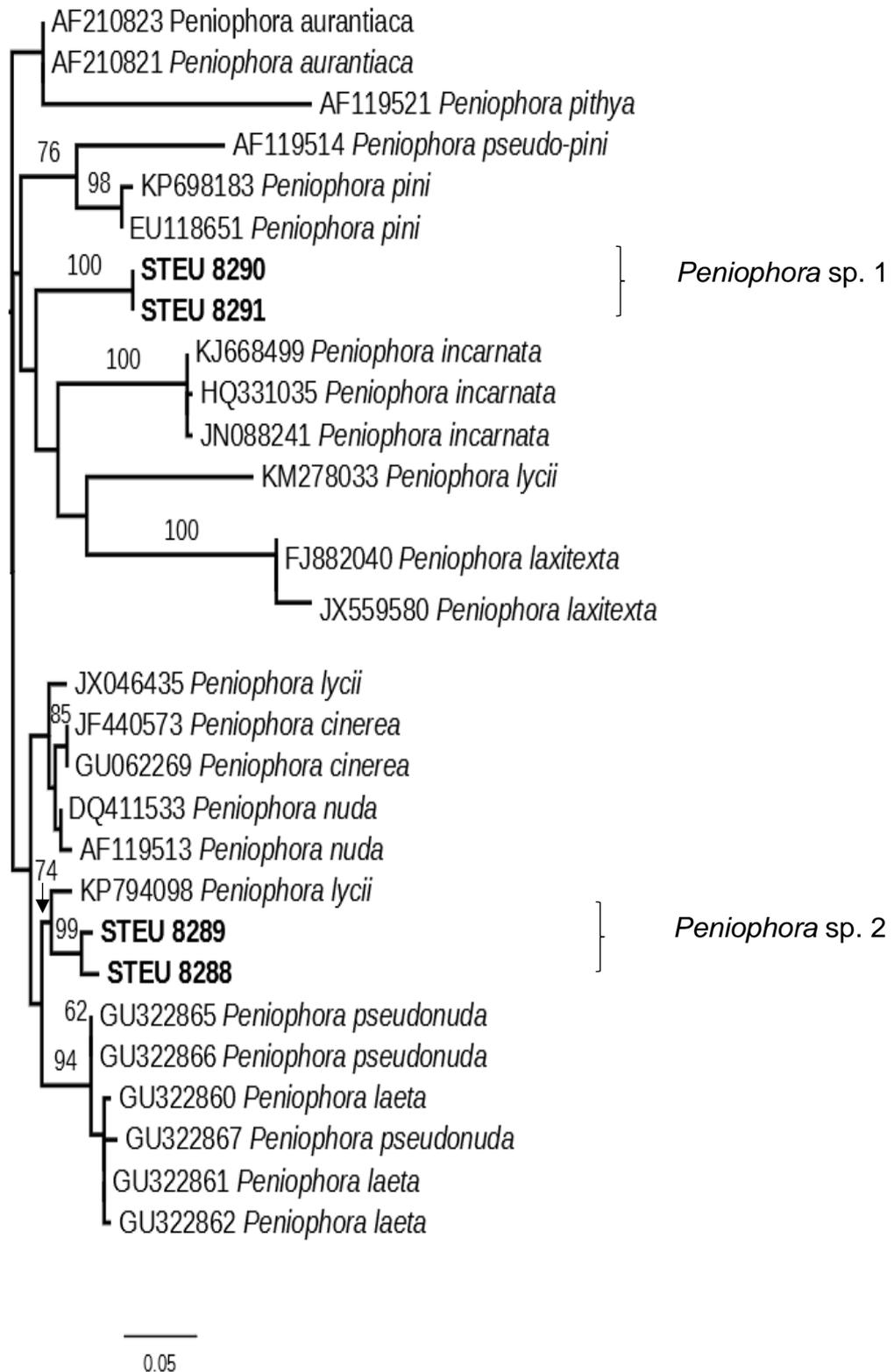
Figure 4. Maximum likelihood phylogenetic tree of wood rotting fungi of the Basidiomycetes specifically species of *Peniophora* which was based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Peniophora pithya* and *Peniophora aurantiaca* were used as the outgroup. Isolates obtained in this study are indicated in bold.
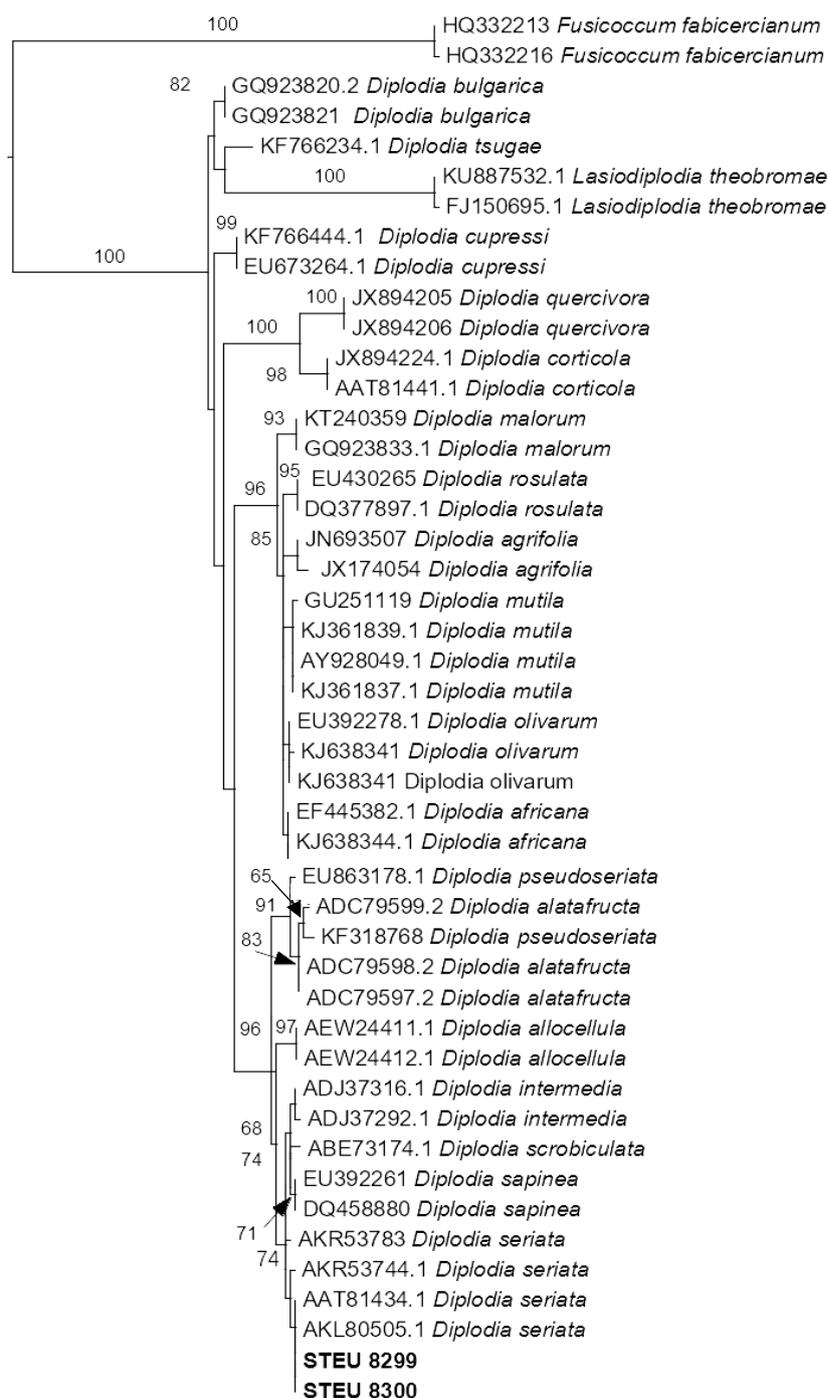
Figure 5. Maximum likelihood phylogenetic tree of *Diplodia* species which was based on elongation factor 1-alpha sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Fusicoccum fabicercianum* was used as the outgroup. Isolates obtained in this study are indicated in bold.
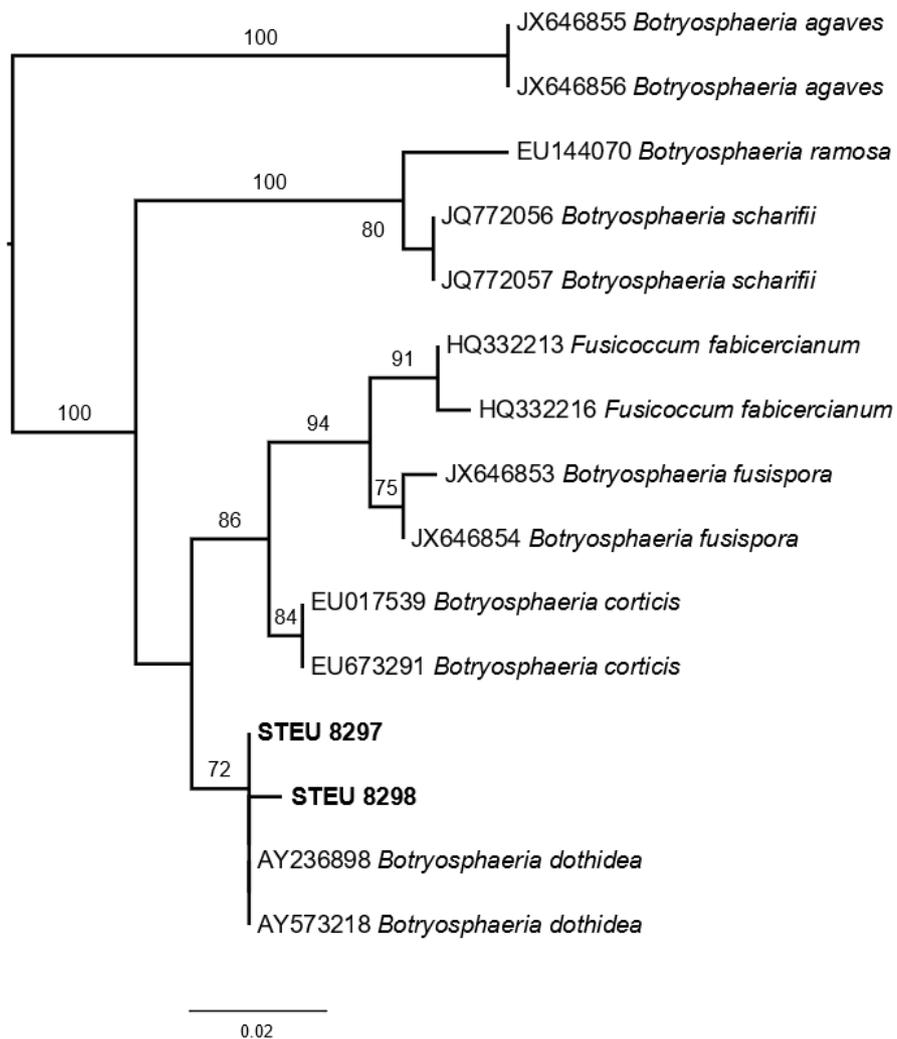
Figure 6. Maximum likelihood phylogenetic tree of *Botryosphaeria* species which was based on elongation factor 1-alpha sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Botryosphaeria agaves* was used as the outgroup. Isolates obtained in this study are indicated in bold.
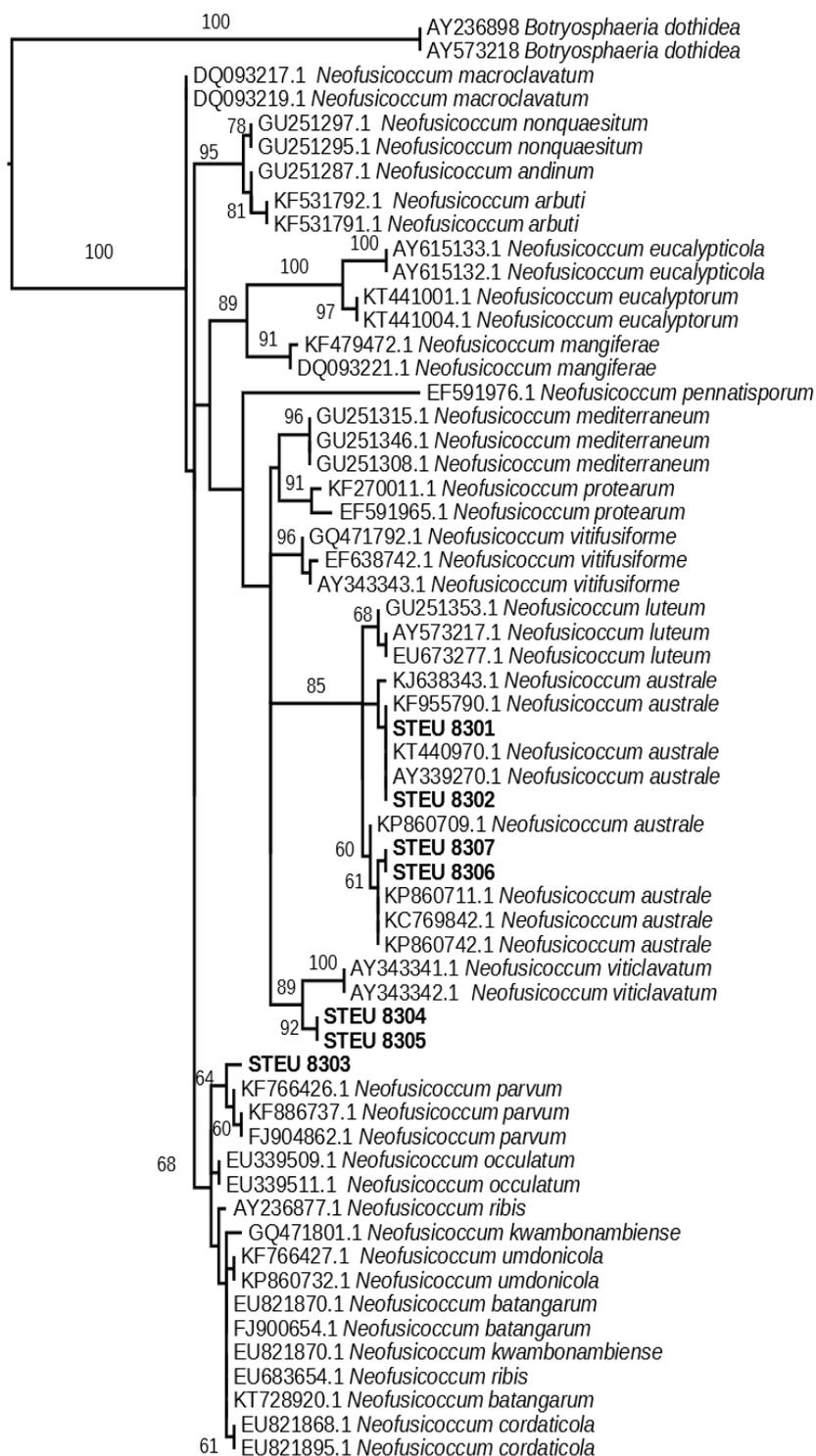
Figure 7. Maximum likelihood phylogenetic tree of *Neofusicoccum* species which was based on elongation factor 1-alpha sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Botryosphaeria dothidea* was used as the outgroup. Isolates obtained in this study are indicated in bold.
