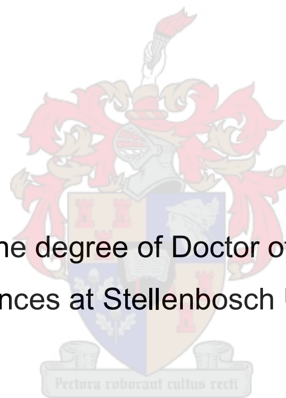
CHARACTERISATION, EPIDEMIOLOGY AND MANAGEMENT OF OLIVE TRUNK DISEASE PATHOGENS IN SOUTH AFRICA

by

MEAGAN VAN DYK



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Supervisor: Prof. F. Halleen
Co-supervisors: Prof. L. Mostert and Dr. C.F.J. Spies

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DECLARATION

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SUMMARY

The Olive Sector Development Plan of the Department of Trade and Industry identified low production and the lack of local research as weaknesses of the olive industry in South Africa. The management of trunk diseases forms an integral part of practices aimed at increasing olive production. A recent olive trunk disease survey performed in the Western Cape Province, South Africa, identified an undescribed *Pseudophaeomoniella* sp. as the most prevalent fungus associated with the trunk disease symptoms, with other fungal species occurring at much lower frequencies. In the current study, 40 of these isolates were selected for a pathogenicity study. The species forming lesions included several Botryosphaeriaceae, *Phaeoacremonium* and Phaeomoniellaceae species, as well as *Biscogniauxia mediterranea*, *Coniochaeta velutina, Diaporthe foeniculina*, *Didymocyrtis banksiae*, *Eutypa lata*, *Pleurostoma richardsiae*, *Symbiotaphrina buchneri*, isolates of the *Cytospora pruinosa* complex, and a *Cytospora* sp., *Fomitiporella* sp., *Geosmithia* sp. and *Punctularia* sp. The *Pseudophaeomoniella* sp. formed among the longest lesions, affirming its status as a potentially important trunk pathogen.

Long distance dispersal of olive trunk pathogens is expected to occur via infected nursery material, similar to that found in other systems such as in grape and fruit trees. Nurseries as an inoculum source was investigated by making isolations from asymptomatic cuttings from mother blocks (Stage 1), rooted cuttings (Stage 2) and 1–2-year-old trees (Stage 3) of eight cultivars in two nurseries. Known olive trunk pathogens of the Botryosphaeriaceae, Diaporthaceae, Nectriaceae, Phaeomoniellaceae, Pleurostomataceae and Togniniaceae were recovered. *Neofusicoccum australe* was detected in a single Stage 1 cutting. Stage 3 material showed the highest incidence of fungi from these families, with *P. richardsiae* having the highest incidence in both nurseries (82.2% and 36.7% of the 1–2-year-old trees). *Phaeoacremonium parasiticum* was present in 28.9% of the trees from one nursery (Stage 3). The remaining pathogens occurred in 13.3% or less of the material. *Pseudophaeomoniella* sp. was present in the nurseries but at low frequencies. This suggests that alternative inoculum sources of this pathogen exists.

A nested species-specific PCR was developed for the detection of *Pseudophaeomoniella* sp. from spore washes of pruning debris collected from established olive orchards. Pruning debris identified with a positive PCR was evaluated microscopically. Pycnidia of *Pseudophaeomoniella* sp. were observed on the pruning debris. Based on previous research, it is expected that the spore release coincides with rainfall and that the spores can be dispersed onto pruning wounds. The susceptibility of wounds from winter and spring pruning

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to *Pseudophaeomoniella* sp. was compared. Two-year-old olive branches of 16-year-old olive trees were pruned and inoculated with spore suspensions of *Pseudophaeomoniella* sp. at different time-points after pruning. The pruning wounds were susceptible for up to 42 days, with no difference between seasons (winter vs. spring). The wounds were the most susceptible within the first week after pruning. Eleven pruning wound protectants were evaluated and applied on pruning wounds made on 16–17-year-old trees directly after pruning. The treated wounds and positive (non-treated) controls were challenged with spore suspensions of *Pseudophaeomoniella* sp. at 1 or 7 days after pruning. Under low inoculum pressure (first season), Garrison, MT1, Neocil Plus and Tree Seal, reduced *Pseudophaeomoniella* sp. infections, while the *Trichoderma*-based protectant, MT1, was considered the most effective water-based protectant. Under higher inoculum pressure (during the second season), Tree Seal and Coprox Super/Bendazid consistently performed the best.

In conclusion, several fungal species were identified as olive trunk pathogens, with *Pseudophaeomoniella* sp. being identified as one of the most important olive trunk pathogens. The propagation process was identified as a source of inoculum for some pathogens, including *Pseudophaeomoniella* sp. Inoculum sources of *Pseudophaeomoniella* sp. were also identified in established orchards. Olive pruning wounds are susceptible to *Pseudophaeomoniella* sp. for prolonged periods. MT1 was highly effective under lower inoculum pressure, while Tree Seal and Coprox/Bendazid were highly effective under high inoculum pressure. This study led to new knowledge with regards to olive trunk diseases, their pathogenicity, detection, epidemiology and control which can be used for the development of improved management strategies of olive trunk diseases in South Africa.

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OPSOMMING

Die Olyf Sektor Ontwikkelingsplan van die Departement van Handel en Nywerheid het lae produksie en die tekort aan plaaslike navorsing as gebrekke in die Suid-Afrikaanse olyf bedryf geïdentifiseer. Die bestuur van stamsiektes vorm 'n integrale deel van praktyke gemik op verhoogde olyfproduksie. 'n Onlangse opname van olyfboom stamsiektes in die Weskaap, Suid-Afrika, het 'n onbekende *Pseudophaeomoniella* sp. geïdentifiseer as die mees algemene swam wat met stamsiekte simptome geassosieer word, terwyl ander swam spesies teen baie laer frekwensies voorkom. Tydens die huidige studie is 40 van hierdie isolate vir patogenisiteit studies gekies. Die isolate wat letsels gevorm het, sluit verskeie Botryosphaeriaceae, *Phaeoacremonium* en Phaeomoniellaceae spesies in, sowel as *Biscogniauxia mediterranea*, *Coniochaeta velutina*, *Didymocyrtis banksiae*, *Diaporthe foeniculina*, *Eutypa lata*, *Pleurostoma richardsiae*, *Symbiotaphrina buchneri*, die *Cytospora pruinosa* kompleks, *Cytospora* sp., *Fomitiporella* sp., *Geosmithia* sp. en *Punctularia* sp. Die *Pseudophaeomoniella* sp. het van die langste letsels gevorm. Dit beklemtoon die status van hierdie stamsiekte patogeen as potensieel belangrik.

Daar word verwag dat langafstandverspreiding van hierdie olyfboom stamsiekte patogene via besmette kwekery materiaal kan plaasvind. Hierdie moontlikheid is ondersoek deur isolasies uit asimptomatiese steggies uit moederblokke (Fase 1), gewortelde steggies (Fase 2) en 1-2-jaar oue bome (Fase 3) van agt kultivars in twee kwekerye. Bekende olyfboom stampatogene van die Nectriaceae, Diaporthaceae, Botryosphaeriaceae, Togniniaceae, Phaeomoniellaceae en Pleurostomataceae is uit kwekery materiaal geïsoleer. *Neofusicoccum australe* is in 'n enkele Fase 1 snit opgespoor. Fase 3 materiaal het die hoogste voorkoms van swamme van hierdie families getoon, met *P. richardsiae* as die mees algemene swam in beide kwekerye (82.2% en 36.7% van die 1-2-jaar-oue bome). *Phaeoacremonium parasiticum* was in 28.9% van die bome uit een kwekery (Fase 3) teenwoordig. Die oorblywende patogene het in minder as 13.3% van die material voorgekom. *Pseudophaeomoniella* sp. is uit kwekery materiaal verkry, maar teen lae frekwensies. Dit dui daarop dat alternatiewe inokulumbronne van hierdie patogeen waarskynlik belangriker is.

'n dubbel inleier spesie spesifieke polimerase kettingreaksie (PKR) is ontwikel vir die opsporing van *Pseudophaeomoniella* sp. in spoorwasse vanaf snoeirommel uit gevestigde olyfboorde. Snoeirommel wat d.m.v. 'n positiewe PKR geïdentifiseer is, is ook mikroskopies ondersoek. Vrugstrukture van *Pseudophaeomoniella* sp. is op die snoeirommel ontdek. Dit word verwag dat spoorvrystelling met reënval gepaard gaan en dat die spore na snoeiwonde versprei kan word. Die vatbaarheid van olyfboom wonde vir *Pseudophaeomoniella* sp. is

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vergelyk tussen winter en lente snoei. Twee-jaar oue olyftakke van 16-jaar oue olyfbome is gesnoei en met spoorsuspensies van *Pseudophaeomoniella* sp. op verskillende tyd-punte na snoei geïnokuleer. Die snoeiwonde was vatbaar vir tot en met 42 dae en daar was geen verskil tussen seisoene (winter vs. lente) nie. Die wonde was die mees vatbaar binne die eerste week. Elf snoeiwond beskermmiddels is geëvalueer vir toepassing direk na snoei. Die behandelde wonde en positiewe (onbehandelde) kontrole is met *Pseudophaeomoniella* sp. geïnokuleer 1 of 7 dae na snoei. Onder lae inokulumdruk het Garrison, MT1, Neocil Plus en Tree Seal die *Pseudophaeomoniella* sp. infeksies verminder, terwyl die *Trichoderma*-gebaseerde produk, MT1, die doeltreffendste water-gebaseerde produk was. Onder hoë inokulumdruk het Tree Seal en Coprox Super/Bendazid konstant die beste resultate getoon.

Ter afsuiting, verskeie swamspesies is as olyfboom stamsiekte patogene geidentifiseer. *Pseudophaeomoniella* sp. is as een van die mees belangrikste olyfstamsiekte patogene geidentifiseer. Die voortplantingsproses is as 'n bron van inokulum vir sommige patogene geidentifiseer, insluitend vir *Pseudophaeomoniella* sp. Inokulumbronne van *Pseudophaeomoniella* sp. is ook in gevestigde boorde geïdentifiseer. Snoeiwonde van olywe is vatbaar vir *Pseudophaeomoniella* sp. vir verlengde periodes. MT1 was hoogs effektief onder lae inokulumdruk, terwyl Tree Seal en Coprox/Bendazid hoogs effektief was onder hoë inokulumdruk. Hierdie studie het gelei tot nuwe kennis aangaande olyfstamsiektes, hul patogenisiteit, opsporing, epidemiologie en beheer wat gebruik kan word vir die ontwikkeling van verbeterde bestuurstrategieë van olyfstamsiektes in Suid-Afrika.

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Chapter 1

The management of fungal trunk diseases of olive trees (*Olea europaea* subsp. *europaea*) – literature review

INTRODUCTION

The South African olive industry is relatively new and small, but it has a high growth potential. It was only after 1935 when olive production in the Western Cape Province, South Africa started to expand. This was when Ferdinando Costa cultivated olives for the first time large scale and established the first olive oil mill in South Africa (Costa, 1998). Today, South Africa is cultivated with approximately 2 616 ha of olive trees with most of this area covered with 'Frantoio' and 'Mission' olive cultivars (Hortgro census data of 2016). 'Frantoio' is predominately cultivated for the production of olive oil or as cross pollinators, whilst Mission is cultivated for black table olive and oil production (Anonymous, 2016). In 2013, the total production of olives in South Africa was approximately 10 000 tons and approximately 60% of this was used for the production of olive oil (Costa, 2015). The demand for olive products in South Africa has been increasing since the appreciation of the Mediterranean diet and the health benefits of olive products (van Heerden, 2013; Anonymous, 2018). Over 95% of South African olive produce is marketed locally and approximately 60% of extra virgin olive oil is still imported to satisfy the local demand. South Africa produces an excellent quality olive oil and has an excellent reputation (Costa, 2015). Furthermore, it is a labour intensive industry. It is for these reasons that the National Development Plan (NDP) recognises the olive industry in South Africa as one with a high growth potential (Taylor and Atkinson, 2013).

The Olive Sector Development Plan of the Department of Trade and Industry identified low production and the lack of local research as weaknesses of the olive industry in South Africa. Olive production in South Africa is estimated at approximately 3–5 t/ha, which is less than half of the optimal olive production benchmark of 12–15 t/ha (Anonymous, 2013). The management of pest and diseases, including trunk diseases, forms an integral part of increasing olive production. In South Africa, peacock spot and anthracnose are the two major olive diseases threatening olive production (Costa, 1998). However, trunk diseases of olive trees are often overlooked due to its slow development and therefore only recently started receiving attention globally (Moral *et al*., 2010; Úrbez-Torres *et al*., 2013; Carlucci *et al*., 2015).

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OLIVE TRUNK DISEASES AND PATHOGENS

Olive trunk disease symptoms appear as a generalised decline of trees that may be comprised of cankers, foliar browning and leaf drop, wilting of apical shoots and dieback of twigs and branches. Internal symptoms include streaking under the bark, internal wood necrosis and black vascular streaking of the diseased wood (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012a; Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013; Triki *et al*., 2015). No studies have been published regarding the mechanisms of host-pathogen interactions of olive trunk diseases. In grapevine studies, infection with trunk pathogens interfere with the water and nutrient transport within the tree due to various factors, including the congestion of the vascular system by tyloses and chemical substances (such as phenols) as part of the plant's natural defence against further colonisation of the pathogen (Edwards *et al*., 2001; Lorena *et al*., 2001; Mutawila *et al*., 2011). This occlusion as well as the phytotoxins produced by the pathogens can lead to the external symptoms resembling water and nutrient deficiencies, such as leaf chlorosis, stunted growth, dieback, wilting and the reduction in the quantity and quality of the fruit (Deswarte *et al*., 1996; Sparapano *et al*., 2000; Edwards *et al*., 2001; Lorena *et al*., 2001; Mahoney *et al*., 2003; Mutawila *et al*., 2011). Furthermore, dead or dying branches in trees represent a reduced fruit-bearing capacity. Although these symptoms are known to develop slowly and do not necessarily seem as a threat initially, the financial loss can become significant as infections spread within the tree or across to neighbouring trees.

Approximately 36 olive trunk pathogens have been identified (Table 1). These are Asomycota, falling within the Botryosphaeriaceae, Calosphaeriaceae, Diaporthaceae, Diatrypaceae, Didymellaceae, Nectriaceae, Phaeomoniellaceae, Pleosporaceae, Togniniaceae and Valsaceae family groups as well as four Basidiomycota spp. (*Athelia rolfsii*, *Fomitiporia mediterranea*, *Trametes versicolor* and *Schizophyllum commune*) (Rumbos, 1988; Sánchez Hernández *et al*., 1998; Taylor *et al*., 2001; Romero *et al*., 2005; Ivic *et al*., 2010; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012a; Úrbez-Torres *et al*., 2012, 2013; Carlucci *et al*., 2013, 2015; Krid Hadj Taieb *et al*., 2014; Triki *et al*., 2014, 2015; Frisullo *et al*., 2015; Markakis *et al*., 2019; Nigro *et al*., 2019). The majority of the fungal species that have been identified as olive trunk pathogens are Ascomycota with most of these species falling within the *Neofusicoccum* and *Phaeoacremonium* genera. Recently, an olive trunk disease survey conducted in the Western Cape revealed a fungal complex dominated by an undescribed *Pseudophaeomoniella* species (Spies *et al*., unpublished). Many of the species recovered, including the *Pseudophaeomoniella* sp., have not been previously associated with olive trees and the pathogenicity of these fungi on olive trees are unknown.

Ascomycota

Botryosphaeriaceae

The Botryosphaeriaceae species that have been identified as olive trunk pathogens include *Botryosphaeria dothidea*, *Diplodia mutila*, *Diplodia seriata*, *Dothiorella iberica*, *Lasiodiplodia theobromae*, *Neofusicoccum australe*, *Neofusicoccum luteum*, *Neofusicoccum mediterraneum*, *Neofusicoccum parvum*, *Neofusicoccum ribis* and *Neofusicoccum vitifusiforme* (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012a; Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013; Triki *et al*., 2015). *Neofusicoccum mediterraneum* has been the most virulent olive trunk pathogen in several pathogenicity studies. It was the most virulent on 'Gordal Sevillana' in glass house trials (Moral *et al*., 2010, 2017) and it was the most virulent on 'Manzanillo' and 'Sevillano' together with *D. mutila* in a Californian field trial (Úrbez-Torres *et al*., 2013). The remaining Botryosphaeriaceae species tested in the study by Úrbez-Torres *et al*. (2013) (i.e. *B. dothidea*, *D. seriata*, *D. iberica*, *L. theobromae*, *N. luteum* and *N. vitifusiforme*) had an intermediate level of virulence on 'Manzanillo' and 'Sevillano'. *Diplodia seriata* was also reported as an olive trunk pathogen in Spain (Moral *et al*., 2010) and Croatia (Kaliterna *et al*., 2012a). In a diseased orchard in Croatia, *D. seriata* was detected in all surface-sterilised wood chips of symptomatic tissue of 165 trees (Kaliterna *et al*., 2012a). *Neofusicoccum luteum* (=*Fusicoccum luteum*) was also reported as an olive trunk pathogen in New Zealand on 'Barnea', 'Manzanillo' and 'J5' (Taylor *et al*., 2001). The virulence of the remaining Botryosphaeriaceae olive trunk pathogens [i.e. *N. parvum*, *N. australe* and *N. ribis* (=*Botryosphaeria ribis*)], were determined in Italy (Carlucci *et al*., 2013), Tunisia (Triki *et al*., 2015) and Spain (Romero *et al*., 2005), respectively.

Calosphaeriaceae

Pleurostoma richardsiae (=*Pleurostomophora richardsiae*) was the second most frequently isolated fungus from cankers and sub-cortical necrotic streaking of wilted olive trees during a survey to determine the cause of a serious decline of olive trees in Italy (Carlucci *et al*., 2013). During this study, *P. richardsiae* was suggested as the primary causal organism because of its high frequency in symptomatic wood and because of its highest virulence response when compared to *N. parvum* and *Phaeoacremonium minimum*, in pathogenicity field trials.

Diaporthaceae

Diaporthe ambigua, Diaporthe foeniculina (as well as other closely related *D. foeniculina* spp.) and *Diaporthe viticola* have been identified as olive trunk pathogens from pathogenicity field trials. *Diaporthe ambigua* and *D. foeniculina* were identified as pathogens of 'Corantina' olive trees in Italy (Frisullo *et al*., 2015). In this study, the *D. foeniculina* isolates were recorded as *Diaporthe neotheicola*. However, through further phylogenetic analyses, these *D. neotheicola*

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isolates were identified as *D. foeniculina.* Other *Diaporthe* spp., closely related to *D. foeniculina* (referred to as '*Phomopsis* group 1' and '*Phomopsis* group 2') and *D. viticola* were identified as trunk pathogens causing lesions on 'Manzanillo' and 'Sevillano' in California (Úrbez-Torres *et al*., 2013).