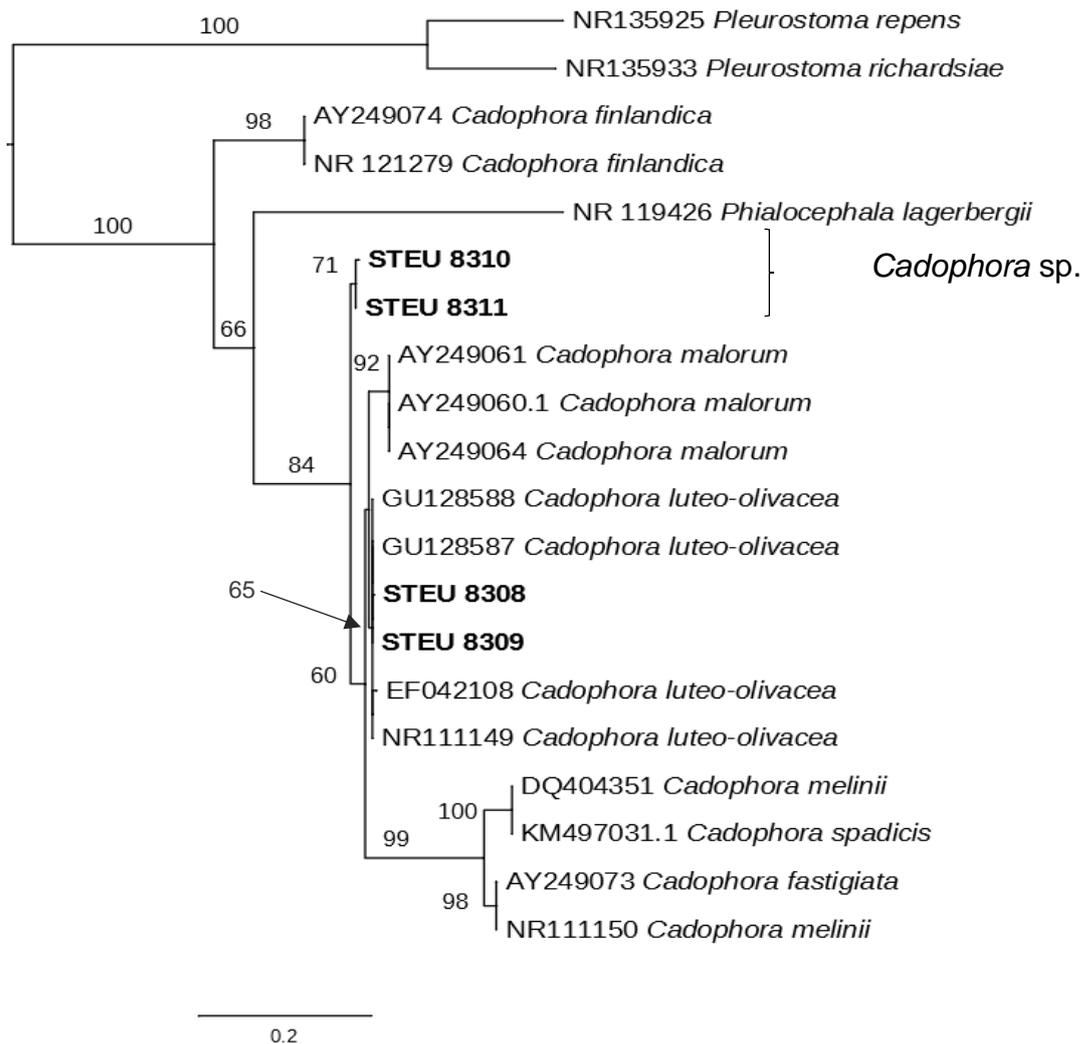
Figure 8. Maximum likelihood phylogenetic tree of *Cadophora* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Pleurostoma repens* and *Pleurostoma richardsiae* were used as the outgroup. Isolates obtained in this study are indicated in bold.
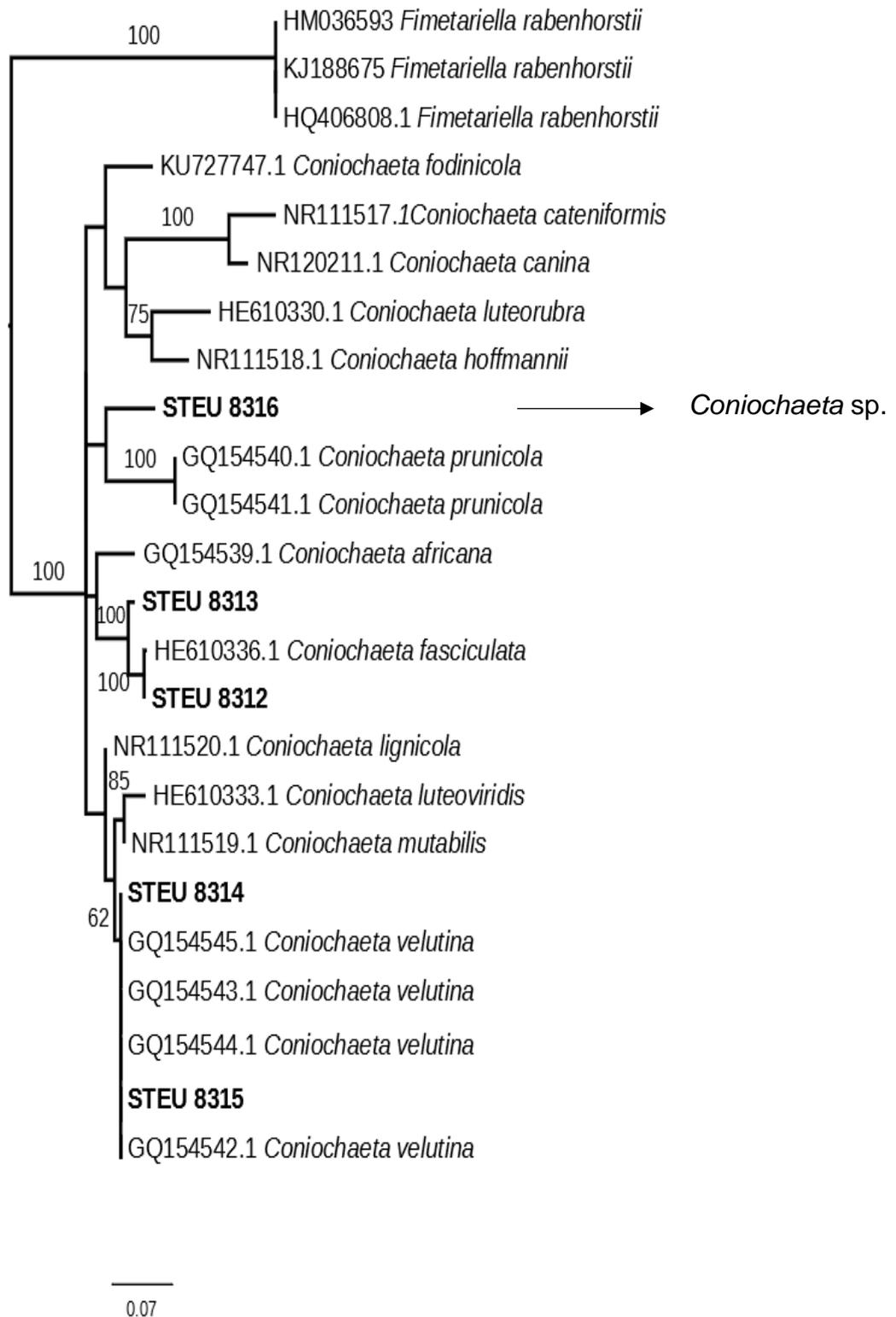
Figure 9. Maximum likelihood phylogenetic tree of *Coniochaeta* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Fimetariella rabenhorstii* was used as the outgroup. Isolates obtained in this study are indicated in bold.
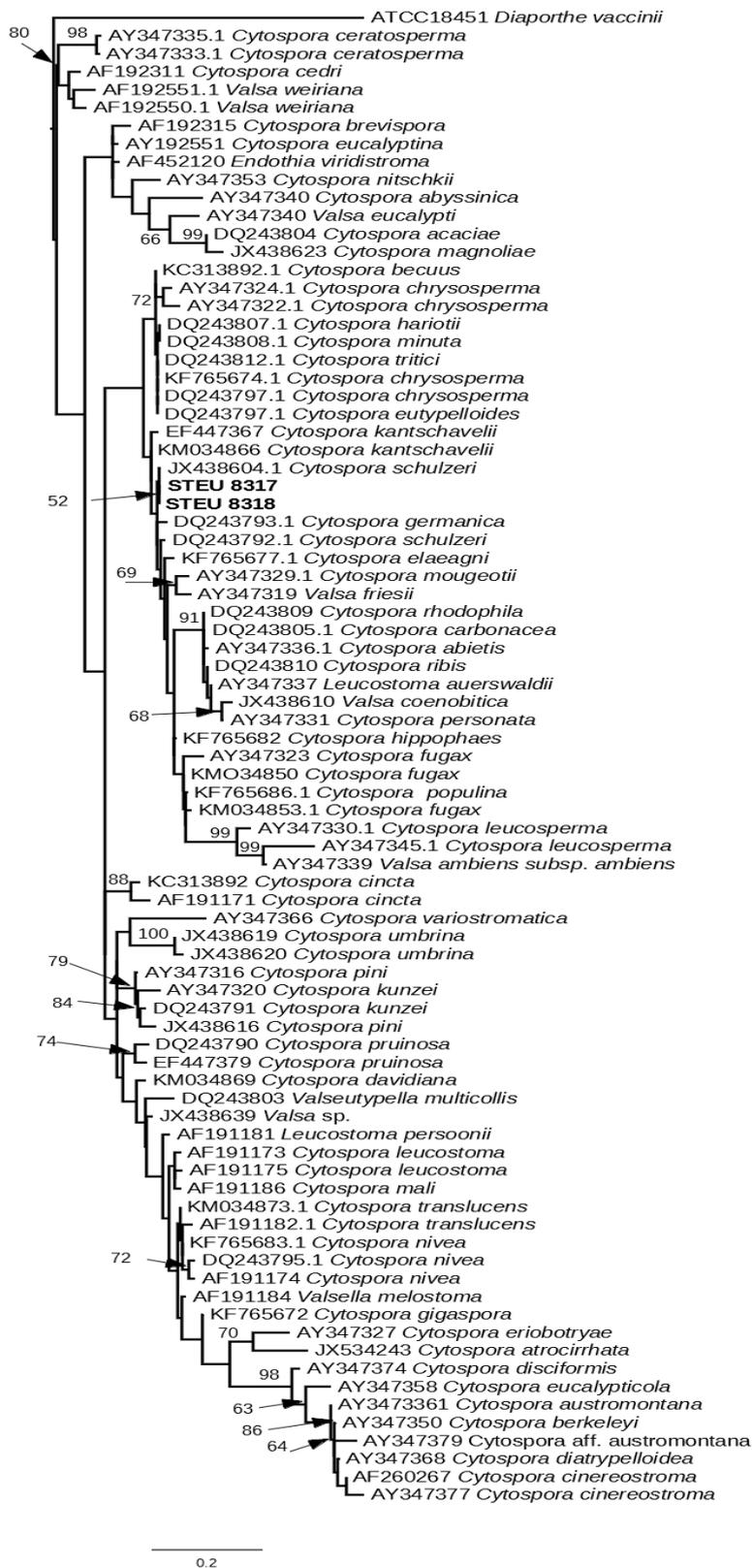
Figure 10. Maximum likelihood phylogenetic tree of *Cytospora* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 52% and higher are shown. *Diaporthe vaccinia* was used as the outgroup. Isolates obtained in this study are indicated in bold.
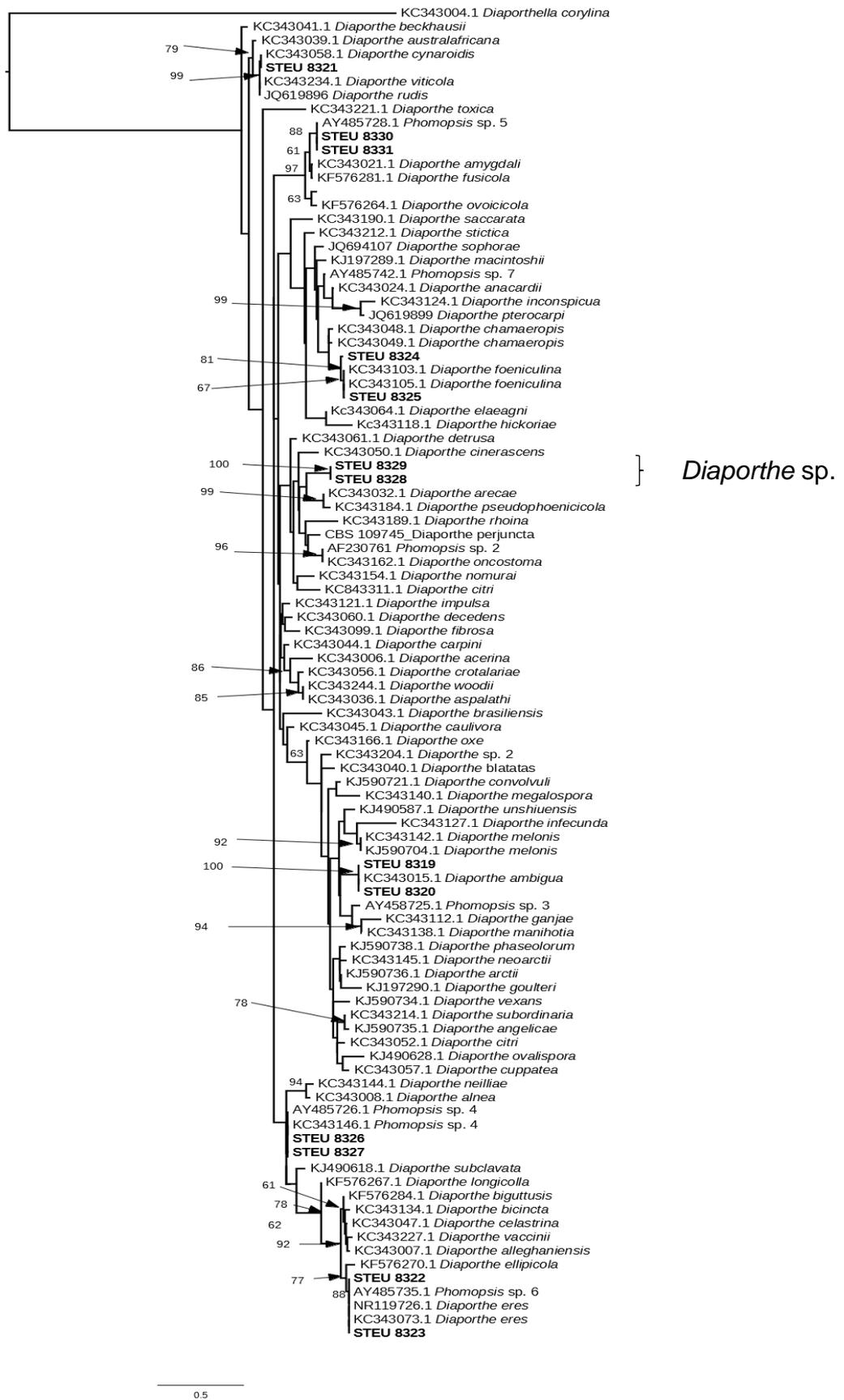
Figure 11. Maximum likelihood phylogenetic tree of *Diaporthe* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 59% and higher are shown. *Diaporthella corylina* was used as the outgroup. Isolates obtained in this study are indicated in bold.
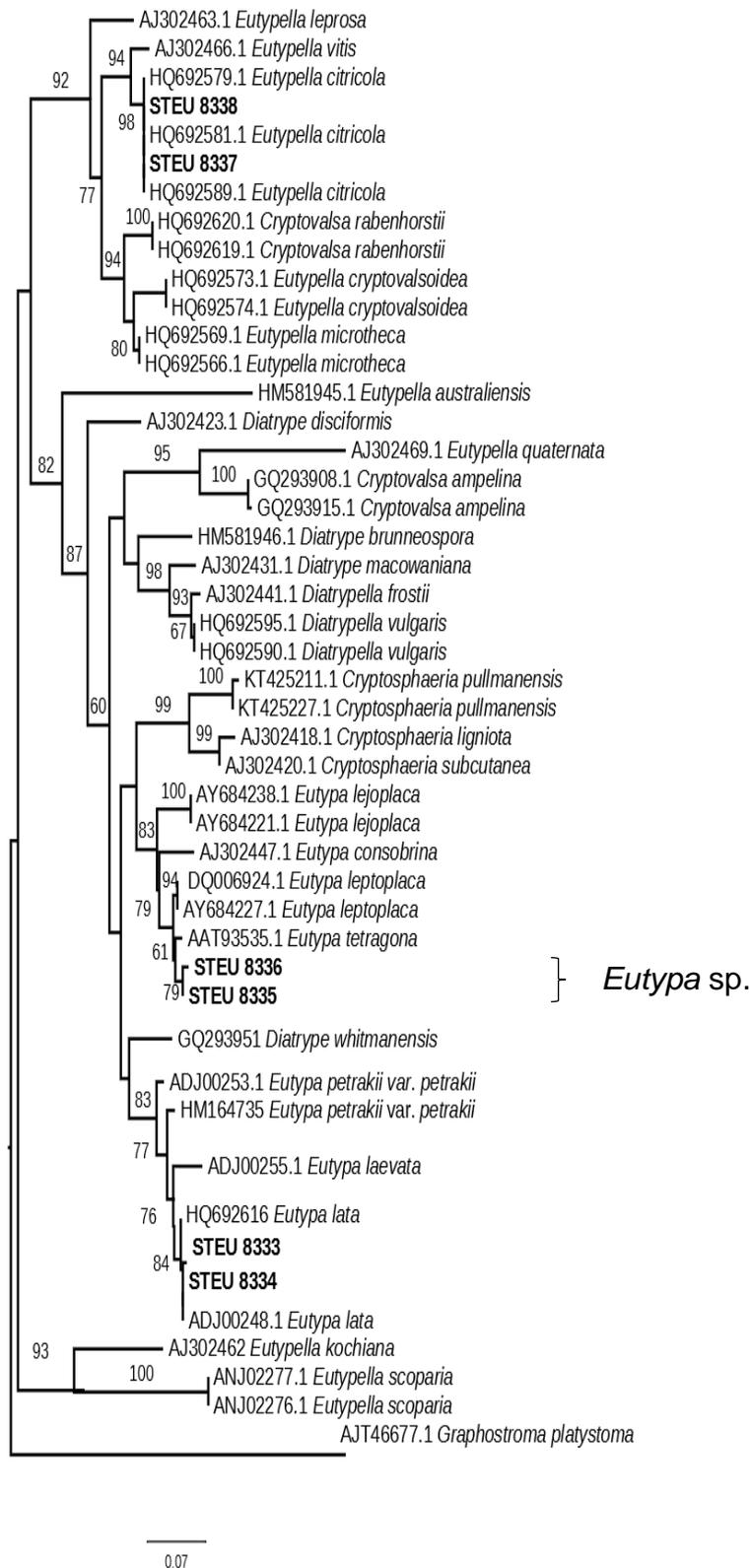
Figure 12. Maximum likelihood phylogenetic tree of the Diatrypaceae based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Graphostroma platystroma* was used as the outgroup. Isolates obtained in this study are indicated in bold.
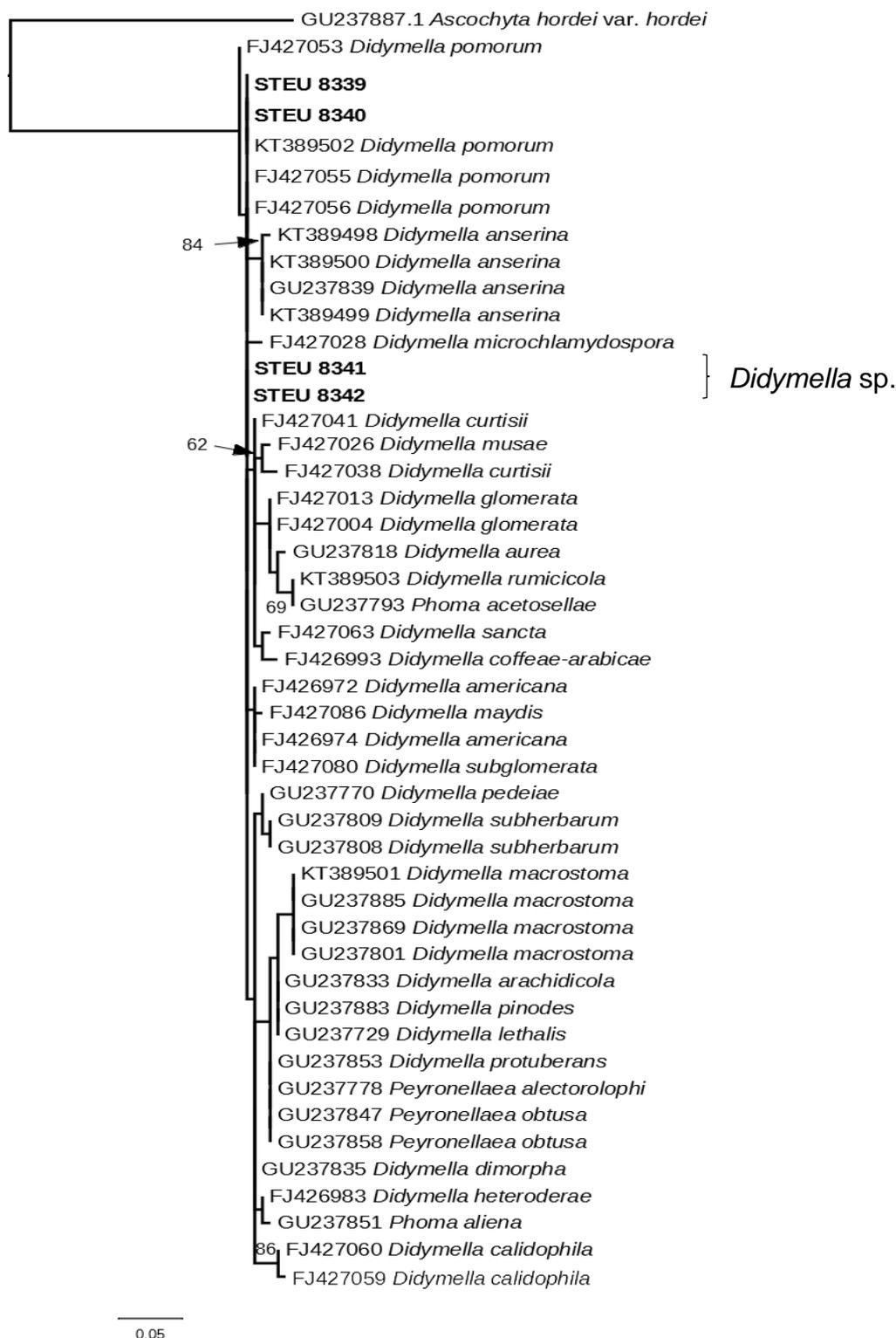
Figure 13. Maximum likelihood phylogenetic tree of *Didymella* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Ascochyta hordei* var. *hordei* was used as the outgroup. Isolates obtained in this study are indicated in bold.