Diatrypaceae

The Diatrypaceae spp. tested for pathogenicity on olives had an intermediate to low virulence on olives. *Eutypa lata* had an intermediate level of virulence, while *Diatrype stigma* and *Diatrype oregonensis* had the lowest level of virulence in the study by Úrbez-Torres *et al*. (2013).

Didymellaceae

The Didymellaceae comprises a broad range of plant pathogenic, saprophytic and endophytic species occurring on a wide range of hosts (Chen *et al*., 2015). *Nothophoma quercina* (=*Phoma fungicola*) was associated with branch cankers in Tunisia and has been confirmed as a pathogen on young 'Chemlali' olive trees (Krid Hadj Taieb *et al*., 2014). Contradictory, studies showed that *N. quercina* had very low to no pathogenicity on olive trees (Moral *et al*., 2017).

Nectriaceae

Many species falling within the '*Cylindrocarpon'*-complex are known to cause root rot symptoms (Halleen *et al*., 2006; Cabral *et al*., 2012). *Dactylonectria macrodidyma* (=*Ilyonectria macrodidyma*) *and Dactylonectria torresensis* caused root rot symptoms on 'Koroneiki', 'Arbosana', and 'Arbequina' in California (Úrbez-Torres *et al*., 2012) and 'Leccino' and 'Picholine' in Southern Italy (Nigro *et al*., 2019), respectively. *Ilyonectria destructans* (=*Cylindrocarpon destructans*) was one of the organisms responsible for Dying Syndrome of 'Picual' olive trees in Spain (Sánchez Hernández *et al*., 1998) and *Ilyonectria radicicola* (=*Neonectria radicicola*) caused wilting and necrotic lesions in the roots of 'Chemlali' olive trees in Tunisia (Triki *et al*., 2014).

Phaeomoniellaceae

Many of the fungal species associated with olive trunk diseases in South Africa were Phaeomoniellaceae spp., indicating the potential importance of these species in causing trunk diseases in South Africa (Spies *et al*., unpublished). Only *Phaeomoniella chlamydospora* has been tested and confirmed as an olive trunk pathogen with an intermediate level of virulence in field trials in California (Úrbez-Torres *et al*., 2013), while *Pseudophaeomoniella oleae* and

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Pseudophaeomoniella oleicola were associated with extensive wood discolouration in olive trees (Crous *et al*., 2015).

Pleosporaceae

Comoclathris incompta (=*Phoma incompta*) has been reported as an olive trunk pathogen (Ivic *et al*., 2010; Moral *et al*., 2017). It was associated with severe trunk disease symptoms of olive trees in southern Croatia (Ivic *et al*., 2010). The symptoms were characterised by reddish-brown lesions on young shoots, withering of leaves, cankers and cracks on older shoots and shoot necrosis (Ivic *et al*., 2010). The pathogenicity of this fungus was confirmed on young 'Oblica' and 'Leccino' olive trees in greenhouse trials (Ivic *et al*., 2010).

Togniniaceae

Phaeoacremonium alvesii, *Phaeoacremonium italicum*, *P. minimum*, *Phaeoacremonium parasiticum*, *Phaeoacremonium scolyti* and *Phaeoacremonium sicilianum* have been confirmed as olive trunk pathogens on potted 'Coratina' olive trees, with *P. italicum*, *P. minimum* and *P. sicilianum* being the most virulent (Carlucci *et al*., 2015). *Phaeoacremonium minimum* was also identified as an olive trunk pathogen on 'Coratina' in glasshouse trials (Carlucci *et al*., 2013) and on 'Manzanillo' and 'Sevillano' in a Californian field trial (Úrbez-Torres *et al*., 2013).

Valsaceae

Cytospora oleina was isolated from dead twigs of olive trees with old cankers in the Mount Pelion region of central Greece (Rumbos, 1988). This fungus caused dieback of olive trees especially when inoculations were carried out in Autumn (Rumbos, 1988). This species was also isolated from olive trees in Italy (Carlucci *et al*., 2015).

Basidiomycota

Atheliaceae

Athelia rolfsii (=*Sclerotium rolfsii*) produce 'Drying Syndrome' in rooted cuttings of 'Picual' (Sánchez Hernández *et al*., 1998).

Hymenochaetaceae

Fomitiporia mediterranea caused trunk disease symptoms of olive trees with the 'Amfissis' cultivar being the most susceptible and 'Koroneiki' and 'Kalamon' being the most tolerate (Markakis *et al*., 2019).

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Polyporaceae

Trametes versicolor has been reported on olives in California and was determined to have an intermediate level of virulence on olives (Úrbez-Torres *et al*., 2013).

Schizophyllaceae

Schizophyllum commune was reported to occur on olives for the first time in California and through pathogenicity studies found to have an intermediate level of virulence (Úrbez-Torres *et al*., 2013).

DETECTION AND IDENTIFICATION OF OLIVE TRUNK PATHOGENS

The traditional plating method on a general growth medium is an invaluable approach for the detection and identification of fungal species. However, the efficacy of this method is at times compromised, due to over-growth by fungal species that are of little interest (Retief *et al*., 2005). Selective media can be used to improve the recovery of specific pathogens from environmental samples (Swart *et al*., 1987; Cilliers *et al*., 1994; Blodgett *et al*., 2003) but often, the problem of difficult morphological identification often remains.

PCR is the basis of many molecular techniques that have been adopted for the detection and identification of species from environmental samples. These include species-specific PCRs and qPCRs, DNA fingerprinting, microarrays, *in situ* hybridisation and DNA sequencing (Pereira *et al*., 2008).

Species-specific PCR and qPCR

Species-specific PCRs and qPCRs provide a simple, rapid and cost effective approach for accurate identification of fungi to species level. Species-specific primers have been developed for a wide range of fungal species and can be used to detect specific fungal species from various environmental samples. Of the known olive trunk pathogens, species-specific PCRs have been developed for *B. dothidea* (Ma *et al*., 2003), *D. macrodidyma* (AgustÍ-Brisach *et al*., 2014), *D. seriata* (Martín *et al*., 2014), *D. foeniculina* (Lesuthu *et al*., 2019), *E. lata* (Lecomte *et al*., 2000; Catal *et al*., 2007; Rouzoulet *et al*., 2017), *I. destructans* (=*C. destructans*) (Hamelin *et al*., 1996; Nascimento *et al*., 2001), *L. theobromae* (Ni *et al*., 2012), *N. parvum* (Ni *et al*., 2012; Xu *et al*., 2016), *P. alvesii* (Mostert *et al*., 2006), *P. chlamydospora* (Tegli *et al*., 2000), *P. minimum* (Tegli *et al*., 2000; Mostert *et al*., 2006), *P. parasiticum* (Mostert *et al*., 2006), *P. scolyti* (Mostert *et al*., 2006), *S. commune* (Buzina *et al*., 2001) and *T. versicolor* (García-Mena *et al*., 2005). These species-specific PCR primers have been developed for specific applications, but can be adapted where necessary. A potential disadvantage of species-specific PCRs, especially when considering qPCR, is the inability to distinguish dead

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microbial cells from viable cells, subsequently affecting the ability to accurately quantify infection from environmental samples. However, the detection of species-specific PCRs designed to amplify RNA, which degrades soon after cell death, can be used to improve the detection of live material in infected wood samples (Retief *et al*., 2005).

Fingerprinting

Fingerprinting is particularly useful for the rapid and cost-effective identification of fungi to species level. Fingerprinting is usually used for species identification rather than detection. Yet, a nested multi-species primer pair (BOT100F/BOT472R) was used to detect *N. australe*, *N. luteum*, *D. mutila* and *D. seriata* in environmental water samples to study the dispersal patterns of conidia of these species in rainwater across seasons (Ridgway *et al*., 2011). Furthermore, olive trunk pathogens can be identified by Inter Simple Short Sequence Repeat (ISSR) analysis (Zhou et al. 2001), PCR-Restriction Fragment Length Polymorphism (RFLP) (Dupont *et al*., 2002; Rolshausen *et al*., 2004; Mohali *et al*., 2007), Random Amplified Polymorphic DNA (RAPD) (Alves *et al*., 2007), Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Alves *et al*., 2005) and Matrix-assisted Laser Desorption Ionization-time of Flight Mass Spectrometry (MALDI-TOF MS) (Alshawa *et al*., 2012).

Hybridization

Fluorescence *in situ* Hybridization (FISH), Peptide Nucleic Acid (PNA)-FISH, DNA microarrays and DNA macroarrays can be used for species identification. A DNA macroarray was designed for the rapid identification and detection of young vine decline pathogens, which included several olive trunk pathogens, namely *D. macrodidyma*, *I. radicicola*, *P. alvesii*, *P. scolyti* and *P. sicilianum* (Úrbez-Torres *et al*., 2015). This method can potentially be designed exclusively for the detection of olive trunk pathogens for use as a diagnostic tool in olive nurseries. The disadvantage of using hybridization for detection and identification is that it is expensive.

Sequencing

Extensive phylogenetic studies, based on sequence data involving multiple loci in combined cluster analyses, can be used as a backbone for species identification and for new species descriptions. Some of the extensive phylogenetic studies of significance to the identification of olive trunk pathogens include those developed for the Botryosphaeriaceae (Crous *et al*., 2006), Calosphaeriaceae (Réblová et al. 2004), Diaporthales (including the Diaporthaceae and Valsaceae) (Senanayake *et al*., 2017), Diatrypaceae (Trouillas *et al*., 2011; de Almeida *et al*., 2016), Nectriaceae (Lombard *et al*., 2014, 2015) and Togniniaceae (Mostert *et al*., 2006), as well as for *Phoma* and related pleosporalean genera (Aveskamp *et al*., 2010; Chen *et al*., 2015). Sequence data used for species identification and new species descriptions specifically

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involving trunk pathogens, include studies such as those identifying Botryosphaeriaceae and Diaporthaceae spp. associated with trunk diseases of walnut trees (Chen *et al*., 2014) and identifying Diaporthaceae spp. associated with dieback of various hosts including grapevine trunk diseases (Kaliterna *et al*., 2012b; Yang *et al*., 2018).

In South Africa, sequencing and phylogeny were used to identify several olive trunk pathogens (*D. ambigua*, *D. foeniculina*, *D. seriata*, *E. lata*, *N. australe*, *N. vitifusiforme*, *P. minimum*, *P. parasiticum*, *P. richardsiae*, *P. scolyti* and *S. commune*) associated with olive trunk disease symptoms of commercial olive trees (Spies *et al*., unpublished). Several species (*Cytospora pruinosa*, an undescribed *Cytospora* sp., *Neofusicoccum capensis*, *Neofusicoccum stellenboschiana*, *Neofusicoccum* sp. 4, *Neofusicoccum* sp. 8, *Neophaeomoniella niveniae*, *Neophaeomoniella zymoides*, *Phaeoacremonium africanum* and undescribed *Phaeomoniella* and *Xenocylindrosporium* spp. and an undescribed *Pseudophaeomoniella* sp.) were reported first on commercial olive trees worldwide, but could be considered as potential olive trunk pathogens due to their association with trunk disease symptoms of other woody hosts or due to their closely related species' association with trunk disease symptoms of olive trees or other woody hosts (Spies *et al*., unpublished).

EPIDEMIOLOGY AND MANAGEMENT OF OLIVE TRUNK DISEASES

No studies have been published regarding the epidemiology and management of olive trunk diseases. Nurseries and established vineyards have, however, shown to be important inoculum sources of grapevine trunk pathogens (Fourie and Halleen, 2002, 2004a; Whiteman *et al*., 2007; van Niekerk *et al*., 2010; Gramaje and Armengol, 2011). This section covers the epidemiology and management strategies of olive trunk pathogens with respect to nurseries and established orchards since nurseries and orchards are expected to carry major inoculum sources of these pathogens.

Nurseries

The olive propagation process in South Africa is initiated by selecting semi-hard olive wood cuttings (4–6 mm in diameter) from well-maintained mother blocks. The majority of olive trees produced in South Africa are not grafted, except for cultivars with lower rooting ability such as 'Kalamata'. The non-grafted cuttings are made by first stripping the lower leaves of the cuttings. These are surface sterilised using a fungicide dip such as captab-benomyl (Costa, 1998; Fabbri *et al*., 2004). The basal end of the cuttings are dipped in a rooting hormone, such as auxin, before placing these in a green house in heated troughs containing a growing medium (Costa, 1998; Fabbri *et al*., 2004). In the glasshouse, fine mist sprayers deliver filtered water at a rate determined by the environmental conditions, while the growing medium is kept

at 19–23°C (Costa, 1998; Fabbri *et al*., 2004). Infections of olive cuttings in the glasshouse can be managed by regular fungicide spays, while a foliar nutrient spray is recommended after 2 months in the glasshouse (Costa, 1998). After 3 months, the rooted cuttings can be hardened before transplanting into plastic bags containing soil mixtures tested and treated for harmful salts and pathogens (Costa, 1998). Beneficial microbes can be added to the soil to further improve the soil health (Costa, 1998). After planting into soil, these trees are hardened off such that the trees are ready for planting in the orchard after 12–18 months (Costa, 1998).

The introduction of trunk pathogens from grapevine nurseries into established vineyards appears to be one of the major means of long distance dispersal. Trunk pathogens have been detected from asymptomatic grapevine rootstock mother block material (Fourie and Halleen, 2002, 2004a; Whiteman *et al*., 2007), and at lower incidences in scion mother block material (Whiteman *et al*., 2007). The infected asymptomatic mother block material can be used unintentionally to produce asymptomatic plants. In grapevine nurseries, it is possible to partially eliminate internal fungal infections of cuttings or grafted plants by performing a hot water treatment of the plant material (Fourie and Halleen, 2004b; Halleen *et al*., 2007; Bleach *et al*., 2013). Hot water treatment and hot air treatments of olive propagation material has been used to eradicate internal infestations of *Verticillium dahlia*, sometimes with adverse effects of the success of tree survival (Morello *et al*., 2016). These heat treatments have not yet been studied to eliminate trunk pathogens from the olive propagation material.

Propagation material, such as grapevine material, free of trunk pathogens and other pathogens can still become infected later during the propagation process (Halleen *et al*., 2003). *Phaeomoniella chlamydospora* and *P. minimum* are able to infect healthy grapevine cuttings during the hydration stages (Gramaje *et al*., 2009). Wounds created during propagation can potentially expose the susceptible plant tissues to trunk pathogen propagules. These propagules may occur on nursery propagation tools (e.g. hands, pruning shears and grafting tools), in the propagation material (e.g. callusing medium, soil, water and pots) and in the air (Halleen *et al*., 2003; Retief *et al*., 2006; Aroca *et al*., 2010; Agustí-Brisach *et al*., 2015). The repeated soaking of propagation material in fungicide chemicals, such as benomyl, captan and didecyldimetylammonium chlorine, during the hydration stages has shown to reduce pathogen infection in grapevine plantlets with no serious effects on the plant growth (Fourie and Halleen, 2006; Fourie and Halleen, 2004b; Halleen and Fourie 2016).

Established orchards

Trunk pathogens can occur as endophytes or latent pathogens in healthy plant material (Úrbez-Torres, 2011). These pathogens can continue to grow in the wood with symptoms

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often only developing after the plant has been exposed to harsh conditions (Schoeneweiss, 1981). The pathogens can multiply within orchards/vineyards by proliferating from pruning debris and other dead wood (van Niekerk *et al*., 2010; Gramaje and Armengol, 2011). The spore release and the quantity of conidia released from pycnidia of trunk pathogens are influenced by weather conditions prior to and during the spore events (Eskalen and Gubler, 2001; van Niekerk *et al*., 2010). Spores of *P. chlamydospora* and *Phaeoacremonium inflatipes* were released during and after rainfall events during late winter and early spring, while the release of *P. minimum* spores were not always associated with rainfall (Eskalen and Gubler, 2001). Similarly, spores of *E. lata*, *Phomopsis* spp. and Botryosphaeriaceae spp. were also release during or after rainfall and/or high relative humidity (van Niekerk *et al*., 2010). Spores of trunk pathogens can be dispersed via various means such as through the movements of water (e.g. rain) and wind (Ahimera *et al*., 2004; van Niekerk *et al*., 2010) and insects (Moyo *et al*., 2014). These spores can infect wounds, including pruning wounds, via short distance dispersal mechanisms (Munkvold and Marois, 1995; Eskalen *et al*., 2007; van Niekerk *et al*., 2011; Úrbez-Torres and Gubler, 2011; Elena and Luque, 2016). The inoculum levels in established orchards can be managed by maintaining good plant health and sanitation within and around orchards. Olive trunk diseases can potentially be managed further by timely pruning and by applying effective pruning wound protectants.

Plant material

The development of trunk disease symptoms on woody crops have been associated with plant stress as a result of drought (water stress) and freezing conditions (Schoeneweiss, 1981; Ferreira *et al*., 1999). Under prolonged drought or freezing conditions, mortality can occur as a result of expanding trunk disease symptoms, but the thresholds of specific host species and cultivars to environmental stresses differ (Schoeneweiss, 1981). It is for these reasons that crops or cultivars could potentially be selected based on production area to improve the general health of the plants, which should in turn improve the plants defence mechanisms against pathogens including trunk pathogens.

Disease resistance is generally governed by several plant defence traits that can be harnessed through plant breeding strategies. For example, increased resistance of apple tree pruning wounds to trunk pathogens have been associated with certain cultivars and can be measured by the incidence of infection, length of cankers and the rate at which the pruning wounds heal or become resistant (Xu *et al*., 1998). Olive trees with resistance to trunk diseases has been observed in potted 'Aloreña de Atarfe', 'Hojiblanca', 'Manzanilla de Sevilla', 'Morona', and 'Verdial de Huévar' olive cultivars inoculated with *N. mediterraneum.* These cultivars formed significantly shorter cankers compared to those formed in the 'Gordal

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Sevillana' and 'Manzanilla Cacereña' olive cultivars (Moral *et al*., 2017). Recently, 'Koroneiki' and 'Kalamon' olive cultivars were shown to be the most tolerant against *F. mediterranea*, compared to the 'Amfissis', 'Chalkidikis' and 'Mastoidis' olive cultivars (Markakis *et al*., 2019). It was further shown that the most tolerant olive cultivars also had the highest lignin content in the trunk wood (Markakis *et al*., 2019).

The genes conferring resistance to olive trunk diseases can potentially be identified by quantitative trait loci mapping such that olive tree germplasm can be selected based on marker-assisted selection during plant breeding programs. Thus far, the susceptibility of cultivars used in South Africa against trunk pathogens are unknown.

Orchard sanitation

In South Africa, branches with dieback symptoms are pruned off during routine pruning (Costa, 2019). In some production systems, the large branches removed during pruning are burned, while the rest are shredded. Trunk disease symptoms on other hosts in close proximity to olive orchards should also be managed, since several olive trunk pathogens have been associated with trunk disease symptoms of other hosts, including the native wild olive (Spies *et al*., 2018) and Proteaceae species (Marincowitz *et al*., 2008). Furthermore, trunk pathogens can be eradicated from infected pruning debris by composting. *Diplodia seriata* (=*Botryosphaeria obtusa*), *P. chlamydospora*, *P. minimum* and *E. lata* were eradicated from infected grapevine wood by a composting technique executed by Lecomte *et al*. (2006).