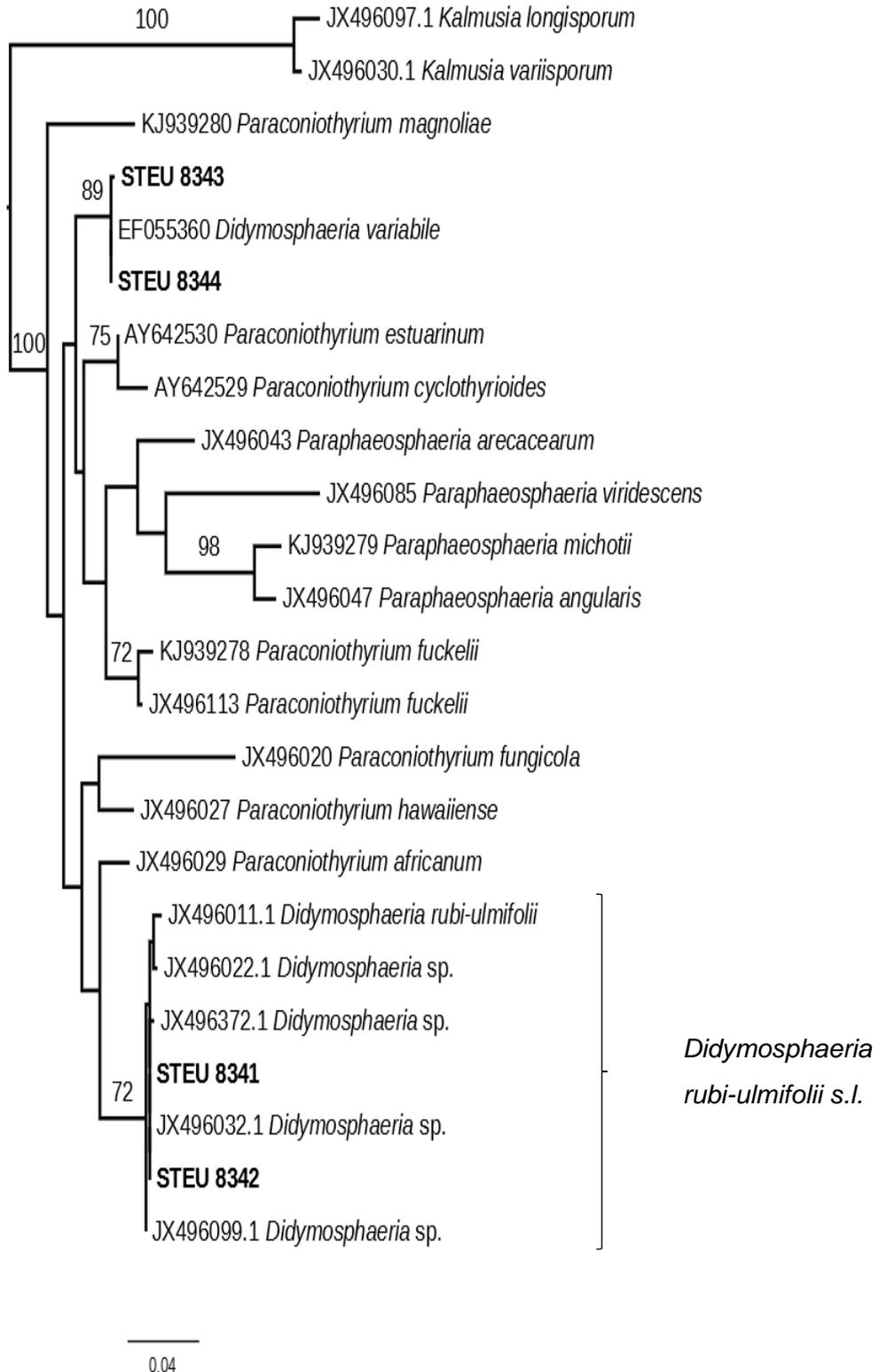
Figure 14. Maximum likelihood phylogenetic tree of *Didymosphaeria rubi-ulmifolii s.l.* species based on ITS sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Kalmusia variisporum* was used as the outgroup. Isolates obtained in this study are indicated in bold.
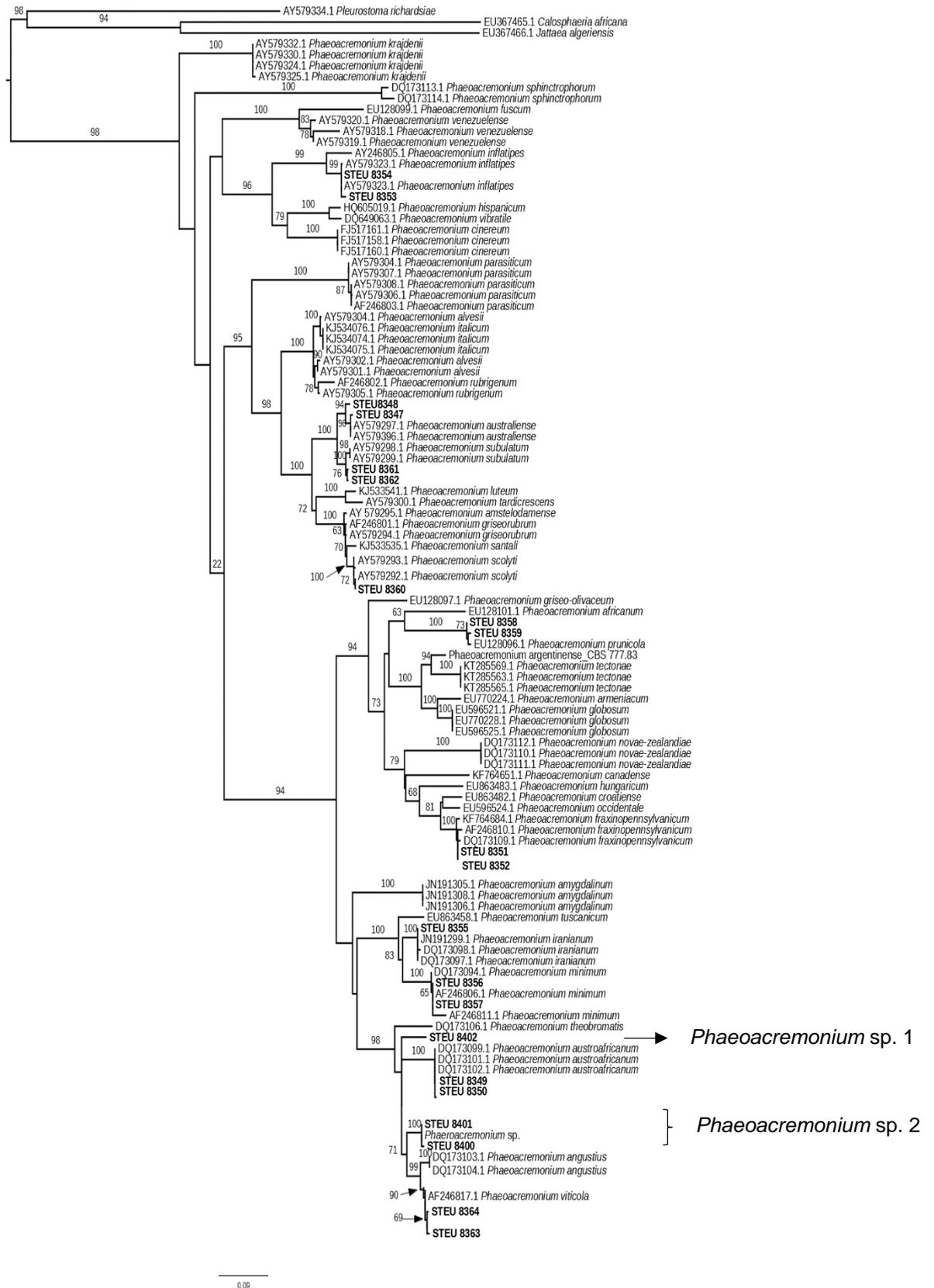
Figure 15. Maximum likelihood phylogenetic tree of *Phaeoacremonium* species based on β-tubulin sequence data. Bootstrap support values were calculated from 100 replicates and bootstrap support of 60% and higher are shown. *Jattaea algeriensis* and *Calosphaeria Africana* were used as the outgroup. Isolates obtained in this study are indicated in bold.
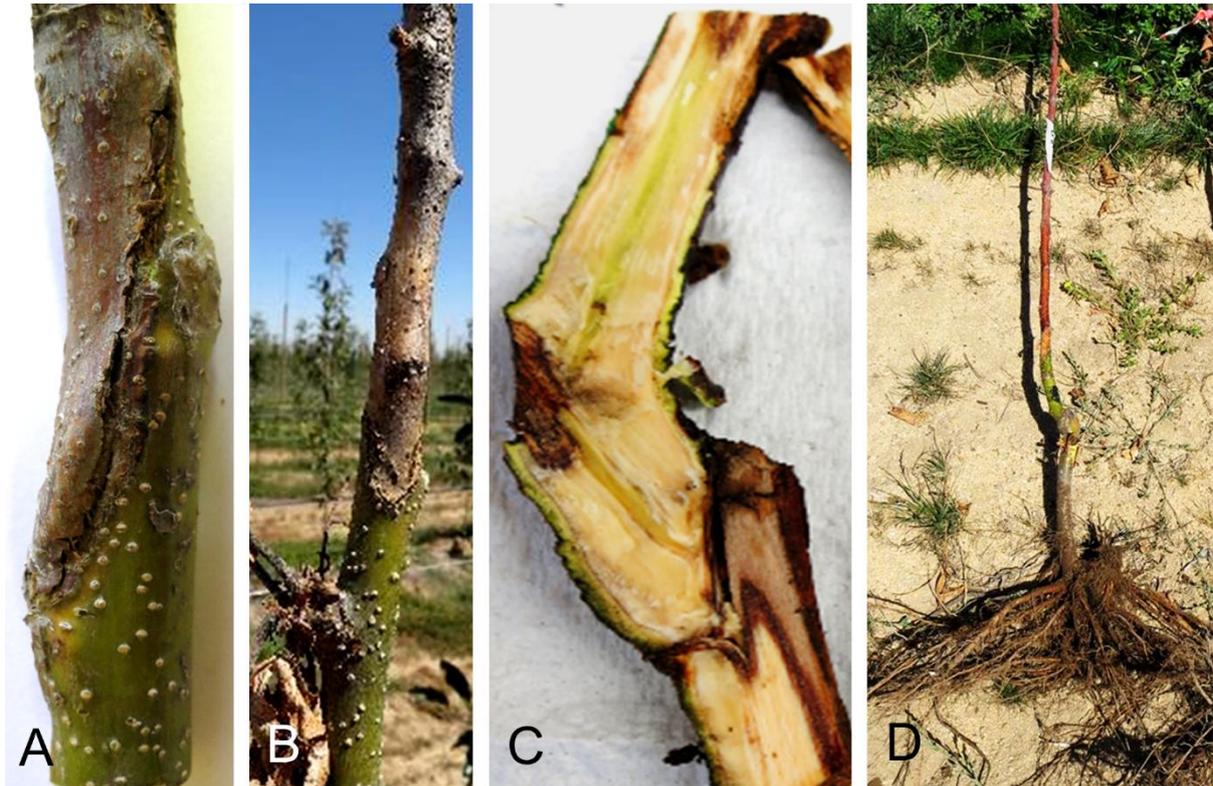
Figure 16. External symptoms exhibited on 1-year-old apple trees showing canker on the scion shoot (A, B). A cross section through the canker showing dark brown vascular discoloration (C). The tree has a well-developed root system (D).

Figure 17. Sections through the scion shoots of 1-year-old apple trees show vascular discoloration. *Diaporthe eres* (A), *Diplodia seriata* (B) and *Didymosphaeria rubi-ulmifolii s.l.* (C) were the causal organisms found on these symptomatic material.
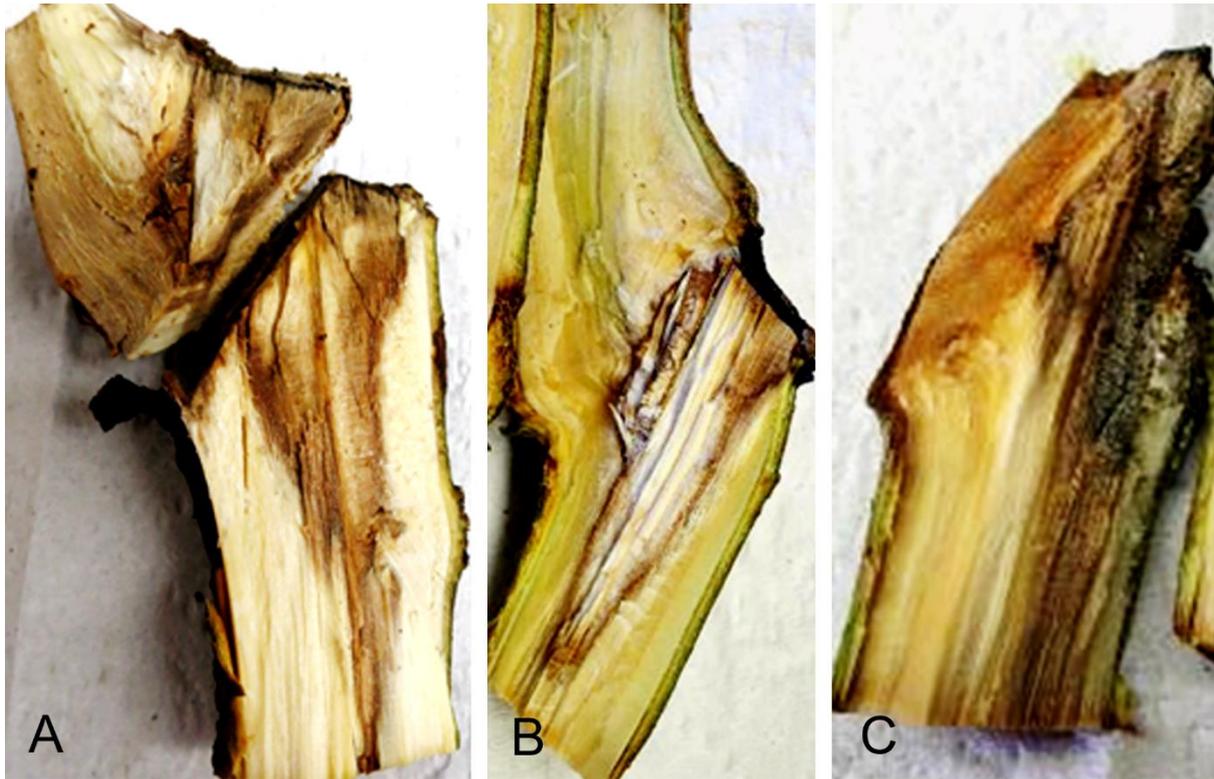
Figure 18. Sections through 1-year-old apple trees shows vascular discoloration originating from the pruning wounds. *Diplodia seriata* (A), *Trametes versicolor* (B) and *Didymosphaeria rubi-ulmifolii s.l.* (C) were the causal organisms isolated from the symptomatic material.