Cultural practises

The pruning of olive trees is an essential part of maintaining olive production. It improves the light distribution within the orchard, which allows the trees to harness sunlight optimally for photosynthesis (Costa, 2019). It improves the aeration through the orchard, which lowers the risk of disease (Costa, 2019). Skirting of the trees (i.e. pruning of the lower branches of the tree) can be done to avoid branches being exposed to weeds, shade and dew, which further reduce the risk of disease (Costa, 2019). Furthermore, pruning can be performed to create a tree and orchard structure that facilitates general farming practises, such as harvesting, mulching and spraying and can improve spray distribution, including the spray distribution of fungicides (Costa, 2019). In South Africa, it is recommended that large branches are pruned during mid-winter to remove large diseased branches and branches or parts of branches obstructing sunlight, as well as branches that have grown too tall (Costa, 2019). Skirting can also be performed during this time (Costa, 2019). During spring after the buds have differentiated into flower buds, regrowth from the winter pruning can be managed by removing the most upright suckers, while some suckers are cut back and headed, and the canopy

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thinned by secateurs cuts (Costa, 2019). No olive pruning wound susceptibility studies have been performed before, but it is expected that olive pruning wounds remain susceptible to infection by trunk pathogens for prolonged periods based on pruning wound susceptibility studies performed on other hosts including apricot (Ramos *et al*., 1975), apple (Chen *et al*., 2016) and grapevine (Chapuis *et al*., 1998; Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011). In these studies, pruning wound susceptibility tended to decrease as the pruning wounds aged, but often remained susceptible for prolonged periods. The oldest pruning wounds that were evaluated for susceptibility were 4 months old grapevine pruning wounds and these wounds were still susceptible at that time-point (Eskalen *et al*., 2007). Other studies showed significant differences of pruning wound susceptibility between seasons.

Apricot pruning wounds made during spring were susceptible to *Eutypa armeniacae* for a shorter duration compared to those made in autumn in California (Ramos *et al*., 1975). These authors suggested that the metabolic activity of the woody tissues may be similar to that of adjacent developing buds and that during spring, flowering and resumption growth deplete the nutrient reserves (starch and sugars), thereby also reducing nutrients available for infection and fungal growth (Ramos *et al*., 1975). In California, the majority of grapevine pruning wound studies showed that pruning wounds were less susceptible when made during late-winter, when the temperature starts to rise and the buds begin to break, compared to those made during early winter (Munkvold and Marois, 1995; Chapuis *et al*., 1998; Úrbez-Torres and Gubler, 2011). Munkvold and Marois (1995) and Úrbez-Torres and Gubler (2011) suggested that their results were due to an accelerated production of resistance structures, such as suberin and lignin, in the pruning wounds during late winter, since the development of these structures has been associated with increased degree-days (Munkvold and Marois, 1995). In contrast, grapevine pruning wounds were less susceptible during early winter against *E. lata*, *P. clamydospora*, *N. australe* and *D. neoviticola* (=*Phomopsis viticola*) in South Africa (van Niekerk *et al*., 2011) and against *D. seriata* in Spain (Elena and Luque, 2016). No significant difference in susceptibility towards *P. chlamydospora* infection between seasons was found in Spain (Elena and Luque, 2016).

Pruning wound protection

There has been no evidence of complete resistance of pruning wounds of woody crops against trunk pathogens regardless of the pruning season and host evaluated. Furthermore, due to the extended susceptibility of pruning wounds and the presence of airborne trunk pathogen inoculum, pruning wound protectants with prolonged protection against trunk disease pathogens appears to be necessary to manage trunk diseases. There are currently no

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chemical or biological pruning wound protectants registered for olive pruning wounds in South Africa. A similar situation was prevalent in the grapevine industry in several countries for the protection of wounds against *E. lata.* Benomyl was one of the first fungicides to be used as a pruning wound protectant in the U.S.A., and was considered an industry standard, but it was banned from the market in most countries due to its potential risk to human health. This lead to several studies to evaluate the efficacy of alternative pruning wound protectants that could potentially be used to replace benomyl. Following these studies, carbendazim was identified as the most effective fungicide (Sosnowski *et al*., 2008).

Due to the large range of fungal pathogens associated with trunk diseases of olives, an effective fungicide should protect against a broad host range. Water-based fungicides as well as sealants and fungicides-amended sealants have been identified as effective against various trunk pathogens on various woody crops to protect pruning wounds. Water-based chemicals providing protection comparable to carbendazim against *N. luteum* on 'Chardonnay' grapevine pruning wounds in field trials include thiophanate-methyl, flusilazole, tebuconazole and mancozeb (Amponsah *et al*., 2012). Thiophanate-methyl has the same mode of action as benomyl and carbendazim, but falls within the thiophanate chemical group. This chemical provided the highest protection against a range of trunk pathogens of almonds under field conditions in Spain (Olmo *et al*., 2017) and of 'Chardonnay' grapevines in California (Rolshausen *et al*., 2010). Chemicals affecting the sterol biosynthesis in fungal cell membranes include the triazoles, flusilazole and tebuconazole. Flusilazole was comparable to benomyl for the protection of 'Cabernet Sauvignon' vineyards against *E. lata* in South Africa (Halleen *et al*., 2010). However, flusilazole was not as effective as carbendazim on grapevine pruning wounds of 'Shiraz' against *D. mutila* and *D. seriata* under field conditions in Australia (Pitt *et al*., 2012). This compound among other demethylation inhibitor fungicide compounds was shown to not provide the same duration of protection as benomyl and carbendazim (Sosnowski *et al*., 2008). Tebuconazole and mancozeb provided some protection of almond pruning wounds against various *Botryosphaeria* trunk pathogens, but these were not always as effective as the thiophanate-methyl (Olmo *et al*., 2017). Fluazinam also showed potential as it was one of the most effective products used in the study conducted by Pitt *et al*. (2012), but this chemical, as well as pyraclostrobin and pyrimethanil, was not as effective as carbendazim on the 'Cabernet Sauvignon' pruning wounds (Sosnowski *et al*., 2008).

Sealants provide a physical barrier to protect fresh pruning wounds against trunk pathogens by preventing the entry of spores. An impermeable barrier must adhere to the wound surface but this mechanism may be compromised due to the presence of a high moisture content in xylem tissues (Spiers and Brewster, 1997). For these reasons it was

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suggested that a barrier ought to be porous to enable gas exchange but still be impermeable to spores (Spiers and Brewster, 1997). Cracking of the sealants after drying reduced the efficacy of the pruning wound protectants (Spiers and Brewster, 1997). Sealants amended with fungicides reduce the chance of infection though cracks of the sealant or paint (Sosnowski *et al*., 2008).

The efficacy of benomyl, thiophanate-methyl, pyraclostrobin and tebuconazole were tested in liquid (water-based) and paste form (vinyl acrylic paint), with increased protection achieved when chemicals were in paste form (Díaz and Latorre, 2013). In this study, benomyl and thiophanate-methyl provided the highest protection against *D. seriata*, *Inocutis* sp. and *P. chlamydospora* on 'Cabernet Sauvignon' grapevines in field trials. In paste form, iprodione, tebuconazole and boric acid were comparable to a benomyl-amended paste against *N. parvum* on the blue berry 'Duke' cultivar in Chile (Latorre *et al*., 2013). However boric acid in paste had phytotoxic effects towards the plant. Garrison is a commercial product that has often shown excellent efficacy of pruning wound protection. It is a paste with cyproconazole and iodocarb as active ingredients and has been comparable to a carbendazim spray application for protection of 'Shiraz' against *D. mutila* and *D. seriata* (Pitt *et al*., 2012) and 'Cabernet Sauvignon' against *E. lata* in Australia (Sosnowski *et al*., 2008). Garrison was also comparable to thiophanate-methyl for protection of 'Chardonnay' pruning wounds against various trunk pathogens in California, except for *P. parasiticum* (Rolshausen *et al*., 2010).

Due to a heightened public concern for safety and the environment, biological control agents are being developed to minimize the use of harmful chemicals. Biological control is often regarded as being less effective and thought of as a long slow progress towards protection of pruning wounds. However biological control has shown potential towards long-term protection of pruning wounds regardless of rainfall events, unlike many chemical control agents (Munkvold and Marois, 1993). *Fusarium lateritium* and *Cladosporium herbarum* has provided consistent control of *E. lata* infections of 'Thompson Seedless' grapevine pruning wounds, in some cases with no significant difference when compared to benomyl (Munkvold and Marois, 1993). Fresh pruning wounds treated with spores of *F. lateritium*, *Trichoderma harzianum* and Vinevax significantly reduced the recovery of *E. lata* from inoculated wounds (John *et al*., 2005). The commercial *Trichoderma* biological control products, Vinevax and Eco77, did not provide as much control as the benzimidazole products in field trials where treated pruning wounds were challenged with *E. lata* 24 hours after application (Halleen *et al*., 2010). However, these products did significantly reduce *D. mutila*, *D. seriata* and *E. lata* infections and can potentially provide adequate protection under natural infection levels (Halleen *et al*., 2010). Eco77, Vinevax as well as other *Trichoderma* products (Biotricho, USPP

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T1 and USPP T2) and *Bacillus subtilis* were in some cases comparable to benomyl on 'Merlot' and 'Chenin Blanc' grapevine cultivars. This was after spray inoculating with a spore suspension containing either *D. neoviticola* (=*P. viticola*), *E. lata*, *P. chlamydospora*, *N. australe*, *N. parvum*, *D. seriata* or *L. theobromae*, 7 days after applying the pruning wound protectants (Kotze *et al*., 2011). On the contrary, some of the commercial biological control agents such as Serenade Max (*B. subtilis*) and Trichonativa (*T. harzianum*, *Trichoderma parceramosum and Trichoderma virens*,) were not effective in reducing *N. parvum* infection of Blueberry plants in Chile (Latorre *et al*., 2013).

CONCLUSIONS

The olive industry in South Africa produces high quality olive oil and has the capacity to grow due to its high demand, yet olive production per hectare is considered low. The effective management of olive trunk diseases can form part of an integrated strategy to improve the lifespan and yield of olive trees. Globally, little is known about the management of olive trunk diseases. Recently an olive trunk disease survey in South Africa revealed a diverse fungal population associated with olive trunk disease symptoms (Spies *et al*., unpublished). Some of these species are known olive trunk pathogens, while others are first reports on olive trees worldwide and have no known interactions with this host. Pathogenicity tests are therefore necessary to establish whether these species contribute to the olive trunk diseases found in South Africa. Furthermore, no studies have been published regarding the epidemiology of olive trunk pathogens. In the grapevine production system, infected nursery material has shown to be inoculum sources for long distance dispersal, while dead wood infected with trunk pathogens in vineyards can serve as inoculum sources for short distance dispersal of trunk pathogens (Fourie and Halleen 2002; Fourie and Halleen 2004a; Retief *et al*., 2006; Whiteman *et al*., 2007; Aroca *et al*., 2010; van Niekerk *et al*., 2010; Gramaje and Armengol 2011; Agustí-Brisach *et al*., 2015). It is through studies such as these that specific sanitation practices in grapevine nurseries and vineyards have been developed for the management of trunk diseases. Similar studies of olive nurseries and orchards could direct new trunk disease management strategies to improve the trunk disease status of established olive orchards in South Africa. Trunk diseases of woody crops could potentially be managed further by means of timely pruning and by applying effective pruning wound protectants (Munkvold and Marois, 1993; Munkvold and Marois, 1995; Spiers and Brewster, 1997; Chapuis *et al*., 1998; John *et al*., 2005; Sosnowski *et al*., 2008; Rolshausen *et al*., 2010; Halleen *et al*., 2010; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011; Amponsah *et al*., 2012; Pitt *et al*., 2012; Díaz and Latorre, 2013; Latorre *et al*., 2013; Olmo *et al*., 2017). No olive pruning wound susceptibility studies have been published previously and no pruning wound protectants are registered for

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olive trees in South Africa. These studies could potentially direct the management of olive trunk diseases further, to improve the yield and longevity of olive trees grown in South Africa.

AIMS AND OBJECTIVE

The aim of the project is to improve the olive industry's competitive edge by providing clarity on trunk diseases affecting olive production, to accurately detect the main pathogens and to provide the industry with clear guidelines to effectively control these diseases, thereby increasing plant yield, fruit quality and extending the productive lifespan of South African olive trees. The specific objectives of this study were carried out in the Western Cape to:

1. Determine the pathogenicity of the fungi frequently associated with trunk disease symptoms of olive trees;
2. Determine the incidence and distribution of olive trunk pathogens in olive nurseries and propagation material;
3. Investigate the epidemiology of an important olive trunk pathogen in established orchards by determine its potential on pruning debris;
4. Investigate the susceptibility of pruning wounds for infection by important olive trunk pathogens and evaluate potential pruning wound protectants.

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TABLES AND FIGURES

Table 1. Olive trunk pathogens based on pathogenicity studies.

Family	Species	Region	Reference
Atheliaceae	*Athelia rolfsii*	Spain	Sánchez Hernández *et al*., 1998
Botryosphaeriaceae	*Botryosphaeria dothidea*	Spain; California	Moral *et al*., 2010; Urbez-Torres *et al*., 2013
	Diplodia mutila	California	Urbez-Torres *et al*., 2013
	Diplodia seriata	California; Croatia	Urbez-Torres *et al*., 2013; Kaliterna *et al*., 2012a
	Dothiorella iberica	California	Urbez-Torres *et al*., 2013
	Lasiodiplodia theobromae	California	Urbez-Torres *et al*., 2013
	Neofusicoccum australe	Tunisia	Triki *et al*., 2015
	Neofusicoccum luteum (=*Fusicoccum luteum*)	New Zealand, California	Taylor *et al*., 2001, Urbez-Torres *et al*., 2013
	Neofusicoccum mediterraneum	California; Spain	Urbez-Torres *et al*., 2013; Moral *et al*., 2010, 2017
	Neofusicoccum parvum	Southern Italy	Carlucci *et al*., 2013
	Neofusicoccum vitifusiforme	California	Urbez-Torres *et al*., 2013
	Neofusicoccum ribis (=*Botryosphaeria ribis*)	Spain	Romero *et al*., 2005
Calosphaeriaceae	*Pleurostoma richardsiae* (=*Pleurostomophora richardsiae*)	Southern Italy	Carlucci *et al*., 2013
Diaporthaceae	*Diaporthe foeniculina*	California	Urbez-Torres *et al*., 2013

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Family	Species	Region	Reference
Diaporthaceae (cont.)	*Diaporthe viticola*	California	Urbez-Torres *et al.*, 2013
Diatrypaceae	*Diatrype oregonensis*	California	Urbez-Torres *et al.*, 2013
	Diatrype stigma	California	Urbez-Torres *et al.*, 2013
	Eutypa lata	California	Urbez-Torres *et al.*, 2013
Didymellaceae	*Nothophoma quercina* (=*Phoma fungicola*)	Tunisia	Krid Hadj Taieb *et al.*, 2014
Hymenochaetaceae	*Fomitiporia mediterranea*	Greece	Markakis *et al.*, 2019
Nectriaceae	*Dactylonectria macrodidyma* (=*Ilyonectria macrodidyma*)	California	Úrbez-Torres *et al.*, 2012
	Dactylonectria torresensis	Southern Italy	Nigro *et al.*, 2019
	Ilyonectria radicicola (=*Neonectria radicicola*)	Tunisia	Triki *et al.*, 2014
	Ilyonectria destructans (=*Cylindrocarpon destructans*)	Spain	Sánchez Hernández *et al.*, 1998
Phaeomoniellaceae	*Phaeomoniella chlamydospora*	California	Urbez-Torres *et al.*, 2013
Pleosporaceae	*Comoclathris incompta* (=*Phoma incompta*)	South Croatia	Ivic *et al.*, 2010; Moral *et al.*, 2017
Polyporaceae	*Trametes versicolor*	California	Urbez-Torres *et al.*, 2013
Schizophyllaceae	*Schizophyllum commune*	California	Urbez-Torres *et al.*, 2013
Togniniaceae	*Phaeoacremonium alvesii*	Southern Italy	Carlucci *et al.*, 2015
	Phaeoacremonium italicum	Southern Italy	Carlucci *et al.*, 2015

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Family	Species	Region	Reference
Togniniaceae (cont.)	*Phaeoacremonium parasiticum*	Southern Italy	Carlucci *et al*., 2015
	Phaeoacremonium scolyti	Southern Italy	Carlucci *et al*., 2015
	Phaeoacremonium sicilianum	Southern Italy	Carlucci *et al*., 2015
	Phaeoacremonium minimum (=*Phaeoacremonium aleophilum*)	Southern Italy	Carlucci *et al*., 2013; Urbez-Torres *et al*., 2013; Carlucci *et al*., 2015
Valsaceae	*Cytospora oleina*	Greece	Rumbos, 1988

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Chapter 2

Pathogenicity testing of fungal isolates associated with olive trunk diseases in South Africa

ABSTRACT

Olive trunk diseases have a negative impact on the yield and lifespan of olive trees. Recently, an olive trunk disease survey performed in the Western Cape Province, South Africa, revealed an undescribed *Pseudophaeomoniella* sp. as the most prevalent fungus associated with the olive trunk disease symptoms, with other fungal species, including species of the Phaeomoniellaceae, Botryosphaeriaceae, Phaeosphaeriaceae, Diaporthaceae, Togniniaceae, Valsaceae, Coniochaetaceae, Calosphaeriaceae, Diatrypaceae, Symbiotaphrinaceae and Basidiomycota occurring at much lower frequencies. Many of the species recovered had not yet been reported from olive trees and their pathogenicity toward this host needed to be determined. In total, 40 of these isolates were selected for the pathogenicity study. The pathogenicity studies were performed by inoculating 2-year-old olive branches of 15-year-old trees by inserting colonised agar plugs into artificially wounded tissue. Measurements were made of the internal lesions after 8 months. Species that formed lesions significantly larger than the control could be considered as olive trunk pathogens. These include *Biscogniauxia mediterranea*, *Coniochaeta velutina,* isolates of the *Cytospora pruinosa* complex, an undescribed *Cytospora* sp., *Didymocyrtis banksiae*, *Diplodia seriata*, *Diaporthe foeniculina*, *Eutypa lata*, an undescribed *Fomitiporella* sp., an undescribed *Geosmithia* sp., *Neofusicoccum capensis*, *Neofusicoccum stellenboschiana*, *Neofusicoccum vitifusiforme*, *Neofusicoccum* sp. 4, *Neofusicoccum* sp. 8, *Neophaeomoniella niveniae*, *Phaeoacremonium africanum*, *Phaeoacremonium minimum*, *Phaeoacremonium oleae*, *Phaeoacremonium parasiticum*, *Phaeoacremonium prunicola*, *Phaeoacremonium scolyti*, *Phaeoacremonium spadicum*, *Phaeomoniella* sp. CFJS-2015f, *Phaeomoniella* sp. CFJS-2015g, *Phaeomoniella* sp. PMM-2014b, *Phaeomoniella* sp. WVJ-2015a, *Pleurostoma richardsiae*, an undescribed *Pseudophaeomoniella* sp., *Punctularia* sp. (aff. strigosozonata), *Symbiotaphrina buchneri*, *Xenocylindrosporium* sp. CFJS-2015c and *Xenocylindrosporium* sp. CFJS-2015e. This was the first olive trunk pathogenicity study performed in South Africa. *Pseudophaeomoniella* sp. can be regarded as one of the main olive trunk pathogens in South Africa, due to its high incidence from olive trunk disease symptoms in established orchards and its high virulence.