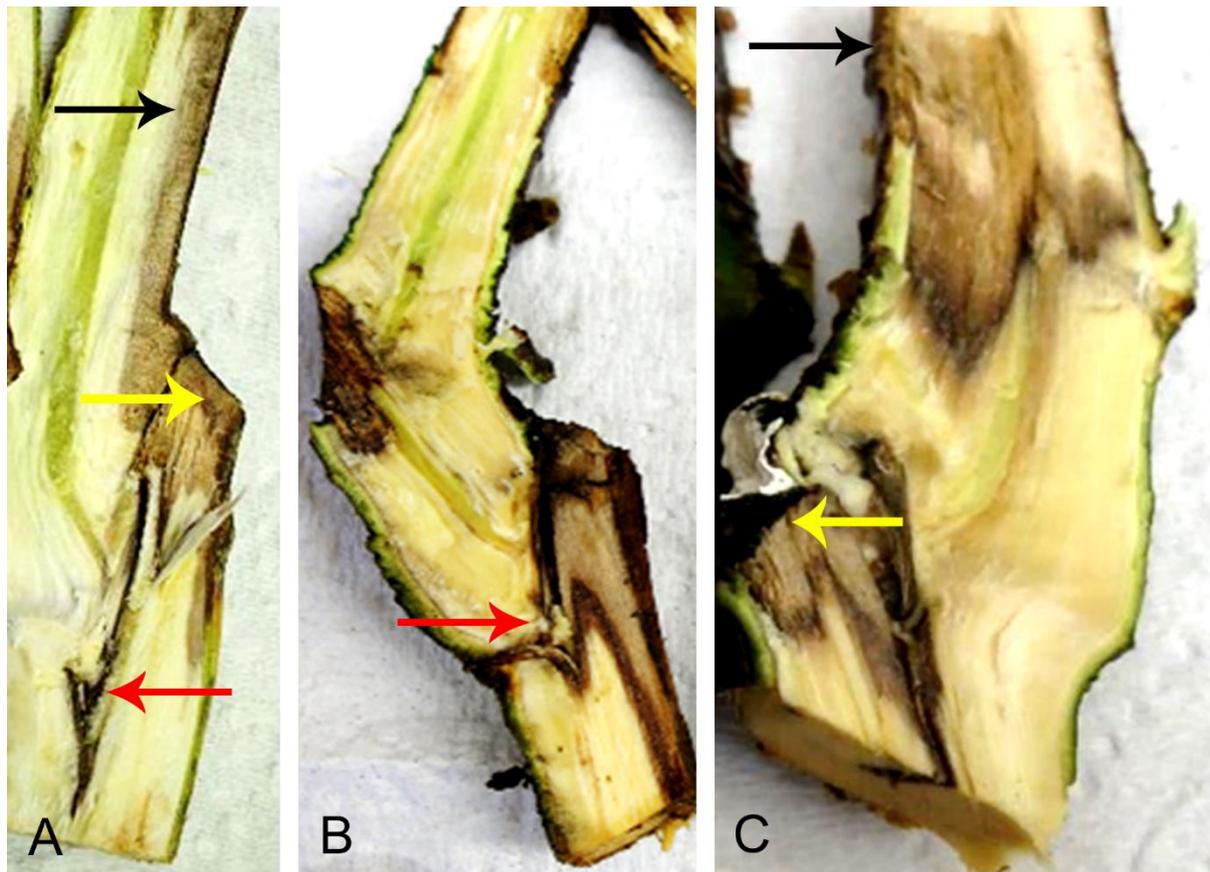
Figure 19. Sections through cankers and pruning wounds found on 1-year-old apple trees showing vascular discoloration. More than one pathogen was isolated from these trees. The causal organisms involved in the symptom expression found were: (A) *Phaeoacremonium austroafricanum* isolated from the bud union and *Diplodia seriata* isolated from the pruning wound; (B) *Phaeoacremonium fraxinopennsylvanicum* isolated from the bud union whereas *Coniochaeta fasiculata* and *Diplodia seriata* were isolated from the canker that formed on the scion shoot and (C) *Diplodia seriata* isolated from the pruning wound and *Didymosphaeria rubi-ulmifolii s.l.* was isolated from the canker that formed on the scion shoot. Red arrow indicate bud union, yellow arrow indicate pruning wound and black arrow scion shoot.

Figure 20. The fruiting structures of *Schizophyllum commune* was observed on a 1-year-old Royal Beaut tree.
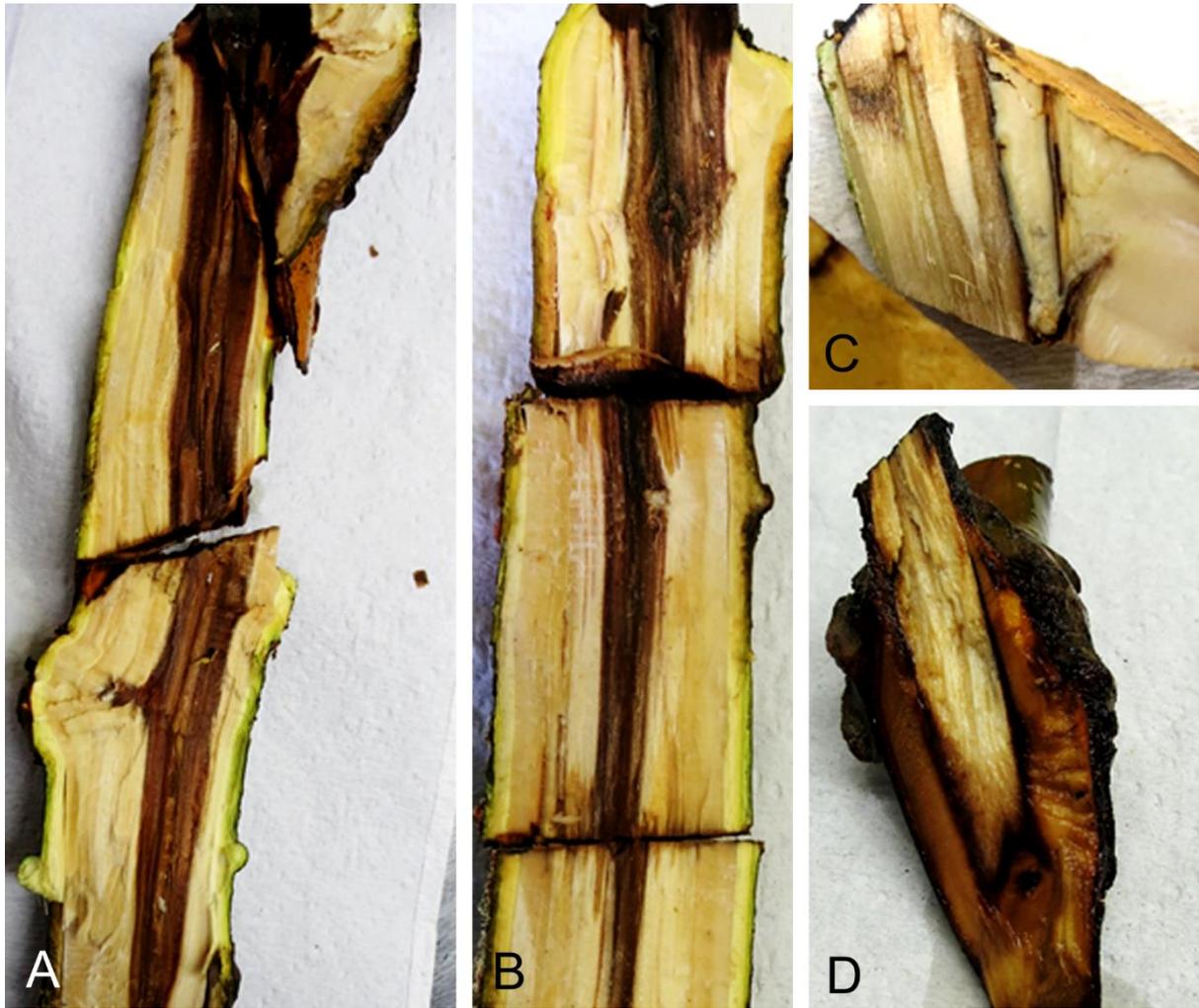
Figure 21. Cross sections through the pruning wound and the bud union of external asymptomatic nursery apple trees. Dark brown discoloration (A, B) and white soft rot (C, D) originating from the pruning wound and bud union.

Figure 22. Sections through the M793 apple rootstock (A) and a section through the pruning wound and bud union (B) showing brown internal discoloration. The causal organisms involved in the vascular discoloration was *Didymosphaeria rubi-ulmifolii s.l.*
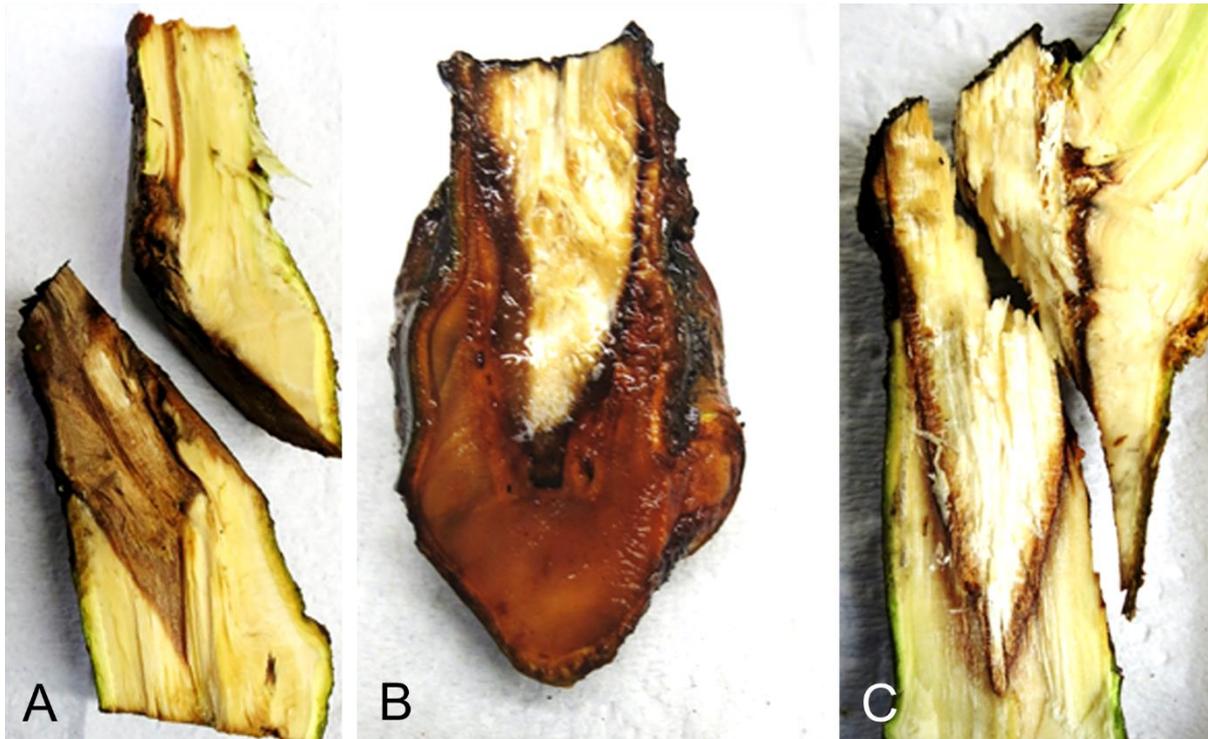
Figure 23. Sections through the bud unions and pruning wounds showing vascular discoloration (A) and white rot (B, C). The causal organism for the brown discoloration was *Schizophyllum commune* (A) and for the typical white rot symptoms were *Bjerkandera adustra* (B) and *Trametes versicolor* (C).