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INTRODUCTION

Olive trunk pathogens can infect olive trees via wounds and cause dieback of twigs and branches, which can lead to a reduced fruit bearing capacity and lifespan of olive trees (Carlucci *et al*., 2013, 2015; Úrbez-Torres *et al*., 2013). These pathogens colonise the cambium, phloem and outer xylem to utilise the free flowing nutrients of the plant as well as nutrients obtained by the degradation of woody cell-wall materials (Manion, 1981). It was only fairly recently that extensive studies regarding the causal organisms of olive trunk diseases were initiated (Ivic *et al*., 2010; Moral *et al*., 2010; Carlucci *et al*., 2013, 2015; Úrbez-Torres *et al*., 2013). During these studies, the suspected olive trunk pathogens were inoculated into green (0.50–1.50 cm in diameter; 1 to 2-year-old shoots) (Ivic *et al*., 2010; Carlucci *et al*., 2015) or older shoots (2–3-year-old) (Úrbez-Torres *et al*., 2013) of olive trees that were kept in glasshouses or that were growing in orchards (Moral *et al*., 2010; Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013). The virulence of the fungal isolates were evaluated after 25 days or up to 6 months after inoculation (Ivic *et al*., 2010; Moral *et al*., 2010, 2017; Carlucci *et al*., 2013, 2015; Úrbez-Torres *et al*., 2013). Through these studies, approximately 36 olive trunk pathogens were identified. These were Ascomycota species, falling within the Botryosphaeriaceae, Calosphaeriaceae, Diaporthaceae, Diatrypaceae, Didymellaceae, Nectriaceae, Phaeomoniellaceae, Pleosporaceae, Togniniaceae and Valsaceae family groups as well as four Basidiomycota spp. (*Athelia rolfsii*, *Fomitiporia mediterranea*, *Trametes versicolor* and *Schizophyllum commune*) (Rumbos, 1988; Sánchez Hernández *et al*., 1998; Taylor *et al*., 2001; Romero *et al*., 2005; Ivic *et al*., 2010; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012; Úrbez-Torres *et al*., 2012, 2013; Carlucci *et al*., 2013, 2015; Krid Hadj Taieb *et al*., 2014; Triki *et al*., 2014, 2015; Frisullo *et al*., 2015; Markakis *et al*., 2019; Nigro *et al*., 2019).

A recent survey of olive trunk diseases occurring in the Western Cape Province, South Africa, identified a diverse fungal population associated with olive trunk disease symptoms. *Pseudophaeomoniella* sp. was the most prevalent fungus associated with the olive trunk disease symptoms (van Jaarsveld, 2015). Other fungal species of Botryosphaeriaceae, Calosphaeriaceae, Coniochaetaceae, Diaporthaceae, Diatrypaceae Phaeomoniellaceae, Symbiotaphrinaceae, Togniniaceae and Valsaceae, as well as an undescribed *Geosmithia* sp., *Biscogniauxia mediterranea* and several Basidiomycota spp. occurred at much lower frequencies. Eleven of the fungal species (*Diplodia seriata*, *Diaporthe ambigua*, *Diaporthe foeniculina*, *Eutypa lata*, *Neofusicoccum australe*, *Neofusicoccum vitifusiforme*, *Phaeoacremonium minimum*, *Phaeoacremonium parasiticum*, *Phaeoacremonium scolyti*, *Pleurostoma richardsiae* and *Schizophyllum commune*) identified during this survey were already known as olive trunk pathogens (Ivic *et al*., 2010; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012; Úrbez-Torres *et al*., 2013; Carlucci *et al*., 2013, 2015; Triki *et al*., 2015; Frisullo *et*

al., 2015). *Colletotrichum acutatum* was also isolated from the trunk disease symptoms. However, this fungus is not classified as an olive trunk pathogen. It is an important pathogen occurring in South Africa, causing anthracnose of the olive fruit (Costa, 1998). The dieback symptoms caused by *C. acutatum* are different from those caused by trunk pathogens, since the symptoms are not the result of structural degradation, but rather due to a phytotoxic response (Moral *et al*., 2009). Many of the species recovered during the South African olive trunk disease survey had not yet been reported from olive trees and their pathogenicity toward this host needed to be determined. These included, *B. mediterranea*, *Didymocyrtis banksiae, Cytospora pruinosa*, an undescribed *Cytospora* sp., *Coniochaeta velutina,* an undescribed *Geosmithia* sp., an undescribed *Fomitiporella* sp., *Neofusicoccum capensis*, *Neofusicoccum stellenboschiana*, two undescribed *Neofusicoccum* spp., *Neophaeomoniella niveniae*, *Neophaeomoniella zymoides*, *Phaeoacremonium africanum*, *Phaeoacremonium oleae*, *Phaeoacremonium prunicola*, *Phaeoacremonium spadicum*, four undescribed *Phaeomoniella* spp., an undescribed *Punctularia* sp., an undescribed *Pseudophaeomoniella* sp., *Symbiotaphrina buchneri* and three undescribed *Xenocylindrosporium* spp.

In spite of the *Pseudophaeomoniella* sp. being the most prominent fungus detected from olive trunk disease symptoms in South Africa, this species has not yet been detected from trunk disease symptoms of olive trees in other countries and it has not been detected from any other woody hosts in South Africa, besides the native wild olive tree (*Olea europaea* subsp. *cuspidata*) (Spies *et al*., unpublished). Other Phaeomoniellaceae spp. associated with trunk disease symptoms include *Pseudophaeomoniella oleae* and *Pseudophaeomoniella oleicola*. These species have been associated with brown streaking of the wood of various olive cultivars (Crous *et al*., 2015), while *Phaeomoniella chlamydospora*, a major grapevine trunk pathogen, has been identified as an olive trunk pathogen (Crous and Gams, 2000; Úrbez-Torres *et al*., 2013). *Neophaeomoniella zymoides* (=*Phaeomoniella zymoides*), has not been tested for pathogenicity on olive, but formed an average lesion significantly larger than the control on both peach and plum shoots (Damm *et al*., 2010).

Botryosphaeriaceae and *Phaeoacremonium* spp. have been identified as the predominant olive trunk pathogens occurring in California and Italy, respectively (Nigro *et al*., 2013; Úrbez-Torres *et al*., 2013; Carlucci *et al*., 2015). The Botryosphaeriaceae olive trunk pathogens include *Botryosphaeria dothidea*, *Diplodia mutila*, *D. seriata*, *Dothiorella iberica*, *Lasiodiplodia theobromae*, *N. australe*, *Neofusicoccum luteum*, *Neofusicoccum mediterraneum*, *Neofusicoccum parvum*, *Neofusicoccum ribis* and *N. vitifusiforme* (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012; Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013; Triki *et al*., 2015). Three of these species (*D. seriata*, *N. australe* and *N.*

vitifusiforme) and four other *Neofusicoccum* spp. (*N. capensis*, *N. stellenboschiana* and two undescribed *Neofusicoccum* spp.) were detected from olive trunk disease symptoms in South Africa. One of the undescribed *Neofusicoccum* species are closely related to *Neofusicoccum protearum*. *Neofusicoccum protearum* (=*Botryosphaeria protearum*) is known to cause dieback and cankers of *Protea magnifica* in South Africa (Denman, 2002) and has been isolated from other Proteaceae hosts in Australia, Hawaii, Madeira, New Zealand, Portugal and Tenerife (Denman, 2002, 2003; Marincowitz *et al*., 2008). The known *Phaeoacremonium* olive trunk pathogens include *Phaeoacremonium alvesii*, *Phaeoacremonium italicum*, *P. minimum*, *P. parasiticum*, *P. scolyti* and *Phaeoacremonium sicilianum* (Carlucci *et al*., 2015). Three of these species (*P. minimum*, *P. parasiticum* and *P. scolyti*) and four other *Phaeoacremonium* spp. (*P. africanum*, *P. oleae*, *P. prunicola* and *P. spadicum*) were detected from olive trunk disease symptoms in South Africa. *Phaeoacremonium africanum* and *P. prunicola* have not been screened for pathogenicity on olives previously, but *P. africanum* has been tested virulent on detached shoots of pome fruit trees (Mostert *et al*., 2016), while *P. prunicola* caused lesions on grapevine pruning wounds (Baloyi *et al*., 2018). Furthermore, *P. oleae* and *P. spadicum* are newly described species and have not been tested previously for pathogenicity on any woody hosts.

The remaining potential olive trunk pathogens occurring in South Africa include species falling within the *Biscogniauxia*, *Cytospora*, *Coniochaeta* and *Geosmithia* genera. *Cytospora oleina* caused dieback of olive trees in central Greece (Rumbos, 1988). *Cytospora pruinosa*, on the other hand, did not cause symptoms when tested on 5-year-old potted ‘Gordal Sevillana’ olive trees in a greenhouse at 25–30°C (Moral *et al*., 2017), but caused dieback of ash (*Fraxinus excelsior*) in Southern Poland (Kowalski *et al*., 2017). Furthermore, *Cytospora vinacea* and *Cytospora viticola* caused dieback of grapevine in North America (Lawrence *et al*., 2017). *Coniochaeta africana*, *Coniochaeta prunicola* and *C. velutina* have been isolated from necrotic lesions of *Prunus* trees in South Africa (Damm *et al*., 2010). The pathogenicity of these species were confirmed on detached shoots of apricot, peach and plum. *Coniochaeta africana* and *Coniochaeta prunicola* were pathogenic, causing trunk disease symptoms on peach and apricot, respectively, while *C. velutina* was not pathogenic (Damm *et al*., 2010). The walnut twig beetle (*Pityophthorus juglandis*) acts as a vector for *Geosmithia morbida* infection of walnut trees, causing “thousand cankers disease” in North Carolina (Hadziabdic *et al*., 2014) and in Europe (Montecchio and Faccoli, 2014; Montecchio *et al*., 2014). Similarly, *Geosmithia pallida* can be vectored by *Pseudopityophthorus pubipennis* to cause “foamy bark canker” of coast live oak (*Quercus agrifolia*) in California (Lynch *et al*., 2014). *Biscogniauxia mediterranea* has been considered a secondary pathogen infecting weakened hosts, but under drought conditions in the Mediterranean regions, this pathogen has been shown to be

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aggressive causing charcoal canker of Turkey oak in Slovenia (Jurc and Ogris, 2005) and of young vigorous cork oak trees in Portugal (Henriques *et al*., 2012). The *Fomitiporella* and *Punctularia* spp. fall within the Basidiomycota class. Some of the species falling within these genera have been associated with white rot (Wojewoda, 2000; Ji *et al*., 2017; Knijn and Ferretti, 2018; Pildain *et al*., 2018). White rot fungi utilise the nutrients obtained by the degradation of woody-cell-wall materials, mainly lignin (Manion, 1981). *Fomitiporella americana* was found responsible for white heart-rot of *Austrocedrus chilensis* in the Patagonian forests of southern Argentina and Chile and of decaying wooden tiles of historic churches in Chile (Pildain *et al*., 2018). *Punctularia strigosozonata* and *Punctularia atropurpurascens* on the other hand have not been screened for pathogenicity but rather, these fungi are known as saprophytes that are associated with white rot (Wojewoda, 2000; Knijn and Ferretti, 2018).

The aim of this study was to determine the relevance of the fungal species, associated with olive trunk disease symptoms in the Western Cape Province, in causing olive trunk diseases. The objectives of this study were 1) to perform a detached shoot virulence screening of the known and potential olive trunk pathogens and 2) to evaluate the virulent species in field trials.

MATERIALS AND METHODS

Detached shoot virulence screening

Representative isolates of fungal species associated with trunk disease symptoms of the European and wild olive trees in Western Cape Province (van Jaarsveld, 2015) were selected for virulence screening. In total, 98 isolates comprising 29 fungal species were included (Table 1). The number of isolates selected for the detached shoot assay for each species were related to the frequency that these species were found associated with olive trunk disease symptoms. In South Africa, *Pseudophaeomoniella* sp. occurred the most frequently (Spies *et al*., unpublished), thus 17 *Pseudophaeomoniella* sp. isolates were screened. These cultures are stored in the STE-U culture collection at the Department of Plant Pathology, Stellenbosch University as well as in the culture collections at the Plant Protection Division at ARC Infruitec-Nietvoorbij (Table 1). Before inoculations, the fungi were grown on potato dextrose agar (PDA, Biolab, South Africa) amended with chloromycetin (250 mg/L) (PDA-C) at approximately 25°C for 5 days or up to 4 weeks depending on the growth rate of the fungal species.

Symptomless 2-year-old shoots of the ‘Frantoio’ cultivar were collected from trees grown on a commercial farm in Stellenbosch. The diameter of the shoots ranged between 8 and 12 mm, and the shoots were trimmed to approximately 25 cm in length. These shoots were

surface sterilised with 70% ethanol and a 4-mm diameter wound was made in the middle between the internodes of each shoot using a cordless electric drill with a flame sterilised drill-bit. The wounds were deep enough to penetrate the xylem tissues, but never reached the pith. Plugs were made from the margin of the isolates using a 4-mm diameter cork borer. Fresh wounds were inoculated by placing the plugs fungal-side down into the wounds. Plugs of PDA-C with no fungal growth were included as controls. Parafilm was used to wrap each inoculated wound. The shoots were arranged in sterile humidity chambers according to a balanced incomplete block design with 108 treatments replicated five times over 45 humidity chambers, each containing 12 shoots. The humidity chambers served as blocks with 12 shoots per humidity chamber. The humidity chambers were made by placing double autoclaved moist paper towels inside plastic (28.5 × 23.5 × 5.0 cm) containers with lids. The shoots were incubated at approximately 21°C ± 1°C for 38 days. During the incubation period, shoots were monitored weekly to monitor superficial contamination and to keep the tissue moist. The entire trial was repeated once. After the incubation period, the shoots were split longitudinally through the inoculation hole and the entire length of the lesion measured.

Field trials

Two to four of the most virulent isolates per species were selected from the detached shoot screening for the field trial. If only one isolate existed for a species, this were selected for the pathogenicity experiment provided that the isolates' lesion length was significantly larger than the control. In total, 65 isolates comprising 38 species were used. Isolates that were not screened during the detached shoot assay but were included in the pathogenicity test included the following (Table 1): *D. banksiae* (CSN 1065), *Fomitiporella* sp. (CSN 503 and PMM 2086), *Geosmithia* sp. (CSN 158 and PMM 2037), *Neofusicoccum* sp. 4 (ID 660), *Neofusicoccum* sp. 8 (ID 828), *N. niveniae* (CSN 985), *P. scolyti s.l.* A (CSN 1193 and CSN 1208), *P. scolyti s.l.* C (CSN 676), *P. scolyti s.s.* (CSN 1217), *Phaeomoniella* sp. CFJS-2015f (CSN 1191), *Punctularia* sp. (aff. strigosozonata) (CSN 1060 and CSN 1061), *S. buchneri* (CSN 986), *Xenocylindrosporium* sp. CFJS-2015c (CSN 1180) and *Xenocylindrosporium* sp. CFJS-2015e (CSN 1222).

Inoculations were performed in two locations (approximately 1 km apart) on 15-year-old 'Frantoio' olive trees that were drip irrigated on a commercial farm in Paarl, Western Cape Province, during late-winter in 2016. The two locations served as trial replicates. Symptomless 2-year-old shoots were inoculated using the same method described for the detached shoot virulence screening. However, petroleum jelly was applied to the inoculated wound before sealing the wound with the Parafilm. Ten replicates of 69 treatments were applied, according to a balanced incomplete block design on 15 branches per tree of 46 trees. After 8 months,

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the inoculated shoots were removed from the trees using pruning shears and taken to the laboratory immediately. The shoots were split longitudinally through the inoculation point and the lesions measured after which isolations were made across the entire length of the lesions. The measurements were made in the same manner as for the detached shoot virulence screening.

Re-isolations and fungal identifications

The shoots were triple sterilised by submerging in 70% ethanol for 30 s, 3.5% sodium hypochlorite for 1 min and 70% ethanol for 30 s. Isolations were made from around the inoculation point or at the edge of lesions in accordance to Koch's postulates by aseptically removing eight wood chips (~1 × 1 × 1 mm) from each shoot. These wood chips were plated onto two PDA-C plates. The plates were incubated at 23–25°C for 4 weeks. The plates were monitored each day for fungal growth resembling the inoculated fungal species. The identifications of the fungi re-isolated from the detached shoot virulence screening were based on visual observation of the culture morphology. The fungi re-isolated from field trials were predominately identified based on sequence data. Only *P. richardsiae* was identified based on morphology alone, according to the description provided by Ellis (2016). This fungus was grown on PDA-C for approximately 2 weeks before making slides and viewing with a light microscope (Nikon Eclipse Ni). Images of the collarette, phialides and conidia were captured at 1000× magnification using a Nikon DS-Ri2 camera. *Phaeoacremonium minimum* and *P. parasiticum* were identified based on species-specific PCRs, while the rest were confirmed by comparing sequence data of the fungi re-isolated from inoculated branches to that of DNA sequences of the original isolates used as inoculum. The DNA was extracted using a simplified version of the protocol by Wang *et al*. (1993). To all tubes containing fungal mycelia, 200 µL of 0.5 M of NaOH was added, regardless of the mass of the mycelia. The rest of the protocol was followed as stated in Wang *et al*. (1993). A general SDS DNA extraction method (Lee *et al*., 1988) was used for some of the *Phaeomoniella*-isolates. The DNA extracted using the NaOH method was used as is, while DNA extracted using the SDS method was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and diluted to 25 ng/µL. The species-specific PCR for *P. minimum* and *P. parasiticum* were adapted and set up using T1F (O'Donnell and Gigelnik, 1997), together with either Pbr6-1R or Pbr2-2R for *P. minimum* and *P. parasiticum*, respectively (Mostert *et al*., 2006), according to the protocol described in Appendix A. The PCR products of these reactions were visualised together with Generuler™ 100bp DNA ladders on 1% agarose gels.

The remaining species were identified based on sequence data comparisons. The *Phaeoacremonium* spp. were identified based on the partial beta-tubulin (BTUB) region, the

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Botryosphaeriaceae spp. were identified based on the partial elongation factor (EF) region and the remaining species were identified based on the internal transcribed spacer (ITS) region. The initial PCRs were set up in 20 µL reactions with either 1 × KAPA Taq ReadyMix (Kapa Biosystems) or Taq DNA Polymerase Master Mix RED, 1.5 mM $MgCl_2$ (ampliqon), 2 µL DNA and 0.2 µM of either T1F (O'Donnell and Gigelnik, 1997) and Bt2bR (Glass and Donaldson, 1995), EF1-688F and EF1-1251R (Alves *et al*., 2008) or ITS4 and ITS5 (White *et al*., 1990), respectively. The PCR conditions for BTUB region was 94°C for 5 min, followed with 36 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 1 min 30 s with a final single step of 72°C for 6 min. The PCR conditions for the EF region was 94°C for 5 min followed with 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min with a final single step of 72°C for 7 min. The PCR conditions for the ITS region was 94°C for 5 min followed with 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s with a final step at 72°C for 7 min.