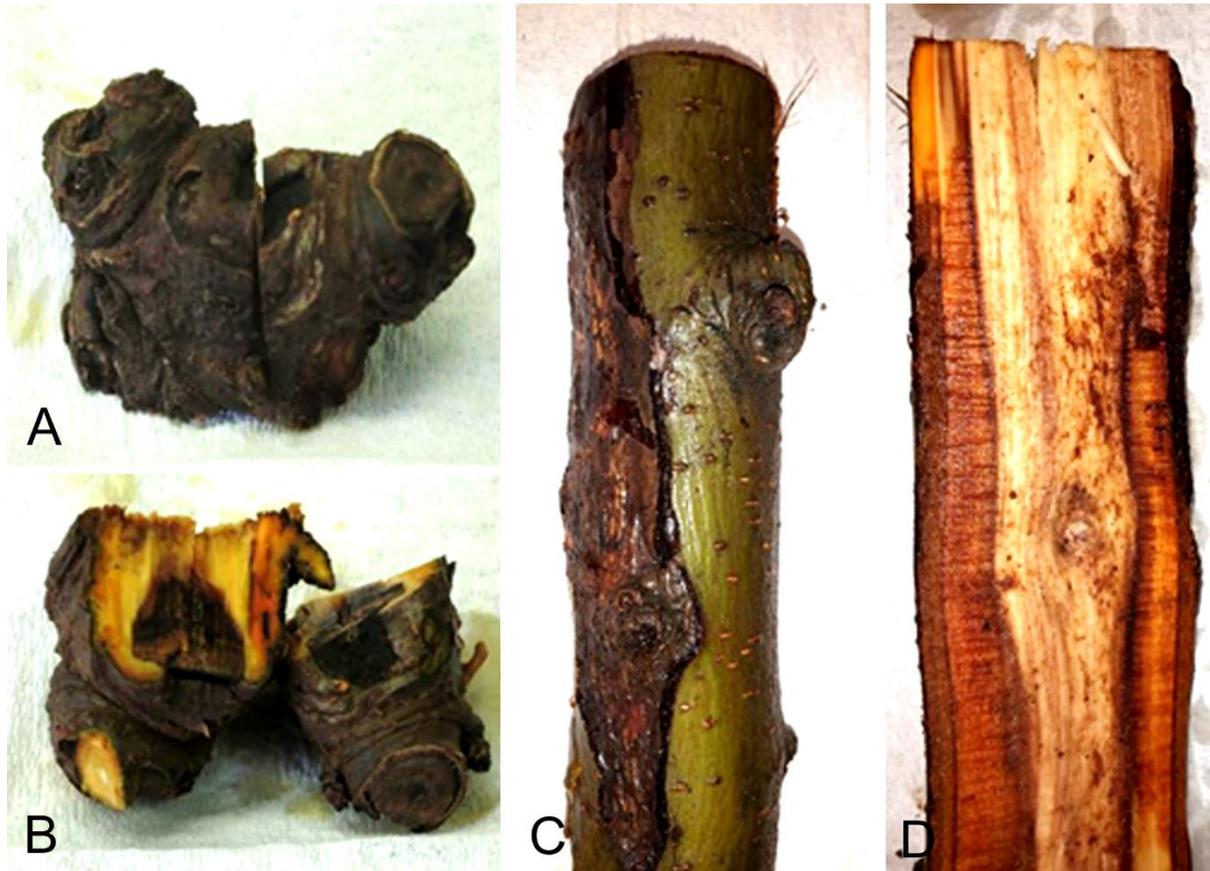
Figure 24. Typical pruning wound (A) sampled from apple scion mother block trees showing vascular discoloration (B) of which *Eutypa lata* was the causal organism. Typical canker (C) with white rot symptoms (D) caused by the wood rotting fungus *Trametes versicolor.*
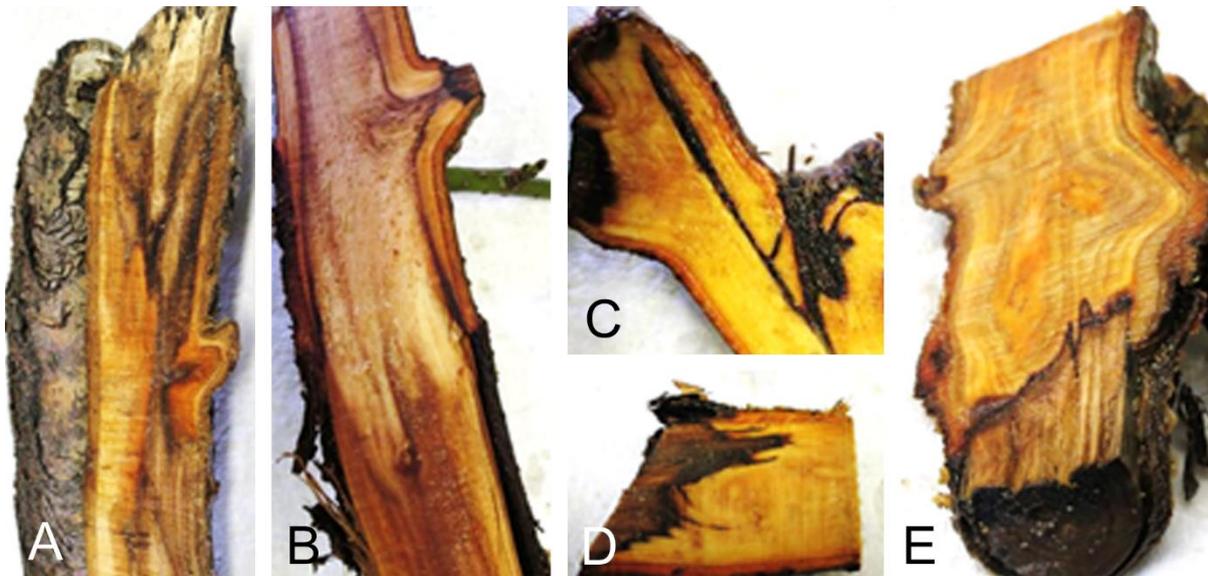
Figure 25. Sections through cankers (A, B) and pruning wounds (C, D, E) found on Golden Delicious trees showing vascular discoloration. *Eutypa lata* (A) and *Trametes versicolor* (B) were the causal organisms found causing brown discolouration (A) and white rot (B) symptoms in these cankers. *Eutypa lata* (C), *Trametes versicolor* (D) and *Didymosphaeria rubi-ulmifolii s.l.* (E) respectively, were the causal organism which infected these pruning wounds.

Figure 26. Fruiting structures of *Trametes versicolor* found in apple scion mother block trees.
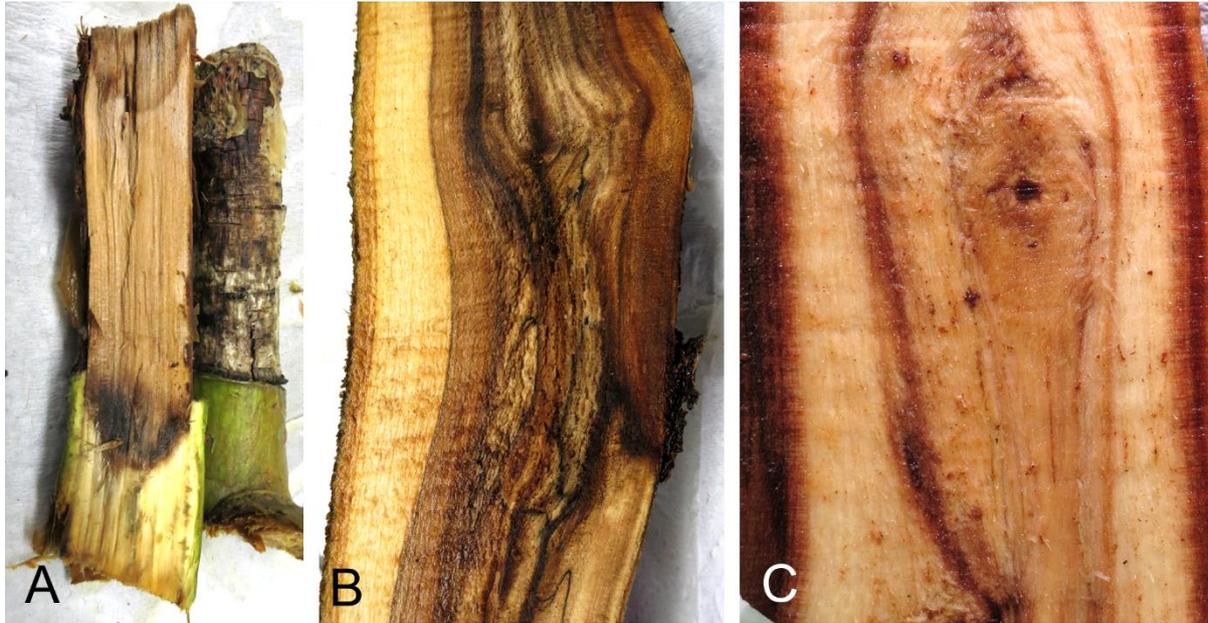
Figure 27. Section though cankers found on scion mother blocks showing brown (A, B) and white (C) vascular discoloration caused by wood rot Basidiomycetes. *Schizophyllum commune* (A) and *Trametes versicolor* (B, C) were the causal organisms involved in these symptoms.
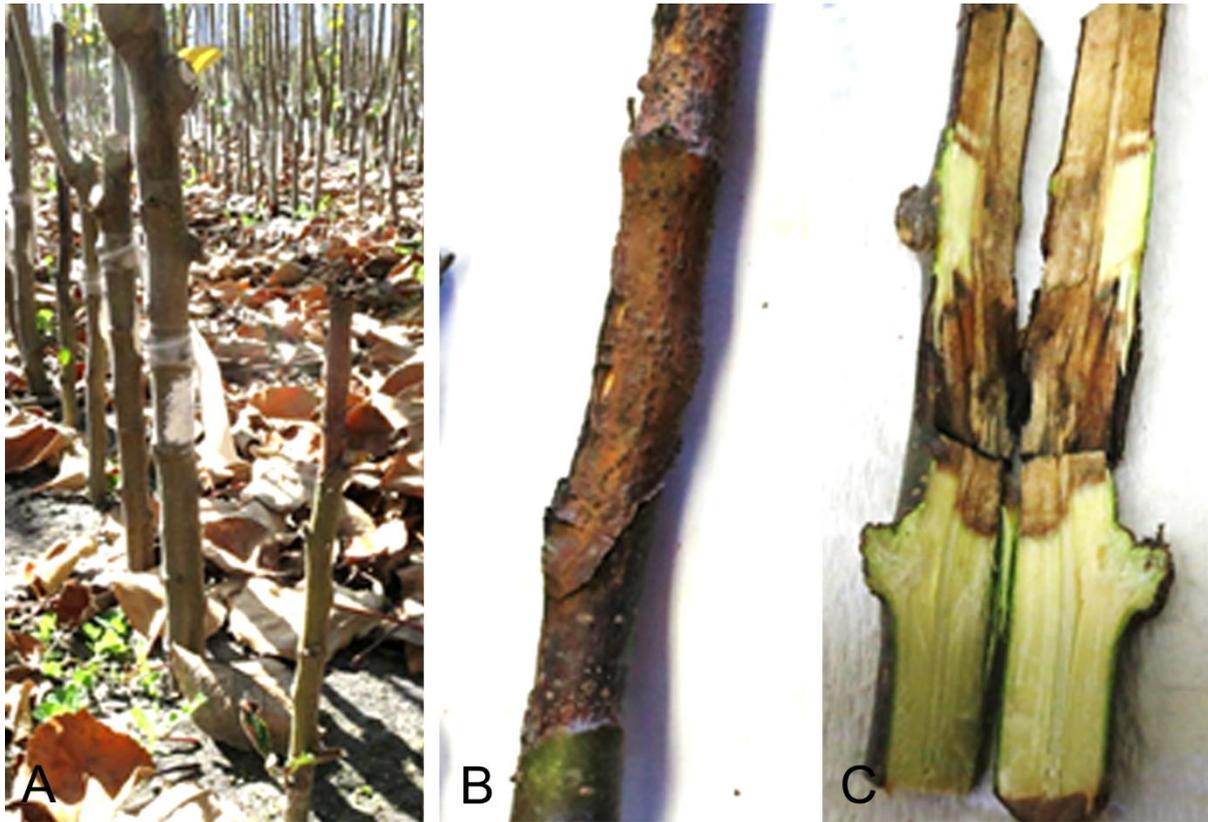
Figure 28. Cankers are observed in 1-year-old nursery blocks next to the already budded nursery tree (A). External symptoms (B) and internal brown vascular discoloration (C) were found on these shoots.

APPENDIX A
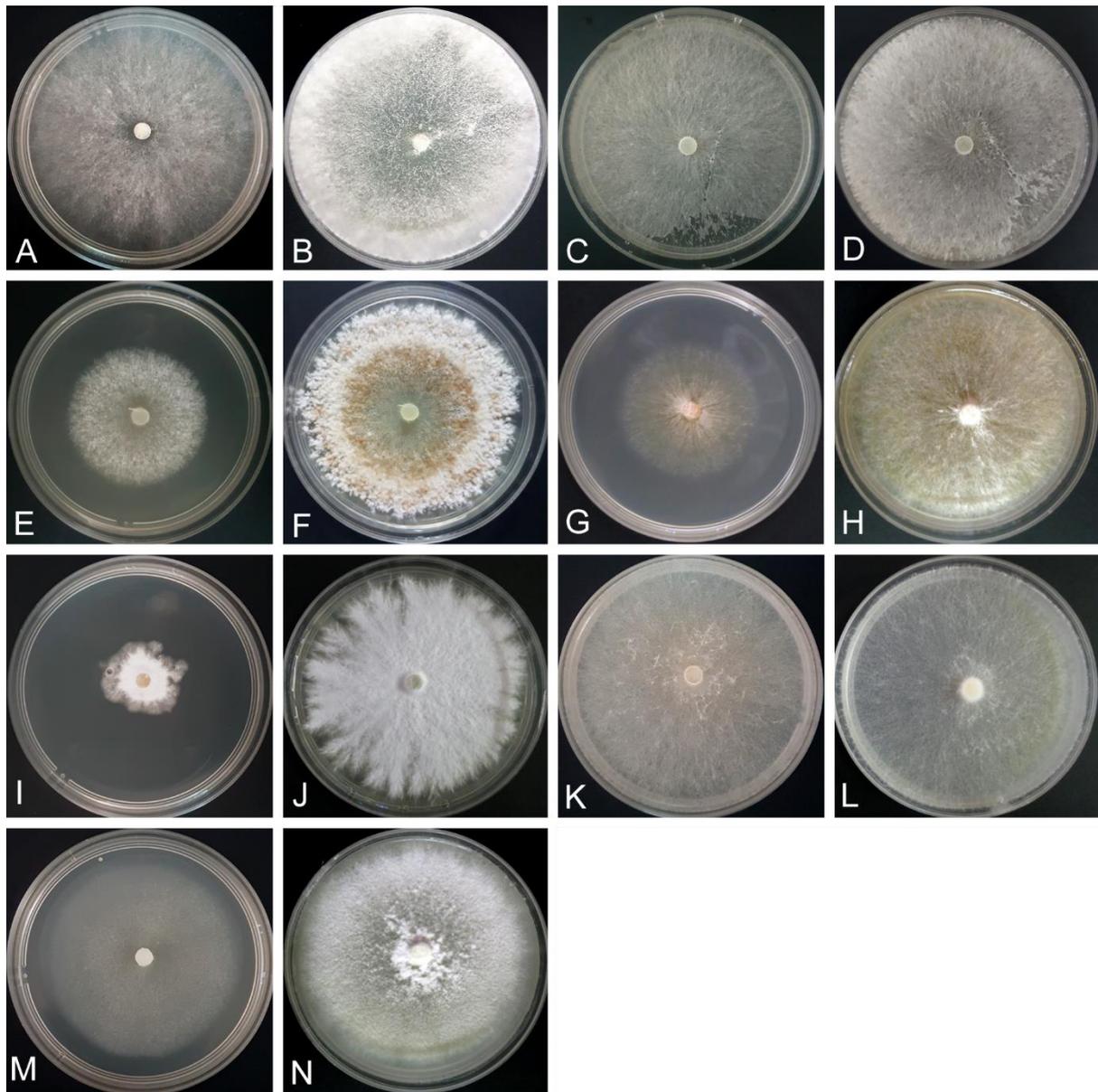
Figure 1. Cultural growth of Basidiomycetes on PDA incubated at 25°C in the dark. *Bjerkandera adustra* (STEU 8285) after 7 days (A) and 14 days (B); *Chondrostereum purpureum* (STEU 8287) after 7 days (C) and 14 days (D); *Peniophora* sp. 1 (STEU 8290) after 7 days (E) and 14 days (F); *Peniophora* sp. 2 (STEU 8289) after 7 days (G) and 14 days (H); *Schizophyllum commune* (STEU 8292) after 7 days (I) and 14 days (J); *Stereum hirsutum* (STEU 8294) after 7 days (K) and 14 days (L); *Trametes versicolor* (STEU 8295) after 7 days (M) and 14 days (N).
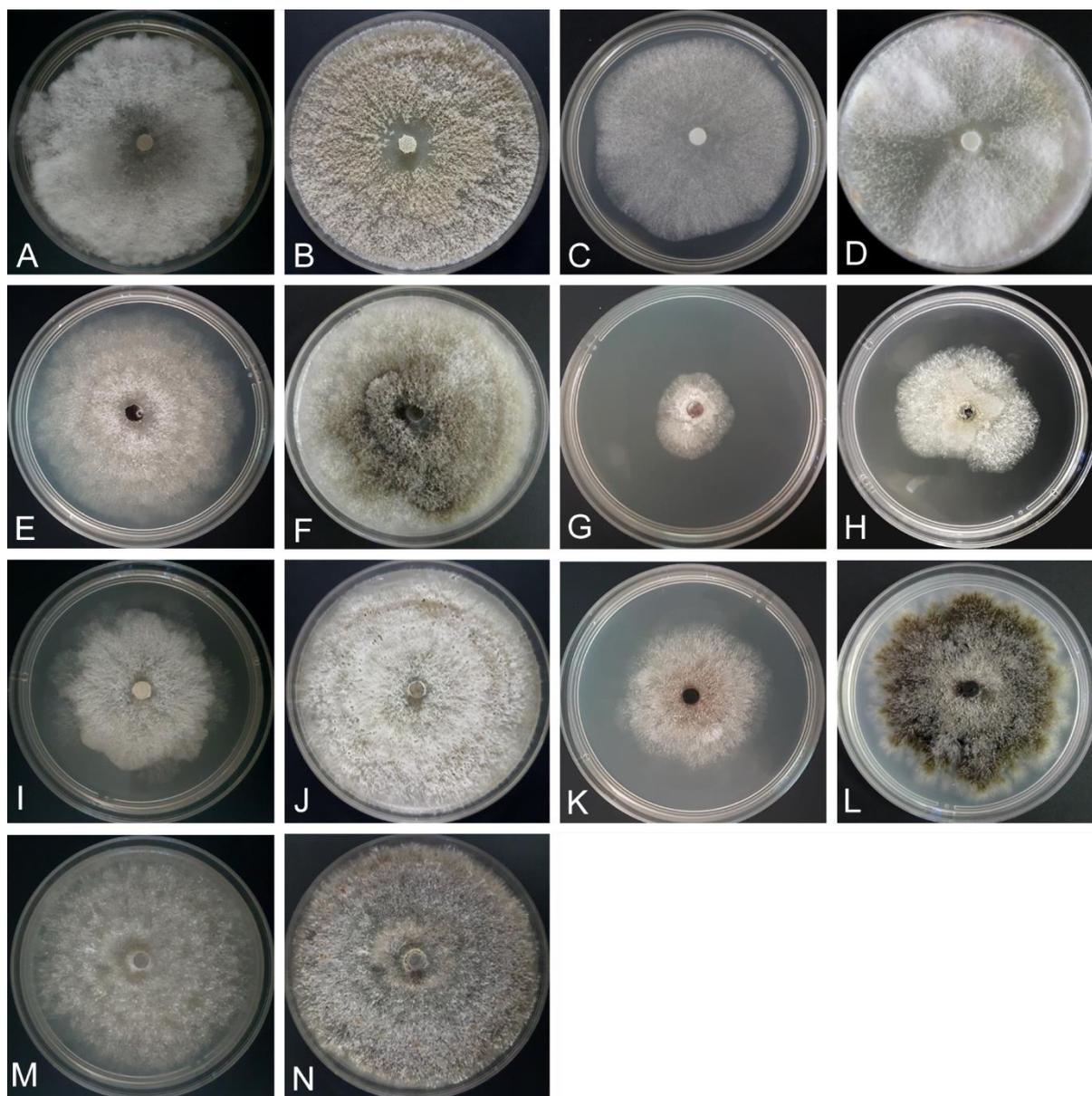
Figure 2. Cultural growth of species within Diaporthales on PDA incubated at 25°C in the dark. *Diaporthe ambigua* (STEU 8319) after 7 days (A) and 14 days (B); *Diaporthe cynaroides* (STEU 8321) after 7 days (C) and 14 days (D); *Diaporthe eres* (STEU 8322) after 7 days (E) and 14 days (F); *Diaporthe foeniculina* (STEU 8324) after 7 days (G) and 14 days (H); *Phomopsis* sp. 4 (STEU 8326) after 7 days (I) and 14 days (J); *Diaporthe* sp. (STEU 8328) after 7 days (K) and 14 days (L); *Phomopsis* sp. 5 (STEU 8330) after 7 days (M) and 14 days (N).
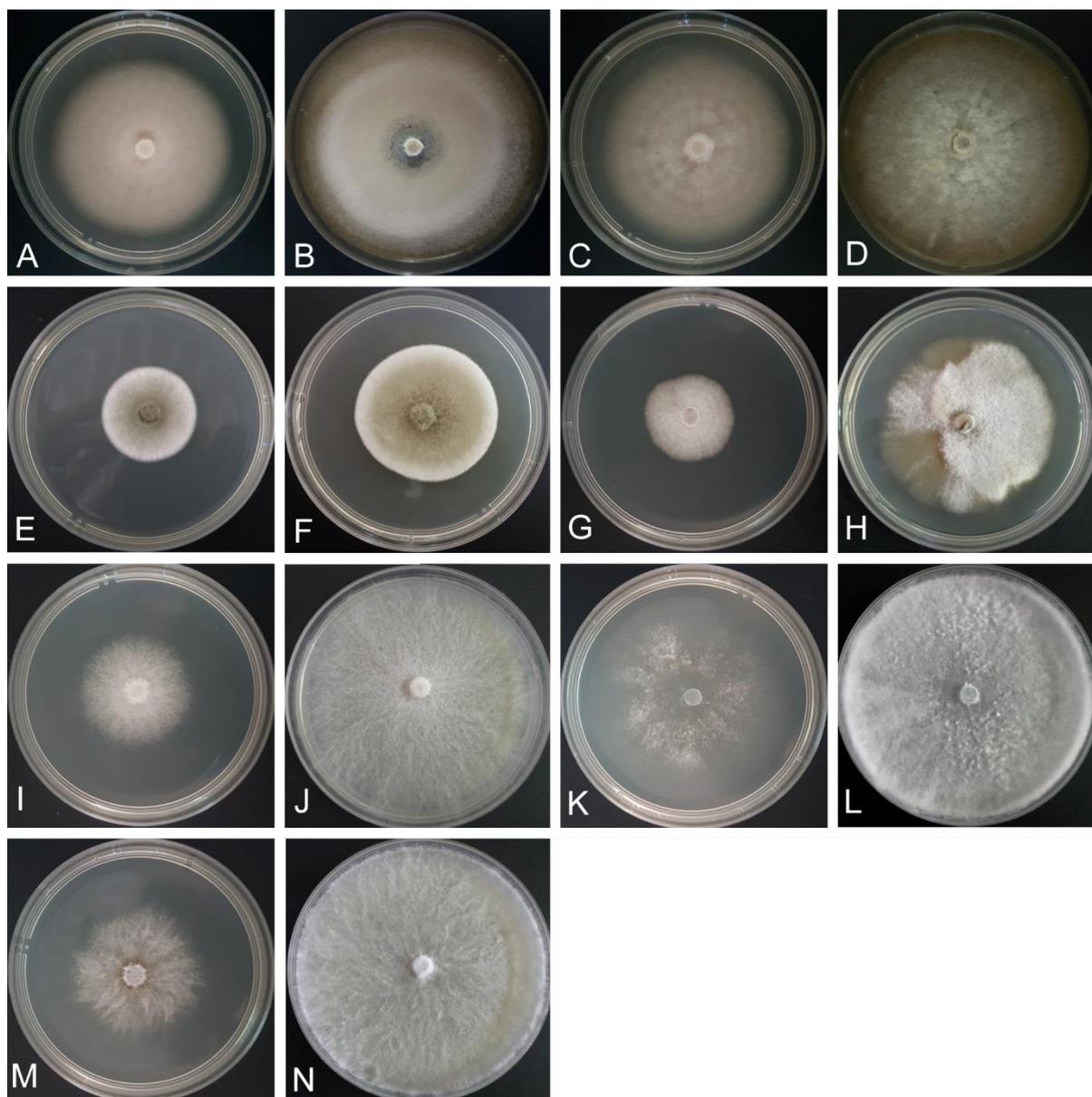
Figure 3. Cultural growth of *Didymella, Didymosphaeria* species and species within Diatrypaceae on PDA incubated at 25°C in the dark. *Didymella pomorum* (STEU 8339) after 7 days (A) and 14 days (B); *Didymella* sp. (STEU 8341) after 7 days (C) and 14 days (D); *Didymosphaeria variabile* (STEU 8343) after 7 days (E) and 14 days (F); *Didymosphaeria rubi-ulmifolii s.l.* (STEU 8345) after 7 days (G) and 14 days (H); *Eutypa lata* (STEU 8333) after 7 days (I) and 14 days (J); *Eutypa* sp. (STEU 8335) after 7 days (K) and 14 days (L); *Eutypella citricola* (STEU 8337) after 7 days (M) and 14 days (N).
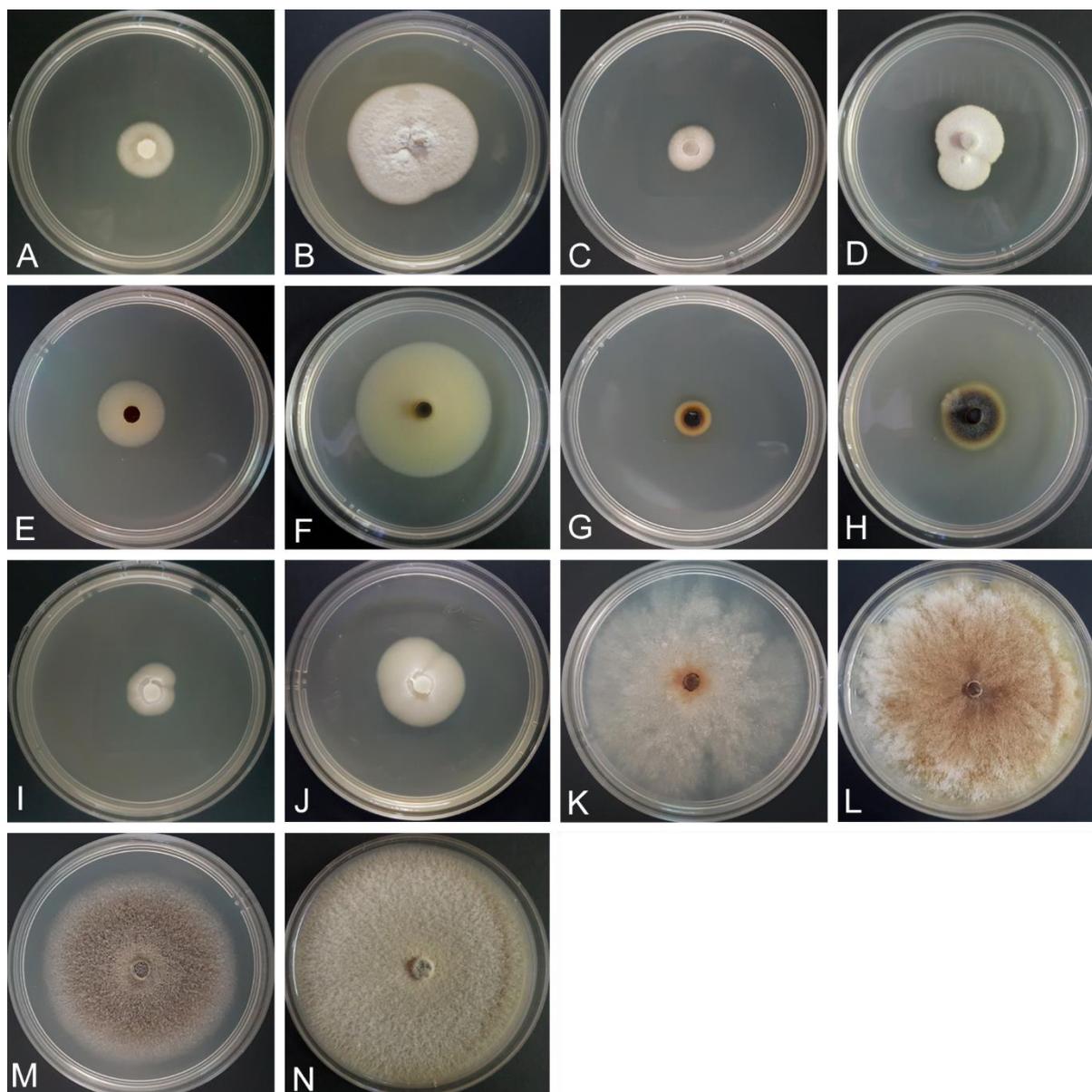
Figure 4. Cultural growth of *Cadophora* and *Coniochaeta* species as well as *Cytospora schulzeri* and *Truncatella angustata* on PDA incubated at 25°C in the dark. *Cadophora luteo-olivacea* (STEU 8308) after 7 days (A) and 14 days (B); *Cadophora* sp. (STEU 8310) after 7 days (C) and 14 days (D); *Coniochaeta fasciculata* (STEU 8312) after 7 days (E) and 14 days (F); *Coniochaeta veluntina* (STEU 8314) after 7 days (G) and 14 days (H); *Coniochaeta* sp. (STEU 8316) after 7 days (I) and 14 days (J); *Cytospora schulzeri* (STEU 8318) after 7 days (K) and 14 days (L); *Truncatella angustata* (STEU 8284) after 7 days (M) and 14 days (N).