The PCR products were subjected to electrophoresis and where a clear PCR product could be observed, the remaining product of these samples were purified using the MSB® Spin PCRapace kit (Stratec molecular, Berlin). Sequencing reactions were carried out with 2 µL 5 × sequencing buffer, 1 µL BigDye, 0.4 µL of the forward primer of the respective loci, 1 µL of the PCR product to a 10 µL reaction. The PCR conditions of the sequencing reactions were 94°C for 5 min followed with 96°C for 10 s, 50°C for 5 s and 60°C for 4 min with a final single step at 60°C for 30 s. Sequencing products were analysed on an ABI PRISM 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CT, USA) at the Central Analytical Facility of Stellenbosch University. The DNA sequences were trimmed, edited and subjected to pairwise alignment against the DNA sequences of the original isolates using Geneious® 9.1.7.

Statistical analyses

Data for both the detached shoot virulence screening and field experiments were subjected to incomplete blocks analysis of variance (ANOVA), for each trial repeat separately, using GLM (General Linear Models) Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Observations for trials were also combined in one ANOVA after testing for trial homogeneity of variance using Levene's test (Levene, 1960). For the field trial, variances were not equal, therefore weighted ANOVAs were implemented (John and Quenouille, 1977). Shapiro-Wilk test was performed to test for deviation from normality (Shapiro and Wilk, 1965). Lesion lengths in both trials deviated from normality and in the case of the detached shoot assay normality was achieved by subjecting lesion lengths to inverse transformation, while for the field trial ln transformation best amended normality (Snedecor and Cochran, 1989). Fisher's least significant difference (LSD) was calculated at the 5% level to compare means

for significant effects (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

RESULTS

Detached shoot virulence screening

The distribution of the inverse transformed lesion lengths did not deviate from normality according to the Shapiro-Wilk test ($P > 0.05$). Trial variances were equal according to Levene's test ($P > 0.05$) and there was no isolate × trial interaction ($P > 0.05$; Appendix B: Table 1), so the two trials were combined and the isolate effect for inverse transformed lesion lengths evaluated ($P = 0.05$; Table 2). Many of the isolates produced lesions significantly larger than the control. The average lesion length of the back transformed data varied between 40.19 mm (for *C. pruinosa*; PMM 2025) and 2.41 mm (for the control) (Table 2), with an average lesion length of 6.38 mm. The re-isolation percentage ranged between 10.0–100.0% with an average re-isolation percentage of 83.5% (Table 2). *Cytospora pruinosa* (PMM 2025) produced the largest average lesion on the detached olive shoots and was not significantly different from *C. pruinosa* (ID 203, PMM 2030, CSN 623 and PMM 2025), *N. capensis* (PMM 2091, PMM 2090, ID 396 and CSN 180), *N. stellenboschiana* (ID 669 and CSN 179), *D. foeniculina* (CSN 343, CSN 549 and CSN 348), *N. vitifusiforme* (ID 827 and CSN 182), *D. seriata* (PMM 2093 and ID 683), *Pseudophaeomoniella* sp. (CSN 18 and CSN 808), *N. australe* (ID 500) and *C. acutatum* (CSN 1066) (Table 2).

The isolates that caused lesions that were both significantly different from *C. pruinosa* (PMM 2025) and from the control included, *E. lata* (ID 305, ID 318, PMM 2907, ID 322, PMM 3064, ID 319, PMM 2905 and PMM 3071), *B. mediterranea* (PMM 2071 and CSN 1052), *P. oleae* (CSN 721, PMM 1980, CSN 403, ID 231 and CSN 1154), *D. banksiae* (CSN 1071, CSN 1067, CSN 1049 and CSN 587), *Pseudophaeomoniella* sp. (CSN 754, PMM 2484, CSN 185, CSN 441, CSN 41, CSN 186, CSN 183, CSN 334, CSN 960, CSN 314, CSN 806 and CSN 401), *Phaeomoniella* sp. CFJS-2015g (CSN 1174), *P. minimum* (PMM 2073), *C. pruinosa* (CSN 577 and CSN 627), *P. africanum* (CSN 946), *P. parasiticum* (CSN 476 and CSN 418), *N. australe* (ID 403), *P. richardsiae* (PMM 2011 and PMM 2012), *D. foeniculina* (CSN 867, CSN 297 and CSN 307), *Phaeomoniella* sp. WVJ-2015a (CSN 1091 and CSN 801), *Cytospora* sp. WVJ-2015a (CSN 620, CSN 621 and CSN 619), *Neofusicoccum* sp. 8 (ID 847), *Ca. velutina* (PMM 2035) and *Xenocylindrosporium* sp. CFJS-2015c (CSN 1203) (Table 2).

The isolates that caused lesions that were not significantly different from the control included, *P. spadicum* (ID 208), *P. richardsiae* (CSN 493, CSN 1101, CSN 144, PMM 2013 and CSN 500), *B. mediterranea* (CSN 1054, CSN 1056 and CSN 1055), *P. oleae* (PMM 2440,

CSN 703, CSN 945 and PMM 2239), *D. banksiae* (CSN 588 and CSN 1072), *N. zymoides* (CSN 743), *Pseudophaeomoniella* sp. (CSN 973, CSN 737 and PMM 1192), *C. pruinosa* (PMM 2029), *Phaeomoniella* sp. PMM-2014b (PMM 1193), *P. prunicola* (ID 230), *N. niveniae* (CSN 742) and *Xenocylindrosporium* sp. CFJS-2015b (CSN 1216 and CSN 1179) (Table 2).

Field trials

The distribution of the inverse transformed lesion lengths did not deviate from normality according to the Shapiro-Wilk test ($P > 0.05$). Levene's test indicated that trial variances were not equal ($P < 0.05$), so a weighted ANOVA was done for the combined analysis. The isolates × trial interaction was not significant ($P > 0.05$; Appendix B: Table 2), so the two trials were combined and the isolate effect for ln transformed lesion lengths evaluated ($P = 0.05$; Table 3). One isolate of each of *C. pruinosa*, *E. lata*, *N. niveniae* and *Punctularia* sp. (aff. strigosozonata) was significantly different from the remaining isolate of the respective species. The Botryosphaeriaceae spp., *E. lata* and *D. foeniculina* produced prominent lesions, while *Phaeoacremonium* spp., *Phaeomoniella* spp., *Pseudophaeomoniella* sp., *P. richardsiae* and some of the *Xenocylindrosporium* spp. formed lesions around the point of inoculation as well as streaking upward and downward into the vascular tissue (Fig. 1). Many of the remaining species, including an undescribed *Cytospora* sp., *C. velutina*, an undescribed *Geosmithia* sp., *B. mediterranea* and an undescribed *Fomitiporella* produced small indistinct lesions that were still significantly different from the control (Fig. 1). The average lesion lengths of the back transformed data varied between 73.11 mm (for *C. pruinosa*; PMM 2025) and 15.34 mm (for the control). The average lesion length was 36.32 mm (Table 3). The re-isolation percentage ranged between 0.0–100.0%, with an average re-isolation percentage of 61.5% (Table 3). *Cytospora pruinosa* (PMM 2025) produced the largest average lesion (73.11 mm) on the inoculated olive branches, which was not significantly different from the lesions made by *N. capensis* (ID 396, CSN 180, PMM 2091 and PMM 2090), *Neofusicoccum* sp. 4 (ID 660), *P. africanum* (CSN 946), *E. lata* (ID 318), *Pseudophaeomoniella* sp. (CSN 183 and CSN 18), *P. parasiticum* (CSN 418), *D. foeniculina* (CSN 549), *N. vitifusiforme* (ID 827 and CSN 182), *Nm. stellenboschiana* (ID 669), *P. richardsiae* (PMM 2011) and *Neofusicoccum* sp. 8 (ID 847) (Table 3). This group of isolates can be regarded as having a high virulence towards olive trees.

The isolates that were both significantly different from *C. pruinosa* (PMM 2025) and the control included *D. seriata* (ID 683 and PMM 2093), *P. richardsiae* (PMM 2012), *N. niveniae* (CSN 985), *P. oleae* (CSN 721 and PMM 1980), *B. mediterranea* (PMM 2071 and CSN 1052), *Phaeomoniella* sp. WVJ-2015a (CSN 1091 and CSN 801), *P. spadicum* (ID 208), *P. parasiticum* (CSN 476), *Xenocylindrosporium* sp. CFJS-2015c (CSN 1203), *C. velutina* (PMM

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2035), *Fomitiporella* sp. (Taxon 1) (CSN 503 and PMM 2086), *P. scolyti s.l.* A (CSN 1208), *P. prunicola* (ID 230), *Phaeomoniella* sp. PMM-2014 (PMM 1193), *P. minimum* (PMM 2073), *C. pruinosa* (ID 203), *E. lata* (ID 305), *Geosmithia* sp. (CSN 158 and PMM 2037), *N. stellenboschiana* (CSN 179), *Punctularia* sp. (aff. strigosozonata) (CSN 1061), *D. banksiae* (CSN 1071, CSN 1049 and CSN 1067), *Neofusicoccum* sp. 8 (ID 828), *Cytospora sp.* WVJ-2015a (CSN 621 and CSN 620), *D. foeniculina* (CSN 343), *S. buchneri* (CSN 986), *Xenocylindrosporium* sp. CFJS-2015e (CSN 1222), *Phaeomoniella* sp. CFJS-2015f (CSN 1191) and *Phaeomoniella* sp. CFJS-2015g (CSN 1174) (Table 3). This group can be regarded as having an intermediate level of virulence.

The isolates that were not significantly different from the control include, *Xenocylindrosporium* sp. CFJS-2015c (CSN 1180), *Xenocylindrosporium* sp. CFJS-2015b (CSN 1216 and CSN 1179), *P. scolyti s.l.* C (CSN 676), *P. scolyti s.s.* (CSN 1217), *P. scolyti s.l.* A (CSN 1193), *N. niveniae* (CSN 742), *C. acutatum* (CSN 1066), *D. banksiae* (CSN 1065), *Punctularia* sp. (aff. strigosozonata) (CSN 1060) and *N. zymoides* (CSN 743) (Table 3). This group can be regarded as the low virulence group and are either non-pathogenic or weakly pathogenic.

The majority of the highly virulent strains were isolated from European olive trees opposed to the wild olive trees (Table 1). The wild olive strains, *C. pruinosa* (PMM 2025), *D. foeniculina* (CSN 549), *N. capensis* (ID 396, PMM 2091 and PMM 2090), *N. stellenboschiana* (ID 669), *N. vitifusiforme* (ID 827), *Neofusicoccum* sp. 4 (ID 660), *Neofusicoccum* sp. 8 (ID 847), *P. parasiticum* (CSN 418), *P. richardsiae* (PMM 2011) and *P. africanum* (CSN 946) were isolated from European olive trees. *Cytospora pruinosa* (PMM 2025), *N. capensis* (ID 396, PMM 2091 and PMM 2090), *P. parasiticum* (CSN 418) and *P. richardsiae* (PMM 2011) were isolated from areas within the Winelands, *N. stellenboschiana* (ID 669), *N. vitifusiforme* (ID 827), *Neofusicoccum* sp. 4 (ID 660) and *Neofusicoccum* sp. 8 (ID 847) was isolated from Hermanus, while *D. foeniculina* (CSN 549) and *P. africanum* (CSN 946) was isolated from Somerset West and Durbanville, respectively. Highly virulent isolates isolated from wild olives include *E. lata* (ID 318), *N. capensis* (CSN 180), *N. vitifusiforme* (CSN 182) and *Pseudophaeomoniella* sp. (CSN 183 and CSN 18). *Neofusicoccum capensis* (CSN 180), *N. vitifusiforme* (CSN 182) and *Pseudophaeomoniella* sp. (CSN 18) were isolated from Franschhoek, while *E. lata* (ID 318) and *Pseudophaeomoniella* sp. (CSN 183) were isolated from the wild olive trees growing in Ceres and Calitzdorp, respectively.

The representative isolates of *P. richardsiae* that were re-isolated during the pathogenicity study was confirmed by microscopic observation (Fig. 2). Representatives of *P. minimum* and

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P. parasiticum were confirmed by species-specific primers. The PCR product of *P. minimum* and *P. parasiticum* was approximately 548 and 446 bp (Fig. 2). The successful establishment of the remaining isolates in the olive tree wood was confirmed based on sequence alignments against the DNA sequences of the original respective fungi that was used as inoculum. The pairwise percentage identity between these DNA sequences was 100.0% for all isolates evaluated, except for *P. scolyti s.s., Pseudophaeomoniella* sp. and *S. buchneri* (Table 4). The pairwise percentage identity of the re-isolate of *P. scolyti s.s.* (CSN 1217) inoculated branches was 98.7%, *Pseudophaeomoniella* sp. (CSN183) was 99.4%, while *S. buchneri* could not be confirmed since the original isolate could not be retrieved. These results suggest that *P. scolyti* and *Pseudophaeomoniella* sp. also infected the olive wounds naturally.

DISCUSSION

A selection of fungal species associated with olive trunk disease symptoms in the Western Cape Province were tested for their pathogenicity on olive trees. A detached shoot assay was conducted to confirm the pathogenicity and to assess the virulence where multiple isolates per species were used. Thereafter, a field study was conducted on olive trees using multiple isolates (where possible) of each of the known olive trunk pathogens and potential olive trunk pathogens, to determine the relevance of these fungi in causing the olive trunk diseases in South Africa. In this study, it was observed that the virulence of isolates within a species may differ significantly. This is not uncommon, as it is expected that genetic diversity among isolates of the same species could affect the virulence of fungal pathogens (Baskarathevan *et al*., 2012). The genetic variance between isolates of the same species can be expected to be higher where isolates were obtained from different hosts or from distant areas (Baskarathevan *et al*., 2012). In this study, the virulence of *C. pruinosa*, *E. lata* and *N. niveniae* was diverse. Furthermore, one isolate each of *C. pruinosa*, *E. lata* and *N. niveniae* was obtained from European olive trees, while the other isolate representing these species was obtained from wild olive trees (Table 1). For *C. pruinosa* and *N. niveniae*, the isolates that originated from the European olive trees produced larger lesions than those that were isolated from the wild olive trees, which is expected, since the isolates originating from the European olive trees may be better adapted and able to cause disease in this host. However, this was not always the case. The *E. lata* isolate that originated from the wild olive tree produced a larger average lesion length compared to the isolate originated from a European olive tree. Furthermore, the lesions produced by the *Punctularia* sp. (aff. strigosozonata) isolates were significantly different, yet both isolates were obtained from wild olive trees, one grown in Durbanville and the other in Wellington.

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The Botryosphaeriaceae spp., *C. pruinosa*, *E. lata* and *D. foeniculina* formed large prominent lesions, while the *Phaeoacremonium* spp., *Phaeomoniella* spp., *Pseudophaeomoniella* sp., *P. richardsiae* and some of the *Xenocylindrosporium* spp. formed lesions around the point of inoculation as well as streaking upward and downward into the vascular tissue. These results are complimentary to previous studies. For example, Botryosphaeriaceae spp. as well as *E. lata* and *D. foeniculina* formed large prominent lesions in olive shoots in California (Úrbez-Torres *et al*., 2013) and a *Cytospora* sp. (i.e. *C. oleina*) produced large prominent lesions on olive shoots in Greece (Rumbos, 1988). Botryosphaeriaceae spp. have also shown to cause dark prominent lesions in inoculated grapevine shoots. This was found in Australia (Pitt *et al*., 2013), California (Úrbez-Torres and Gubler, 2009; Úrbez-Torres 2011) and Italy (Mondello *et al*., 2013). The *Phaeoacremonium* spp., *Phaeomoniellaceae* spp. and *P. richardsiae* on the other hand produced streaking of the vascular system of olive tree wood in California and Italy (Carlucci *et al*., 2013, 2015; Úrbez-Torres *et al*., 2013). The remaining species of our study that formed lesions (including *B. mediterranea*, *C. velutina*, *Cytospora* sp. WVJ-2015a, *D. banksiae*, *Fomitiporella* sp. (Taxon 1), *Geosmithia* sp., *Punctularia* sp. (aff. strigosozonata), *N. zymoides* and *S. buchneri* were small and indistinct.

Cytospora pruinosa (PMM 2025) formed the largest average lesion length of all the isolates used in the field trial. Contradictory to our study, *C. pruinosa* were not able to form lesions on potted olive trees in glasshouse trials (Moral *et al*., 2017). Furthermore, significant variation in the virulence of *C. pruinosa* isolates was found in this study. The genetic variance of *C. pruinosa* is expected to be high, since *C. pruinosa* forms part of a species complex (Adams *et al*., 2006). Further phylogenetic analysis can be used to clarify the relationship between virulence and the genetic composition of isolates of the *C. pruinosa* complex. The *Neofusicoccum* spp. were also generally highly virulent. This was also found in other similar pathogenicity studies that included diverse fungal species, on olives (Úrbez-Torres *et al*., 2013) and English Walnut in California (Chen *et al*., 2014), as well as on grapevines in Australia (Pitt *et al*., 2013). Other species that were able to cause long lesions included *D. foeniculina*, *E. lata*, *P. africanum*, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp. In California, closely related *D. foeniculina* species (=*Phomopsis* sp. group 1 and *Phomopsis* sp. group 2) and *E. lata* were described as intermediately virulent olive trunk pathogens (Úrbez-Torres *et al*., 2013), while the pathogenicity of *P. parasiticum* and *P. richardsiae* have not been compared to a diverse group of trunk pathogens, but these species have shown to be important olive trunk pathogens with *P. richardsiae* being considered the primary cause of a severe dieback of olives trees in Italy (Carlucci *et al*., 2013, 2015). In this study, *P. africanum*

could be classified as an olive trunk pathogen with high virulence for the first time on 'Frantoio' olive trees in the Western Cape Province.

Species that consistently formed lesions that were not significantly different from the negative control included *C. acutatum* and *N. zymoides.* It was expected that *C. acutatum* would not produce the typical dieback symptoms in this study, because this species is not known as a trunk pathogen but rather a pathogen causing anthracnose on olive fruit (Moral *et al*., 2009). The pathogenicity of *N. zymoides* was not been tested previously; it was only isolated from trunk disease symptoms of olive and plum trees (van Jaarsveld, 2015; Mostert *et al*., 2016). *Neophaeomoniella zymoides* can now be considered either non-pathogenic or a pathogen with a very low virulence to the 'Frantoio' olive cultivar.