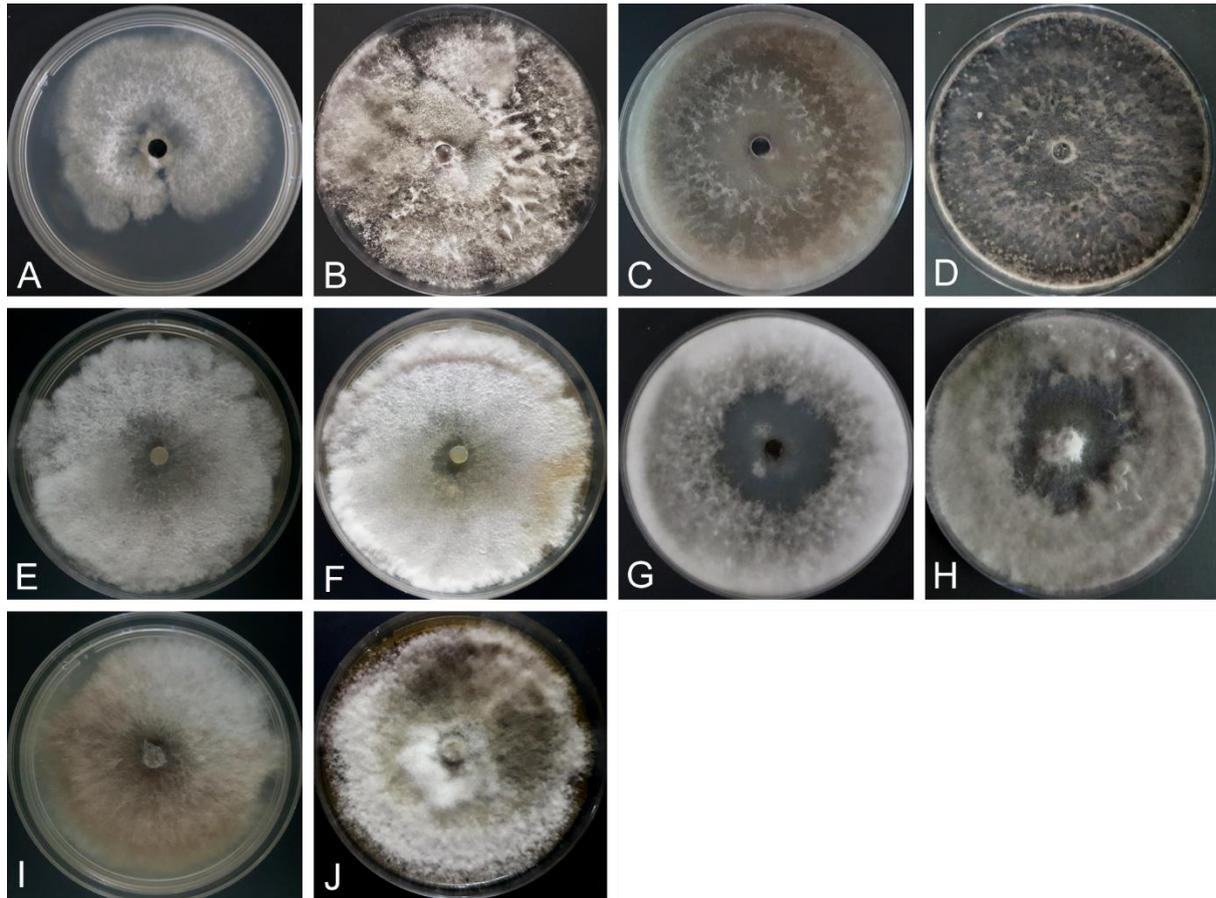
Figure 5. Cultural growth of species within Botryosphaeriaceae on PDA incubated at 25°C in the dark. *Botryosphaeria dothidea* (STEU 8297) after 7 days (A) and 14 days (B); *Diplodia seriata* (STEU 8300) after 7 days (C) and 14 days (D); *Neofusicoccum australe* (STEU 8302) after 7 days (E) and 14 days (F); *Neofusicoccum parvum* (STEU 8303) after 7 days (G) and 14 days (H); *Neofusicoccum viticlavatum* (STEU 8305) after 7 days (I) and 14 days (J).
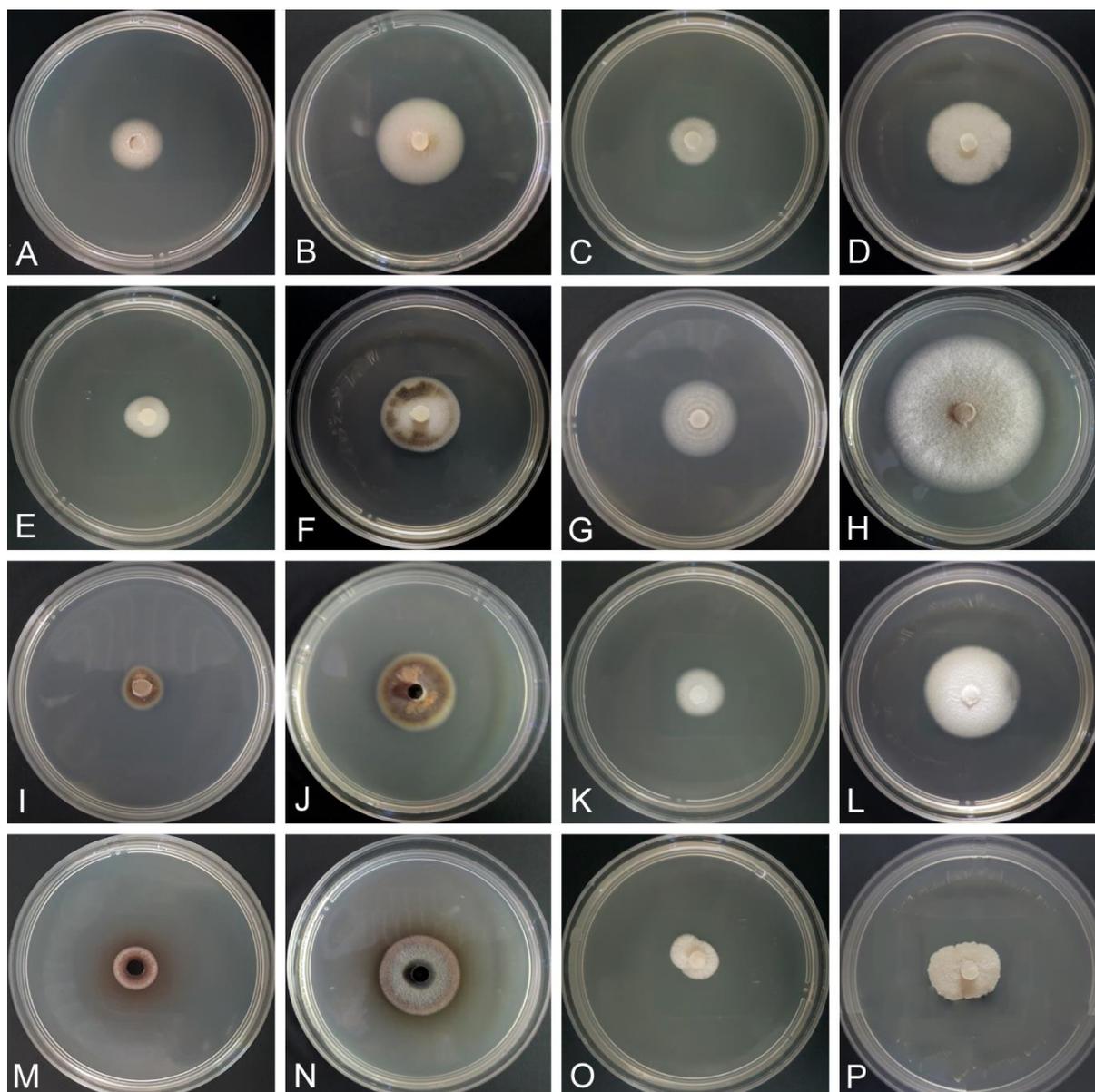
Figure 6. Cultural growth of *Phaeoacremonium* species on PDA incubated at 25°C in the dark. *Phaeoacremonium australiense* (STEU 8348) after 7 days (A) and 14 days (B); *Phaeoacremonium austroafricanum* (STEU 8349) after 7 days (C) and 14 days (D); *Phaeoacremonium fraxinopennsylvanicum* (STEU 8352) after 7 days (E) and 14 days (F); *Phaeoacremonium inflatipes* (STEU 8353) after 7 days (G) and 14 days (H); *Phaeoacremonium iranianum* (STEU 8355) after 7 days (I) and 14 days (J); *Phaeoacremonium minimum* (STEU 8356) after 7 days (K) and 14 days (L); *Phaeoacremonium prunicola* (STEU 8359) after 7 days (M) and 14 days (N); *Phaeoacremonium scolyti* (STEU 8360) after 7 days (O) and 14 days (P).
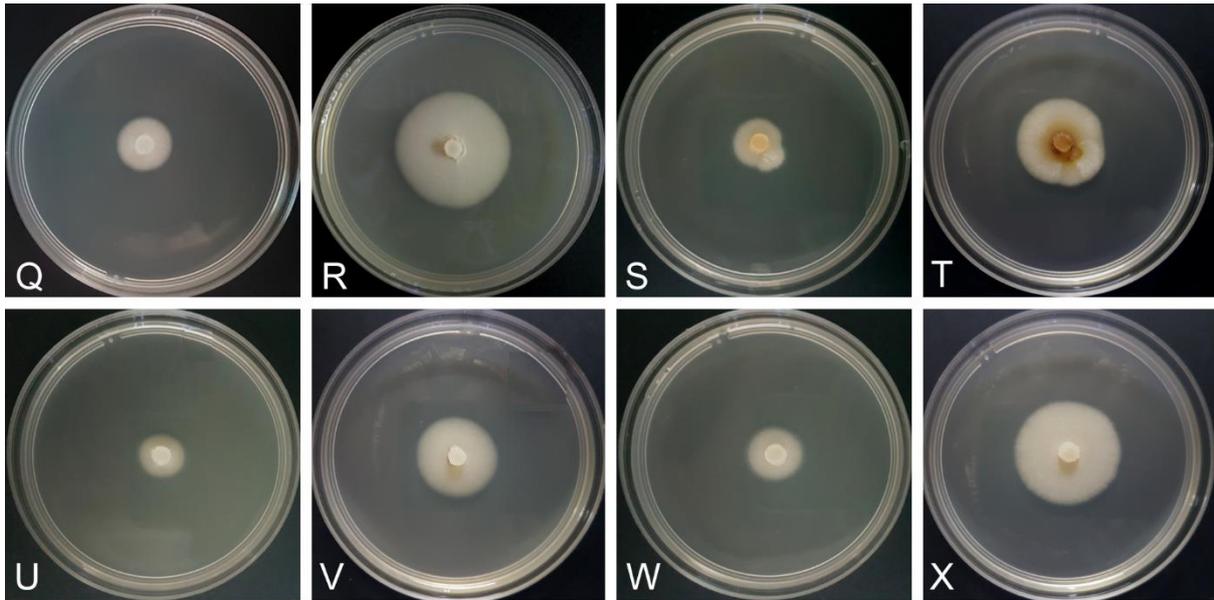
Figure 6. Continue. *Phaeoacremonium subulatum* (STEU 8362) after 7 days (Q) and 14 days (R); *Phaeoacremonium viticola* (STEU 8363) after 7 days (S) and 14 days (T); *Phaeoacremonium* sp. 1 (STEU 8402) after 7 days (U) and 14 days (V); *Phaeoacremonium* sp. 2 (STEU 8400) after 7 days (W) and 14 days (X).