Similar to our study, *D. seriata* also had an intermediate level of virulence in California, while *P. minimum* was intermediate to weakly virulent in California and Italy (Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013). *Phaeoacremonium scolyti* was also found to be a pathogen of olives in Italy (Carlucci et al. 2015). The remaining species (*B. mediterranea*, *Cytospora* sp. WVJ-2015a, *C. velutina*, *D. banksiae*, *Fomitiporella* sp., *Geosmithia* sp., *N. niveniae*, *P. oleae*, *P. prunicola*, *P. scolyti s.l.* A, *P. spadicum*, *Phaeomoniella* sp. CFJS-2015f, *Phaeomoniella* sp. CFJS-2015g, *Phaeomoniella* sp. PMM-2014b, *Phaeomoniella* sp. WVJ-2015a, *Punctularia* sp., *S. buchneri*, *Xenocylindrosporium* sp. CFJS-2015c and *Xenocylindrosporium* sp. CFJS-2015e) had not been screened for pathogenicity previously, but can now be classified as intermediately virulent olive trunk pathogens of 'Frantoio' olive trees.

The re-isolation percentage of *C. pruinosa* (PMM 2025) in the field trial was lower than expected (46.2%), considering that it was the most virulent pathogen for both the detached shoot virulence screening and the field trials. The re-isolation percentage of the *C. pruinosa* (ID 203) isolate was also low (33.3%). *Cytospora pruinosa* also caused long lesions on *Fraxinus excelsior*, but with a low re-isolation percentage of only 54.2% of the inoculated stems (Kowalski *et al*., 2017). The low re-isolation percentage may be due to the production of phytotoxins by the *Cytospora* sp. that migrate beyond the area that the pathogen has colonised. *Cytospora* spp. produce 10-membered lactones which are known to be phytotoxins (Lu *et al*., 2011a,b; Courtial *et al*., 2018). However, in the detached shoot assay, *C. pruinosa* (PMM 2025 and ID 203) had a high re-isolation percentage. Only two *C. pruinosa* isolates (CSN 577 and PMM 2029) of the six isolates included had a low re-isolation percentage of 10.0% and 14.3%, respectively. These were also the least pathogenic in the detached shoot assay. It is expected that the detached shoots have a lower resistance response to the pathogens than the branches inoculated in field trials. It is therefore also expected that there

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would be less resistance to the colonisation of plant tissue, consequently higher re-isolation percentages should be found in the detached shoots. This could explain the lower re-isolation frequency of *C. pruinosa* (PMM 2025 and ID 203) in field trials compared to the detached shoot assay.

Through this study it was established that a high diversity of trunk pathogens contributes to the olive trunk disease symptoms found in the Western Cape Province. This high diversity may be partially due to the Western Cape having one of the world's most diverse plant species (Goldblatt and Manning, 2002; Spies *et al*., 2018), with one of the native wild olive tree species being related to the European olive tree. *Pseudophaeomoniella* sp. can be considered as one of the major olive trunk pathogens in the Western Cape Province, since it occurred the most frequently from olive trunk disease symptoms and it was highly virulent in this study. All the known olive trunk pathogens included in this study, namely *D. seriata*, *D. foeniculina*, *E. lata*, *N. vitifusiforme*, *P. minimum*, *P. parasiticum*, *P. scolyti* and *P. richardsiae* were confirmed as olive trunk pathogens infecting the olive trees in the Western Cape Province. Most of the other fungi [including, *B. mediterranea*, *C. velutina, C. pruinosa*, an undescribed *Cytospora* sp., *D. banksiae*, an undescribed *Fomitiporella*, an undescribed *Geosmithia* sp., *N. capensis*, *N. stellenboschiana*, *Neofusicoccum* sp. 4, *Neofusicoccum* sp. 8, *N. niveniae*, *P. africanum*, *P. prunicola*, *P. oleae*, *P. spadicum*, *Phaeomoniella* sp. CFJS-2015f, *Phaeomoniella* sp. CFJS-2015g, *Phaeomoniella* sp. PMM-2014b, *Phaeomoniella* sp. WVJ-2015a, an undescribed *Pseudophaeomoniella sp.*, *Punctularia* sp. (aff. strigosozonata), *S. Buchneri*, *Xenocylindrosporium* CFJS-2015c and *Xenocylindrosporium* sp. CFJS-2015e] can be considered as newly derived olive trunk pathogens. Some of the slower growing fungi such as the *Neophaeomoniella* and *Phaeomoniella* spp. may require pathogenicity studies of longer duration to resolve the pathogenicity of these species. Furthermore, *P. chlamydospora* (=*Phaeoacremonium chlamydospora*) and *Phaeoacremonium* spp. are known as stress related pathogens (Ferreira *et al*., 1999; Eskalen *et al*., 2004), an aspect that has not been investigated in this study. Several isolates from various areas originally isolated from the wild olive trees were able to cause lesions and survive within olive wood, highlighting the possible role these plants play on the inoculum reservoir and associated threat to the olive industry. Thus far, little information is available regarding the inoculum sources of the pathogens identified in this study and their mode of infection. Further investigations into these aspects will improve our understanding of the biology of these pathogens, which could direct improved management strategies against olive trunk diseases in order to improve olive yields and the productive lifespan of these trees.

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TABLES AND FIGURES

Table 1. Isolates selected for the detached shoot virulence screening and field trials.

Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Biscogniauxia mediterranea	CSN 1052	European olive	Stellenbosch	x	x
	CSN 1054	Wild olive	Wellington	x	
	CSN 1055	Wild olive	Wellington	x	
	CSN 1056	Wild olive	Stellenbosch	x	
	PMM 2071	European olive	Stellenbosch	x	x
Colletotrichum acutatum	CSN 1066	European olive	Durbanville	x	x
Coniochaeta velutina	PMM 2035	European olive	Stellenbosch	x	x
Cytospora pruinosa[d]	CSN 577	European olive	Stellenbosch	x	
	CSN 623	European olive	Riebeek-Kasteel	x	
	CSN 627	European olive	Stellenbosch	x	
	ID 203	Wild olive	Ceres	x	x
	PMM 2025	European olive	Stellenbosch	x	x
	PMM 2029	European olive	Paarl	x	
	PMM 2030	European olive	Paarl	x	
Cytospora sp. WVJ-2015a	CSN 619	European olive	Stellenbosch	x	
	CSN 620	European olive	Stellenbosch	x	x

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Cytospora sp. WVJ-2015a (cont.)	CSN 621	European olive	Durbanville	x	x
Diaporthe foeniculina	CSN 297	European olive	Durbanville	x	
	CSN 307	European olive	Stellenbosch	x	
	CSN 343	Wild olive	Stellenbosch	x	x
	CSN 348	European olive	Paarl	x	
	CSN 549	European olive	Somerset West	x	x
	CSN 867	European olive	Hermanus	x	
Didymocyrtis banksiae	CSN 1049	European olive	Hermanus	x	x
	CSN 1065	Wild olive	Wellington		x
	CSN 1067	European olive	Somerset West	x	x
	CSN 1071	European olive	Durbanville	x	x
	CSN 1072	European olive	Somerset West	x	
	CSN 587	European olive	Paarl	x	
	CSN 588	European olive	Paarl	x	
Diplodia seriata	ID 683	European olive	Hermanus	x	x
	PMM 2093	European olive	Paarl	x	x
Eutypa lata	ID 305	European olive	Ceres	x	x
	ID 318	Wild olive	Ceres	x	x
	ID 319	Wild olive	Ceres	x	

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Eutypa lata (cont.)	ID 322	Wild olive	Ceres	x	
	PMM 2905	European olive	Riebeek-Kasteel	x	
	PMM 2907	Wild olive	Durbanville	x	
	PMM 3064	European olive	Stellenbosch	x	
	PMM 3071	European olive	Hermanus	x	
Fomitiporella sp. (Taxon 1)	CSN 503	European olive	Paarl		x
	PMM 2086	European olive	Paarl		x
Geosmithia sp.	CSN 158	Wild olive	Calitzdorp		x
	PMM 2037	European olive	Paarl		x
Neofusicoccum australe	ID 403	European olive	Stellenbosch	x	
	ID 500	European olive	Durbanville	x	
Neofusicoccum capensis	CSN 180	Wild olive	Franschhoek	x	x
	ID 396	European olive	Riebeek-Kasteel	x	x
	PMM 2090	European olive	Paarl	x	x
	PMM 2091	European olive	Paarl	x	x
Neofusicoccum stellenboschiana	CSN 179	Wild olive	Strand	x	x
	ID 669	European olive	Hermanus	x	x
Neofusicoccum vitifusiforme	CSN 182	Wild olive	Franschhoek	x	x
	ID 827	European olive	Hermanus	x	x

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Neofusicoccum sp. 4	ID 660	European olive	Hermanus		x
Neofusicoccum sp. 8	ID 828	European olive	Hermanus		x
	ID 847	European olive	Hermanus	x	x
Neophaeomoniella niveniae	CSN 742	Wild olive	Stellenbosch	x	x
	CSN 985	European olive	Hermanus		x
Neophaeomoniella zymoides	CSN 743	Wild olive	Stellenbosch	x	x
Phaeoacremonium africanum	CSN 946	European olive	Durbanville	x	x
Phaeoacremonium minimum	PMM 2073	European olive	Stellenbosch	x	x
Phaeoacremonium oleae	CSN 1154	Wild olive	Durbanville	x	
	CSN 403	Wild olive	Paarl	x	
	CSN 703	Wild olive	Stellenbosch	x	
	CSN 721	Wild olive	Wellington	x	x
	CSN 945	Wild olive	Durbanville	x	
	ID 231	Wild olive	Ceres	x	
	PMM 1980	Wild olive	Stellenbosch	x	x
	PMM 2239	Wild olive	Bonnievale	x	
	PMM 2440	Wild olive	Bonnievale	x	
Phaeoacremonium parasiticum	CSN 418	European olive	Paarl	x	x
	CSN 476	European olive	Paarl	x	x

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Phaeoacremonium prunicola	ID 230	Wild olive	Ceres	x	x
Phaeoacremonium scolyti s.l. A	CSN 1193	European olive	Vredendal		x
	CSN 1208	European olive	Somerset West		x
Phaeoacremonium scolyti s.l. C	CSN 676	European olive	Paarl		x
Phaeoacremonium scolyti s.s.	CSN 1217	European olive	Somerset West		x
Phaeoacremonium spadicum	ID 208	Wild olive	Ceres	x	x
Phaeomoniella sp. CFJS-2015f	CSN 1191	European olive	Hermanus		x
Phaeomoniella sp. CFJS-2015g	CSN 1174	European olive	Somerset West	x	x
Phaeomoniella sp. PMM-2014b	PMM 1193	European olive	Durbanville	x	x
Phaeomoniella sp. WVJ-2015a	CSN 1091	European olive	Somerset west	x	x
	CSN 801	Wild olive	Durbanville	x	x
Pleurostoma richardsiae	CSN 1101	European olive	Hermanus	x	
	CSN 144	European olive	Robertson	x	
	CSN 493	European olive	Paarl	x	
	CSN 500	European olive	Durbanville	x	
	PMM 2011	European olive	Stellenbosch	x	x
	PMM 2012	European olive	Paarl	x	x
	PMM 2013	European olive	Paarl	x	
Pseudophaeomoniella sp.	CSN 18	Wild olive	Franschhoek	x	x

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Pseudophaeomoniella sp. (cont.)	CSN 41	Wild olive	Strand	x	
	CSN 183	Wild olive	Calitzdorp	x	x
	CSN 185	European olive	Robertson	x	
	CSN 186	European olive	Calitzdorp	x	
	CSN 314	European olive	Paarl	x	
	CSN 334	Wild olive	Paarl	x	
	CSN 401	Wild olive	Wellington	x	
	CSN 441	Wild olive	Stellenbosch	x	
	CSN 737	European olive	Riebeek-Kasteel	x	
	CSN 754	European olive	Stellenbosch	x	
	CSN 806	Wild olive	Durbanville	x	
	CSN 808	European olive	Durbanville	x	
	CSN 960	European olive	Hermanus	x	
	CSN 973	European olive	Somerset West	x	
	PMM 1192	European olive	Vredendal	x	
	PMM 2484	Wild olive	Bonnievale	x	
Punctularia sp. (aff. strigosozonata)	CSN 1060	Wild olive	Durbanville		x
	CSN 1061	Wild olive	Wellington		x
Symbiotaphrina buchneri	CSN 986	European olive	Hermanus		x

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Species	Isolate	Host[a]	Origin	Detached[b]	Field[c]
Xenocylindrosporium sp. CFJS-2015b	CSN 1179	European olive	Paarl	x	x
	CSN 1216	European olive	Somerset West	x	x
Xenocylindrosporium sp. CFJS-2015c	CSN 1180	European olive	Paarl		x
	CSN 1203	European olive	Hermanus	x	x
Xenocylindrosporium sp. CFJS-2015e	CSN 1222	European olive	Hermanus		x

[a] Host from which isolates were isolated. European olive = *O. europaea* subsp. *europaea*; Wild olive = *Olea europaea* subsp. *cuspidata*

[b] Isolates selected for the detached shoot virulence screening

[c] Isolates selected for the pathogenicity field trial

[d] Isolates of the *C. pruinosa* complex

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Table 2. Average lesion lengths produced by the different isolates during the detached shoot virulence screening.

Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
Cytospora pruinosa	PMM 2025	40.19 (0.02 ± 0.01) C	High	100.0
C. pruinosa	ID 203	36.41 (0.03 ± 0.02) BC	High	87.5
Neofusicoccum capensis	PMM 2091	31.52 (0.03 ± 0.03) BC	High	90.0
N. capensis	PMM 2090	24.18 (0.04 ± 0.05) A–C	High	90.0
Neofusicoccum stellenboschiana	ID 669	21.84 (0.05 ± 0.03) z–C	High	100.0
N. stellenboschiana	CSN 179	20.70 (0.05 ± 0.03) z–C	High	88.9
Diaporthe foeniculina	CSN 343	16.67 (0.06 ± 0.05) y–C	High	90.0
Neofusicoccum vitifusiforme	ID 827	13.66 (0.07 ± 0.06) x–C	High	100.0
N. capensis	ID 396	12.57 (0.08 ± 0.05) w–C	High	80.0
D. foeniculina	CSN 549	11.25 (0.09 ± 0.06) v–C	High	90.0
C. pruinosa	PMM 2030	11.05 (0.09 ± 0.13) u–C	High	90.0
Diplodia seriata	PMM 2093	9.59 (0.10 ± 0.09) t–C	High	90.0
D. seriata	ID 683	9.33 (0.11 ± 0.05) s–C	High	100.0
N. vitifusiforme	CSN 182	7.37 (0.14 ± 0.09) r–C	High	100.0
Pseudophaeomoniella sp.	CSN 18	7.22 (0.14 ± 0.18) r–C	High	100.0
Neofusicoccum australe	ID 500	7.11 (0.14 ± 0.09) r–C	High	100.0
D. foeniculina	CSN 348	6.84 (0.15 ± 0.14) q–C	High	90.0
N. capensis	CSN 180	6.81 (0.15 ± 0.18) q–C	High	88.9

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Species	**Isolate**	**Lesion length (mm) (Transformed lesion length ± SD)[a]**	**Virulence[b]**	**Re-isolation (%)[c]**
Pseudophaeomoniella sp.	CSN 808	6.64 (0.15 ± 0.12) p–C	High	80.0
Colletotrichum acutatum	CSN 1066	6.54 (0.15 ± 0.08) o–C	High	88.9
C. pruinosa	CSN 623	6.45 (0.16 ± 0.19) o–C	High	88.9
Eutypa lata	ID 305	6.24 (0.16 ± 0.14) n–B	Intermediate	90.0
E. lata	ID 318	5.99 (0.17 ± 0.10) m–a	Intermediate	80.0
E. lata	PMM 2907	5.59 (0.18 ± 0.18) l–z	Intermediate	90.0
Biscogniauxia mediterranea	PMM 2071	5.58 (0.18 ± 0.16) l–z	Intermediate	70.0
B. mediterranea	CSN 1052	5.55 (0.18 ± 0.14) l–z	Intermediate	44.4
Phaeoacremonium oleae	CSN 721	5.55 (0.18 ± 0.14) l–z	Intermediate	100.0
E. lata	ID 322	5.43 (0.18 ± 0.15) k–y	Intermediate	80.0
E. lata	PMM 3064	5.43 (0.18 ± 0.16) k–y	Intermediate	100.0
Didymocyrtis banksiae	CSN 1071	5.38 (0.19 ± 0.15) k–y	Intermediate	88.9
Pseudophaeomoniella sp.	CSN 754	5.20 (0.19 ± 0.20) k–y	Intermediate	100.0
P. oleae	PMM 1980	5.15 (0.19 ± 0.13) j–y	Intermediate	100.0
Pseudophaeomoniella sp.	PMM 2484	5.04 (0.20 ± 0.17) j–x	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 185	5.00 (0.20 ± 0.16) j–x	Intermediate	100.0
Phaeomoniella sp. CFJS 2015g	CSN 1174	4.95 (0.20 ± 0.16) j–x	Intermediate	11.1
Phaeoacremonium minimum	PMM 2073	4.94 (0.20 ± 0.19) i–x	Intermediate	100.0

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
C. pruinosa	CSN 577	4.92 (0.20 ± 0.15) i–x	Intermediate	10.0
Phaeoacremonium africanum	CSN 946	4.87 (0.21 ± 0.19) i–x	Intermediate	100.0
E. lata	ID 319	4.79 (0.21 ± 0.22) h–w	Intermediate	80.0
Phaeoacremonium parasiticum	CSN 476	4.72 (0.21 ± 0.13) g–w	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 441	4.70 (0.21 ± 0.18) g–w	Intermediate	100.0
P. oleae	CSN 403	4.68 (0.21 ± 0.17) g–w	Intermediate	100.0
N. australe	ID 403	4.67 (0.21 ± 0.13) g–w	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 41	4.67 (0.21 ± 0.14) g–v	Intermediate	100.0
Pleurostoma richardsiae	PMM 2011	4.63 (0.22 ± 0.16) g–v	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 186	4.48 (0.22 ± 0.16) f–v	Intermediate	90.0
P. oleae	ID 231	4.47 (0.22 ± 0.21) f–u	Intermediate	100.0
D. foeniculina	CSN 867	4.46 (0.22 ± 0.15) f–u	Intermediate	66.7
D. foeniculina	CSN 297	4.44 (0.23 ± 0.18) e–u	Intermediate	100.0
P. richardsiae	PMM 2012	4.41 (0.23 ± 0.16) e–t	Intermediate	90.0
P. parasiticum	CSN 418	4.40 (0.23 ± 0.18) e–t	Intermediate	88.9
D. foeniculina	CSN 307	4.26 (0.23 ± 0.22) d–t	Intermediate	80.0
D. banksiae	CSN 1067	4.25 (0.24 ± 0.21) d–t	Intermediate	70.0
C. pruinosa	CSN 627	4.24 (0.24 ± 0.21) d–t	Intermediate	100.0

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
D. banksiae	CSN 1049	4.22 (0.24 ± 0.13) d–t	Intermediate	80.0
Phaeomoniella sp. WVJ-2015a	CSN 1091	4.21 (0.24 ± 0.18) d–t	Intermediate	88.9
Cytospora sp. WVJ-2015a	CSN 620	4.18 (0.24 ± 0.20) d–s	Intermediate	90.0
Pseudophaeomoniella sp.	CSN 183	4.16 (0.24 ± 0.19) d–s	Intermediate	100.0
Phaeomoniella sp. WVJ-2015a	CSN 801	4.07 (0.25 ± 0.18) d–r	Intermediate	12.5
Neofusicoccum sp. 8	ID 847	4.07 (0.25 ± 0.15) d–r	Intermediate	80.0
D. banksiae	CSN 587	3.98 (0.25 ± 0.19) c–r	Intermediate	70.0
E. lata	PMM 2905	3.96 (0.25 ± 0.12) c–r	Intermediate	90.0
Coniochaeta velutina	PMM 2035	3.94 (0.25 ± 0.18) c–r	Intermediate	80.0
Phaeomoniella sp. CFJS-2015c	CSN 1203	3.92 (0.26 ± 0.19) c–r	Intermediate	70.0
Pseudophaeomoniella sp.	CSN 334	3.92 (0.26 ± 0.17) c–r	Intermediate	90.0
Pseudophaeomoniella sp.	CSN 960	3.86 (0.26 ± 0.18) b–r	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 314	3.86 (0.26 ± 0.13) b–r	Intermediate	100.0
Pseudophaeomoniella sp.	CSN 806	3.82 (0.26 ± 0.21) b–r	Intermediate	100.0
E. lata	PMM 3071	3.81 (0.26 ± 0.21) b–r	Intermediate	100.0
Cytospora sp. WVJ-2015a	CSN 621	3.77 (0.27 ± 0.25) b–r	Intermediate	30.0
Pseudophaeomoniella sp.	CSN 401	3.76 (0.27 ± 0.15) b–r	Intermediate	80.0
Cytospora sp. WVJ-2015a	CSN 619	3.74 (0.27 ± 0.16) b–r	Intermediate	70.0

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
P. oleae	CSN 1154	3.61 (0.28 ± 0.15) b–q	Intermediate	100.0
Phaeoacremonium spadicum	ID 208	3.54 (0.28 ± 0.15) a–p	Low to No	90.0
P. richardsiae	CSN 493	3.52 (0.28 ± 0.14) a–p	Low to No	100.0
B. mediterranea	CSN 1054	3.49 (0.29 ± 0.19) a–o	Low to No	40.0
P. oleae	PMM 2440	3.42 (0.29 ± 0.15) a–n	Low to No	90.0
D. banksiae	CSN 588	3.36 (0.30 ± 0.19) a–m	Low to No	88.9
D. banksiae	CSN 1072	3.35 (0.30 ± 0.15) a–m	Low to No	100.0
Neophaeomoniella zymoides	CSN 743	3.33 (0.30 ± 0.20) a–m	Low to No	50.0
Pseudophaeomoniella sp.	CSN 973	3.29 (0.30 ± 0.14) a–l	Low to No	100.0
C. pruinosa	PMM 2029	3.26 (0.31 ± 0.20) a–l	Low to No	14.3
Phaeomoniella sp. PMM-2014b	PMM 1193	3.26 (0.31 ± 0.23) a–l	Low to No	88.9
Phaeoacremonium prunicola	ID 230	3.25 (0.31 ± 0.15) a–l	Low to No	100.0
Pseudophaeomoniella sp.	CSN 737	3.23 (0.31 ± 0.20) a–l	Low to No	66.7
Pseudophaeomoniella sp.	PMM 1192	3.20 (0.31 ± 0.20) a–l	Low to No	88.9
P. richardsiae	CSN 1101	3.14 (0.32 ± 0.19) a–k	Low to No	100.0
P. oleae	CSN 703	3.05 (0.33 ± 0.17) a–j	Low to No	88.9
P. oleae	CSN 945	2.97 (0.34 ± 0.23) a–i	Low to No	90.0
Neophaeomoniella niveniae	CSN 742	2.93 (0.34 ± 0.14) a–h	Low to No	55.6

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
Phaeomoniella sp. CFJS-2015b	CSN 1216	2.90 (0.35 ± 0.20) a–g	Low to No	55.6
B. mediterranea	CSN 1056	2.84 (0.35 ± 0.20) a–f	Low to No	60.0
P. richardsiae	CSN 144	2.81 (0.36 ± 0.18) a–f	Low to No	100.0
P. oleae	PMM 2239	2.78 (0.36 ± 0.22) a–e	Low to No	100.0
P. richardsiae	PMM 2013	2.78 (0.36 ± 0.18) a–e	Low to No	100.0
P. richardsiae	CSN 500	2.73 (0.37 ± 0.18) a–d	Low to No	100.0
B. mediterranea	CSN 1055	2.60 (0.38 ± 0.19) a–c	Low to No	44.4
Phaeomoniella sp. CFJS-2015b	CSN 1179	2.56 (0.39 ± 0.18) ab	Low to No	60.0
Control		2.41 (0.42 ± 0.17) a	n/a[c]	n/a

[a] Lesion length = back transformed values. The inverse transformed and standard deviation are in brackets. Means with the same letter are not significantly different ($P < 0.05$; LSD = 0.1347). Once all the letters of the alphabet were used, letters proceeded in uppercase.

[b] Virulence was regarded as high if the average lesion length was not significantly different from *C. pruinosa* (PMM 2025); virulence was regarded as intermediate if the lesion length was significantly lower from *C. pruinosa* (PMM 2025), but still significantly longer than the control; low to no virulence was regarded for isolates that did not produce lesions or produced lesions that were not significantly different from the control.

[c] Percentage of branches from which the fungus was isolated.

[d] not applicable

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Table 3. Average lesion lengths produced by the possible olive trunk pathogens during a pathogenicity study conducted on 2-year-old shoots of 15-year 'Frantoio' olive trees in two orchards in Paarl, Western Cape Province, South Africa.

Species	**Isolate**	**Lesion length (mm) (Transformed lesion length ± SD)[a]**	**Virulence[b]**	**Re-isolation (%)[c]**
Cytospora pruinosa	PMM 2025	73.11 (4.29 ± 0.80) a	High	46.2
Neofusicoccum capensis	ID 396	67.43 (4.21 ± 1.06) ab	High	79.0
Neofusicoccum sp. 4	ID 660	61.13 (4.11 ± 0.87) a–c	High	80.0
Phaeoacremonium africanum	CSN 946	60.32 (4.10 ± 0.70) a–d	High	100.0
N. capensis	CSN 180	58.89 (4.08 ± 0.55) a–d	High	89.5
N. capensis	PMM 2091	54.95 (4.01 ± 0.74) a–e	High	70.6
Eutypa lata	ID 318	53.19 (3.97 ± 0.58) a–f	High	100.0
Pseudophaeomoniella sp.	CSN 183	53.04 (3.97 ± 0.71) a–g	High	100.0
Pseudophaeomoniella sp.	CSN 18	52.47 (3.96 ± 0.79) a–h	High	94.4
Phaeoacremonium parasiticum	CSN 418	49.31 (3.90 ± 0.91) a–i	High	95.0
Diaporthe foeniculina	CSN 549	49.06 (3.89 ± 0.75) a–i	High	75.0
Neofusicoccum vitifusiforme	ID 827	48.31 (3.88 ± 0.91) a–j	High	79.0
Neofusicoccum stellenboschiana	ID 669	47.68 (3.86 ± 1.02) a–k	High	68.4
N. vitifusiforme	CSN 182	47.28 (3.86 ± 0.72) a–k	High	80.0
Pleurostoma richardsiae	PMM 2011	46.67 (3.84 ± 1.03) a–k	High	88.9
Neofusicoccum sp. 8	ID 847	46.34 (3.84 ± 0.78) a–k	High	80.0
N. capensis	PMM 2090	45.28 (3.81 ± 1.17) a–l	High	72.2

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
Diplodia seriata	ID 683	43.80 (3.78 ± 0.66) b–m	Intermediate	88.2
P. richardsiae	PMM 2012	41.35 (3.72 ± 1.14) b–n	Intermediate	95.0
Neophaeomoniella niveniae	CSN 985	40.83 (3.71 ± 1.25) c–n	Intermediate	31.3
Phaeoacremonium oleae	CSN 721	40.52 (3.70 ± 0.92) c–n	Intermediate	82.4
Biscogniauxia mediterranea	PMM 2071	39.73 (3.68 ± 0.94) c–o	Intermediate	40.0
Phaeomoniella sp. WVJ-2015a	CSN 1091	38.94 (3.66 ± 0.79) c–o	Intermediate	50.0
Phaeoacremonium spadicum	ID 208	38.76 (3.66 ± 1.07) c–o	Intermediate	47.4
P. parasiticum	CSN 476	38.40 (3.65 ± 1.02) c–p	Intermediate	85.0
Xenocylindrosporium sp. CFJS-2015c	CSN 1203	38.08 (3.64 ± 0.89) c–p	Intermediate	21.4
Coniochaeta velutina	PMM 2035	38.06 (3.64 ± 0.97) c–p	Intermediate	88.2
P. oleae	PMM 1980	37.60 (3.63 ± 0.94) c–q	Intermediate	89.5
B. mediterranea	CSN 1052	36.86 (3.61 ± 1.04) d–q	Intermediate	31.3
D. seriata	PMM 2093	34.43 (3.54 ± 0.83) e–r	Intermediate	89.5
Fomitiporella sp. (Taxon 1)	CSN 503	34.22 (3.53 ± 0.93) e–r	Intermediate	52.9
Phaeoacremonium scolyti s.l. A	CSN 1208	34.00 (3.53 ± 0.98) e–r	Intermediate	36.4
Phaeoacremonium prunicola	ID 230	33.38 (3.51 ± 1.02) f–r	Intermediate	84.2
Phaeomoniella sp. PMM-2014b	PMM 1193	32.94 (3.49 ± 0.95) f–s	Intermediate	0.0
Phaeoacremonium minimum	PMM 2073	32.80 (3.49 ± 0.99) f–t	Intermediate	90.0
C. pruinosa	ID 203	32.44 (3.48 ± 1.22) g–t	Intermediate	33.3

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
E. lata	ID 305	32.06 (3.47 ± 0.79) h–t	Intermediate	68.4
Geosmithia sp.	CSN 158	31.42 (3.45 ± 1.03) i–t	Intermediate	73.3
N. stellenboschiana	CSN 179	30.91 (3.43 ± 1.00) i–u	Intermediate	84.2
Punctularia sp. (aff. strigosozonata)	CSN 1061	30.58 (3.42 ± 0.73) i–u	Intermediate	21.4
Didymocyrtis banksiae	CSN 1071	30.52 (3.42 ± 1.19) i–u	Intermediate	45.5
Phaeomoniella sp. WVJ-2015a	CSN 801	30.52 (3.42 ± 1.45) i–u	Intermediate	81.3
Neofusicoccum sp. 8	ID 828	30.49 (3.42 ± 1.18) i–u	Intermediate	83.3
Fomitiporella sp. (Taxon 1)	PMM 2086	30.28 (3.41 ± 0.91) i–u	Intermediate	41.2
Cytospora sp. WVJ-2015a	CSN 621	30.26 (3.41 ± 0.87) i–u	Intermediate	29.4
D. foeniculina	CSN 343	30.24 (3.41 ± 1.01) i–u	Intermediate	82.4
Cytospora sp. WVJ-2015a	CSN 620	29.57 (3.39 ± 1.16) j–v	Intermediate	50.0
Symbiotaphrina buchneri	CSN 986	29.46 (3.38 ± 1.17) k–v	Intermediate	70.6
Xenocylindrosporium sp. CFJS-2015e	CSN 1222	28.13 (3.34 ± 1.20) l–v	Intermediate	6.7
Phaeomoniella sp. CFJS-2015f	CSN 1191	27.92 (3.33 ± 1.23) l–v	Intermediate	11.1
D. banksiae	CSN 1049	27.17 (3.3 ± 1.07) m–w	Intermediate	58.8
D. banksiae	CSN 1067	26.94 (3.29 ± 0.95) m–w	Intermediate	56.3
Phaeomoniella sp. CFJS-2015g	CSN 1174	26.75 (3.29 ± 1.58) n–w	Intermediate	12.5
Geosmithia sp.	PMM 2037	26.67 (3.28 ± 1.00) n–w	Intermediate	64.3
Xenocylindrosporium sp. CFJS-2015c	CSN 1180	24.64 (3.20 ± 1.02) o–x	Low to No	14.3

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Species	Isolate	Lesion length (mm) (Transformed lesion length ± SD)[a]	Virulence[b]	Re-isolation (%)[c]
Xenocylindrosporium sp. CFJS-2015b	CSN 1216	24.56 (3.2 ± 0.79) o–x	Low to No	0.0
P. scolyti s.l. C	CSN 676	23.58 (3.16 ± 1.26) p–x	Low to No	68.8
P. scolyti s.s.	CSN 1217	23.05 (3.14 ± 1.07) q–x	Low to No	21.4
N. niveniae	CSN 742	22.22 (3.10 ± 0.98) r–x	Low to No	33.3
Colletotrichum acutatum	CSN 1066	20.14 (3.00 ± 0.97) s–x	Low to No	75.0
P. scolyti s.l. A	CSN 1193	20.07 (3.00 ± 1.05) t–x	Low to No	68.4
D. banksiae	CSN 1065	18.89 (2.94 ± 0.98) u–x	Low to No	52.9
Punctularia sp. (aff. strigosozonata)	CSN 1060	18.45 (2.91 ± 0.92) v–x	Low to No	77.8
Xenocylindrosporium sp. CFJS-2015b	CSN 1179	18.36 (2.91 ± 0.9.) v–x	Low to No	46.7
Neophaeomoniella zymoides	CSN 743	16.84 (2.82 ± 1.09) wx	Low to No	61.1
Control		15.34 (2.73 ± 0.70) x	n/a[c]	n/a

[a] Lesion length = back transformed values. The Ln transformed and standard deviation are in brackets. Means with the same letter are not significantly different ($P < 0.05$; LSD = 0.1347).

[b] Virulence was regarded as high if the average lesion length was not significantly different from *C. pruinosa* (PMM 2025); virulence was regarded as intermediate if the lesion length was significantly lower from *C. pruinosa* (PMM 2025), but still significantly longer than the control; low to no virulence was regarded for isolates that did not produce lesions or produced lesions that were not significantly different from the control.

[c] Percentage of branches from which the fungus was isolated.

[d] not applicable

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Table 4. The percentage similarity of the isolates compared with the original isolates used to inoculate the pathogenicity field trials.

Species	**Isolate**	**Locus**	**Coverage (bp)[a]**	**Similarity (%)[b]**
Biscogniauxia mediterranea	CSN 1052	ITS	505	100.0
	PMM 2071	ITS	474	100.0
Colletotrichum acutatum	CSN 1066	ITS	501	100.0
Coniochaeta velutina	PMM 2035	ITS	460	100.0
Cytospora pruinosa	ID 203	ITS	524	100.0
	PMM 2025	ITS	581	100.0
Cytospora sp. WVJ-2015a	CSN 620	ITS	546	100.0
	CSN 621	ITS	534	100.0
Diaporthe foeniculina	CSN 343	ITS	295	100.0
	CSN 549	ITS	278	100.0
Diplodia seriata	ID 683	EF	347	100.0
	PMM 2093	EF	440	100.0
Didymocyrtis banksiae	CSN 1049	ITS	505	100.0
	CSN 1065	ITS	557	100.0
	CSN 1067	ITS	505	100.0
	CSN 1071	ITS	347	100.0
Eutypa lata	ID 305	ITS	444	100.0
	ID 318	ITS	520	100.0
Fomitiporella sp. (Taxon 1)	CSN 503	ITS	482	100.0
	PMM 2086	ITS	442	100.0
Geosmithia sp.	CSN 158	ITS	488	100.0
	PMM 2037	ITS	483	100.0
Neofusicoccum capensis	CSN 180	EF	399	100.0
	ID 396	EF	371	100.0
	PMM 2090	EF	436	100.0
	PMM 2091	EF	444	100.0
Neofusicoccum stellenboschiana	CSN 179	EF	394	100.0
	ID 669	EF	456	100.0
Neofusicoccum vitifusiforme	CSN 182	EF	392	100.0
	ID 827	EF	303	100.0
Neofusicoccum sp. 4	ID 660	EF	351	100.0
Neofusicoccum sp. 8	ID 828	EF	208	100.0
	ID 847	EF	330	100.0
Neophaeomoniella niveniae	CSN 742	ITS	387	100.0
	CSN 985	ITS	552	100.0
Neophaeomoniella zymoides	CSN 743	ITS	553	100.0
Phaeoacremonium africanum	CSN 946	BTUB	706	100.0
Phaeoacremonium prunicola	ID 230	BTUB	610	100.0
Phaeoacremonium spadicum	ID 208	BTUB	645	100.0
Phaeoacremonium oleae	CSN 721	BTUB	677	100.0
	PMM 1980	BTUB	608	100.0
Phaeoacremonium scolyti s.l. A	CSN 1193	BTUB	940	100.0
	CSN 1208	BTUB	712	100.0
Phaeoacremonium scolyti s.l. C	CSN 676	BTUB	699	100.0
Phaeoacremonium scolyti s.s.	CSN 1217	BTUB	614	98.7
Phaeomoniella sp. CFJS-2015f	CSN 1191	ITS	808	100.0
Phaeomoniella sp. CFJS-2015g	CSN 1174	ITS	327	100.0
Phaeomoniella sp. PMM-2014b	PMM 1193	ITS	923	100.0
Phaeomoniella sp. WVJ-2015a	CSN 1091	ITS	536	100.0
	CSN 801	ITS	540	100.0

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Species	Isolate	Locus	Coverage (bp)[a]	Similarity (%)[b]
Punctularia sp. (aff. strigosozonata)	CSN 1060	ITS	568	100.0
	CSN 1061	ITS	550	100.0
Pseudophaeomoniella sp.	CSN 18	ITS	210	100.0
	CSN 183	ITS	337	99.4
Symbiotaphrina buchneri	CSN 986	ITS	nd[c]	nd
Xenocylindrosporium sp. CFJS-2015b	CSN 1216	ITS	548	100.00
Xenocylindrosporium sp. CFJS-2015c	CSN 1180	ITS	619	100.00
	CSN 1203	ITS	368	100.00
Xenocylindrosporium sp. CFJS-2015e	CSN 1222	ITS	663	100.00

[a] Base pair (bp) coverage of the alignment.

[b] Similarity between re-isolate and original sequence based on pairwise % identity (excluding ambiguities).

[C] Not determined. Cannot retrieve the original species.

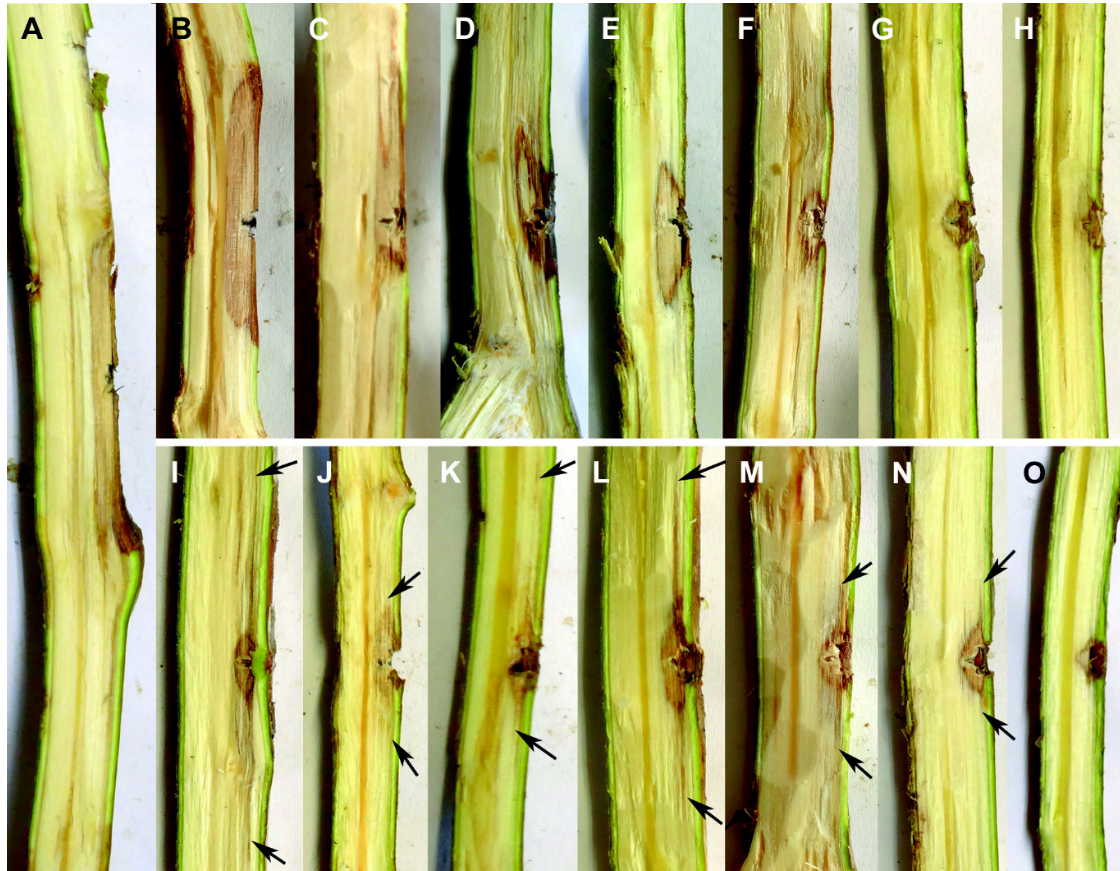

Figure 1. Lesions produced on 2-year-old 'Frantoio' shoots during the pathogenicity field trials assessed after 8 months. Prominent lesions caused by (A) *Cytospora pruinosa* (PMM 2025), (B) *Neofusicoccum capensis* (CSN 180), (C) *Neofusicoccum* sp. 8 (ID 828), (D) *Eutypa lata* (ID 305) and (E) *Diplodia seriata* (PMM 2093). Small lesions caused by (F) *Geosmithia* sp. (CSN 158) and (G) *Biscogniauxia mediterranea* (PMM 2071). Little to no lesions caused by (H) *Colletotrichum acutatum* (CSN 1066). Streaky lesions caused by (I) *Pseudophaeomoniella* sp. (CSN 183), (J) *Phaeoacremonium africanum* (CSN 946), (K) *Pleurostoma richardsiae* (PMM 2011), (L) *Phaeoacremonium parasiticum* (CSN 476), (M) *Phaeoacremonium oleae* (PMM 1980) and (N) *Xenocylindrosporium* sp. CFJS-2015e (CSN 1222). No lesions formed for (O) the control treatment.

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Figure 2. A flared collarette of *Pleurostoma richardsiae* in the process of expelling a fully developed globose conidium.

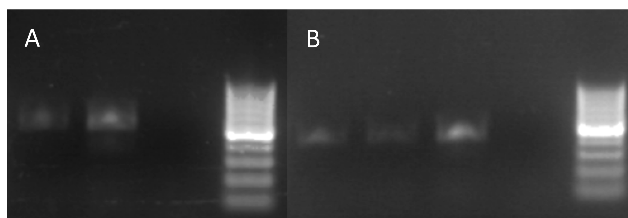

Figure 3. The PCR products of the species-specific PCRs used to confirm the identity of the re-isolates of *Phaeoacremonium minimum* (T1F/Pbr6-1R; 548 bp) and *Phaeoacremonium parasiticum* (T1F/Pbr6-1R; 446) from branches inoculated with these species in field trials. (A) Lane 1: re-isolate of *P. minimum* (PMM 2073); Lane 2: positive control (PMM2073), Lane 3: non-template control; Lane 4: DNA ladder. (B) Lane 1-2: Re-isolate of *P. parasiticum* (CSN 418 and CSN 476); Lane 4: positive control (CSN 418); Lane 5: non-template control, Lane 6: DNA ladder.

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Chapter 3

Survey of trunk pathogens in South African olive nurseries

ABSTRACT

Several fungal trunk pathogens have been identified causing olive trunk diseases in South Africa. An undescribed *Pseudophaeomoniella* sp. was the most prevalent pathogen associated with trunk diseases in the Western Cape Province, South Africa. Little is known regarding the inoculum sources of these trunk pathogens in the olive industry and no specific management strategies are in place. The aim of this study was to investigate the status of trunk pathogens in olive nurseries, in order to determine whether inoculum sources of these pathogens reside within nursery material. Isolations were made from asymptomatic cuttings from mother blocks (Stage 1), rooted cuttings (Stage 2) and 1–2-year-old trees (Stage 3) of eight cultivars in two nurseries. Known olive trunk pathogens of the Nectriaceae, Diaporthaceae, Botrysphaeriaceae, Togniniaceae, Phaeomoniellaceae and Pleurostomataceae were recovered. *Neofusicoccum australe* was detected in a single Stage 1 cutting. Stage 3 material showed the highest incidence of fungi from these families, with *Pleurostoma richardsiae* having the highest incidence in both nurseries (82.2% and 36.7% of the 1–2-year-old trees). *Phaeoacremonium parasiticum* was present in 28.9% of the trees from one nursery (Stage 3). The remaining pathogens occurred in 13.3% or less of the material. These results indicate that nursery propagation material from mother blocks harbour low levels of trunk pathogens, but that additional infections occur during the nursery process. Management strategies should focus on the prevention and elimination of infections in mother blocks, as well as during the propagation process to ensure pathogen-free material is delivered to producers.

INTRODUCTION

The management of pest and diseases, including trunk diseases, forms an integral part of improving olive production. Olive trunk diseases appear as a generalised decline of trees and are often associated with dieback of twigs and branches (Taylor *et al*., 2001; Romero *et al*., 2005; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012; Carlucci *et al*., 2013; Úrbez-Torres *et al*., 2013; Triki *et al*., 2015). The dead or dying branches in the trees represent a reduced fruit-bearing capacity. Several olive trunk pathogens have been identified, with these species falling within the Botryosphaeriaceae, Calosphaeriaceae, Diaporthaceae, Diatrypaceae, Didymellaceae, Nectriaceae, Phaeomoniellaceae, Pleosporaceae, Togniniaceae and Valsaceae as well as Basidiomycota (Rumbos, 1988; Sánchez Hernández *et al*., 1998; Taylor

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et al., 2001; Romero *et al*., 2005; Ivic *et al*., 2010; Moral *et al*., 2010, 2017; Kaliterna *et al*., 2012; Úrbez-Torres *et al*., 2012, 2013; Carlucci *et al*., 2013, 2015; Krid Hadj Taieb *et al*., 2014; Triki *et al*., 2014, 2015; Frisullo *et al*., 2015).

Recently, a diverse olive trunk disease fungal population was identified in the Western Cape Province, South Africa, with an undescribed *Pseudophaeomoniella* sp. being regarded as one of the main olive trunk pathogens that may negatively impact olive production in the Western Cape (van Jaarsveld, 2015; Chapter 2). Limited information is available regarding the inoculum sources of these pathogens and their mode of infection. However, major inoculum sources are expected to reside in olive nurseries and/or established orchards based on previous research of trunk pathogens of other hosts. Latent infection with these pathogens have been detected in apple nursery material (Havenga *et al*., 2019). Furthermore, trunk pathogens have been detected in asymptomatic grapevine rootstock mother block material (Fourie and Halleen, 2002, 2004a; Whiteman *et al*., 2007) and at lower incidences in scion mother block material (Whiteman *et al*., 2007). Partial elimination of the grapevine trunk pathogens during nursery propagation can be achieved by hot water treatment (Fourie and Halleen, 2004b; Halleen *et al*., 2007a; Bleach *et al*., 2013). However, grapevine material free of pathogens, including trunk pathogens, can still become infected later during the nursery propagation process (Halleen *et al*., 2003), such as during the hydration stages (Gramaje *et al*., 2009). The repeated soaking of grapevine propagation material in fungicide chemicals, such as benomyl, captan and didecyldimetylammonium chlorine during the hydration stages has shown to reduce the incidence of trunk pathogens in the material with no serious effects on the plants growth (Fourie and Halleen, 2004b; Fourie and Halleen, 2006; Halleen and Fourie 2016).

Hot water treatments are not practised in olive nurseries in South Africa, although some heat treatments for the elimination of *Verticillium dahliae* from young olive nursery plants in Spain have been studied (Morello *et al*., 2016). Large scale olive nurseries in the Western Cape Province use a fungicide dip such as captab-benomyl to surface sterilise semi-hard wood cuttings (4–6 mm in diameter) collected from well-maintained mother blocks (Costa, 1998; Fabbri *et al*., 2004). The majority of olive trees produced in South Africa are not grafted, except for cultivars with lower rooting ability such as 'Kalamata'. The non-grafted cuttings are made by first stripping the lower leaves of the cuttings. The cuttings are typically surface sterilised and the basal end of the cuttings dipped in a rooting hormone, before placing these in a green house in heated troughs (also known as mist beds) containing a growing medium, such as perlite (Costa, 1998; Fabbri *et al*., 2004). Regular fungicide spays and foliar nutrient sprays are recommended (Costa, 1998). After 3 months, the rooted cuttings can be

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transplanting into plastic bags containing soil mixtures tested and treated for harmful salts and pathogens (Costa, 1998). Beneficial microbes can be added to the soil to further improve the soil health (Costa, 1998; Mercado-Blanco *et al*., 2004). After planting the rooted cuttings in soil, the trees are further hardened-off to be ready for planting in an orchard after 12–18 months (Costa, 1998).

Nursery material is expected to become infected with trunk pathogens via wounds, such as through the top and bottom ends of the cutting. Currently, no studies have been published regarding the trunk disease status of olive nurseries in South Africa, and no recommendations have been established to minimise the risk of infection with these pathogens. The aim of this study was to investigate the status of trunk pathogens in olive nursery trees, in order to determine whether inoculum sources of these pathogens reside within nurseries. Non-grafted nursery material was evaluated for the presence of olive trunk pathogens. The woody plant tissues of mother block cuttings, rooted cuttings and 1–2-year-old trees were screened for the presence of olive trunk pathogens to determine the expected infection sites.

MATERIALS AND METHODS

Plant material

The nursery material was collected from two major olive tree nurseries in the Western Cape Province, during three nursery stages. Stage 1 material was collected as cuttings made directly from the mother block trees, Stage 2 was collected as 3–6-month-old rooted cuttings from rooting medium and Stage 3 was collected from 1–2-year-old trees that were ready to be delivered to producers. The number of samples collected per stage depended on the number of mother blocks. Five cuttings/plants were collected per mother block per cultivar (Table 1). The plant material was washed with tap water and Stage 2 and 3 material was dissected into compartments: the upper parts of the original cutting, the lower parts of the original cutting and roots (Fig. 1). The remaining parts (leaves, new shoots and excess roots) were discarded.

Isolations

The plant material was surface sterilised with 70% ethanol for 30 s, 3.5% sodium hypochlorite for 2 min, followed by 70% ethanol for 30 s. Small wood chips (~1 × 1 × 1 mm) were cut out aseptically and these were placed onto potato dextrose agar (Biolab, Midrand, South Africa) plates containing 250 mg/L chloromycetin (PDA-C). Twelve wood chips were sampled from each Stage 1 cutting. Wood chips from Stage 2 and 3 plants were sampled from the top margin, upper parts of the original cutting, lower parts of the original cutting, the foot of the cutting and from the roots (Fig. 1). Isolations from the upper parts of the original cutting were made at the margin of dead and living tissue as well as from the remaining parts of the plant

stem growing above the perlite and soil of the Stage 2 and Stage 3 plants, respectively. Isolations made from the lower parts of the original cutting were from portions of the stem growing under perlite or soil, while that made from the foot (or basal end) was from the margin of dead and living tissue of the basal end of the Stage 2 and Stage 3 plants. Eight wood chips were sampled from the top margin, while 12 were sampled each from the above-ground and below-ground parts of the plant as well as from the foot (basal end) and the roots of the Stage 2 and 3 plants. The plates were monitored for 4 weeks, during which sub-culturing by hyphal tipping was performed and the transfer of wood chips onto clean PDA-C plates were made where necessary.

Fungal species identifications

Morphology

Isolates of the nursery survey were first evaluated based on macro- and micro-morphological features. Isolates that resembled olive trunk pathogens and other fungi of interest were selected for molecular identification to species level. *Pleurostoma richardsiae* was identified based on microscopic observation of its unique morphological features (Ellis, 2016).

Species-specific PCR

Phaeoacremonium parasiticum was identified using a species-specific PCR. The DNA of these isolates were extracted using a simplified version of the protocol by Wang *et al*. (1993). In all tubes containing mycelia, regardless of the mycelial mass, 200 μL of 0.5 M of NaOH was added. The rest of the protocol was followed as stated in Wang *et al*. (1993). The species-specific PCRs were set up using T1F (O'Donnell and Gigelnik, 1997), together with Pbr2-2R (Mostert *et al*., 2006), according to the protocol in Appendix A for *P. parasiticum*. The PCR products of these reactions were visualised together with Generuler^{TM} 100-bp DNA ladders on 1% agarose gels

Phylogeny

Representative isolates of *P. richardsiae* and *P. parasiticum* as well as the remaining fungal species resembling trunk pathogens were subjected to PCR, sequencing and phylogenetic analyses. The DNA was extracted using the same NaOH DNA extraction method described above, for most isolates, while a general SDS DNA extraction method was used for some of the *Phaeomoniellaceae* spp. (Lee *et al*., 1988). The DNA extracted using the NaOH method was used as is, while DNA extracted using the SDS method was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and diluted to 10 ng/μL. Sequences were generated of the beta-tubulin (BTUB) region for the *Diaporthe foeniculina* and *Phaeoacremonium parasiticum* species, elongation factor (EF) region for the

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Botryosphaeriaceae spp., Histone (H3) region for the '*Cylindrocarpon*' spp. and internal transcribed spacer (ITS) region for the remaining species. The initial PCRs were set up in 20 µL reactions with either 1 × KAPA Taq ReadyMix (Kapa Biosystems) or Taq DNA Polymerase Master Mix RED, 1.5 mM $MgCl_2$ (ampliqon), 2 µL DNA and 0.2 µM of T1F (O'Donnell and Gigelnik, 1997) and Bt2bR (Glass and Donaldson, 1995) to amplify the BTUB, EF1-688F and EF1-1251R (Alves *et al*., 2008) to amplify the EF region or ITS4 and ITS5 (White *et al*., 1990) to amply the ITS region. The PCR conditions used to amplify the BTUB region was 94°C for 5 min, followed with 36 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 1 min 30 s with a final single step of 72°C for 6 min. The PCR conditions used to amplify the EF region was 94°C for 5 min followed with 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min with a final single step of 72°C for 7 min. The PCR conditions used to amplify the ITS region was 94°C for 5 min followed with 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s with a final single step at 72°C for 7 min.

The histone region of the '*Cylindrocarpon*' spp. were sequenced using the CylH3F and CylH3R (Crous *et al*., 2004) primer pair. The 25 µL reaction consisted of the following reagents: 1 × NH_4 buffer (Bioline, Germany), 1 mM $MgCl_2$ (Bioline), 0.2 mg bovine serum albumin (BSA), 0.2 mM of each dNTP (Bioline), 0.25 µM of each primer (CylH3F and CylH3R) and 0.5 U of BIOTAQ™ DNA polymerase (Bioline) and 3 µL DNA. The PCR conditions were 96°C for 5 min, 30 cycles of 96°C for 30 s, 52°C for 30 s and 72°C for 1 min, and then one final cycle of 72°C for 5 min. The PCR products were subjected to electrophoresis together with a Generuler™ 100bp Plus DNA ladder on a 1% agarose gel. Where a clear PCR product could be observed, the remaining PCR product of these samples were purified using the MSB® Spin PCRapace kit (Stratec molecular, Berlin). The sequencing reactions were carried out in 10 µL reactions containing, 1 µL of the PCR product, 2 µL 5 × sequencing buffer, 1 µL BigDye and 0.4 µL of the forward and reverse primers, separately, for each of the respective loci. The PCR conditions of the sequencing reactions were set at 94°C for 5 min followed with 96°C for 10 s, 50°C for 5 s and 60°C for 4 min with a final single step at 60°C for 30 s. Sequencing products were analysed on an ABI PRISM 3130XL DNA sequencer (Perkin-Elmer, USA) at the Central Analytical Facility (CAF) of Stellenbosch University.

The DNA sequences obtained by CAF were trimmed and edited using Geneious® v9.1.7. Preliminary identifications were obtained by BLAST analyses of single strand sequences against the GenBank nucleotide databases using blastn within Geneious® v9.1.7. The BLAST identifications were confirmed and refined by preliminary maximum likelihood phylogenies. The sequences were aligned using MAFFT v7.2.2.2 (Katoh and Standley, 2013) within Geneious® v9.1.7. The sequence alignments were edited manually in Geneious® v9.1.7

before performing the Maximum likelihood phylogenies using the PhyML (Guindon *et al*., 2010) plugin. The phylogenies were performed under the GTR model without bootstrap support calculations. Known olive trunk pathogens and other potential olive trunk pathogens were selected for further analysis. For these strains, both forward and reverse sequences were generated. The double stranded consensus sequences were aligned by single loci respectively (either BTUB, EF, H3 or ITS) using the MAFFT v7.2.2.2 plugin (Katoh and Standley, 2013) against the reference DNA sequences (Appendix C). The E-INS-i algorithm was used for BTUB, EF, H3, and L-INS-i for ITS sequence alignments. The reference sequences were selected based on published articles (Harrington and Mcnew, 2003; Damm *et al*., 2010; Cabral *et al*., 2012; Gomes *et al*., 2013; Lombard *et al*., 2014; Verkley *et al*., 2014; Lombard *et al*., 2015; Lopes *et al*., 2016; Gordillo and Decock, 2017; Guarnaccia and Crous, 2017; Spies *et al*., 2018; Liu *et al*., 2019) or were 'Type' sequences reviewed by NCBI staff. A small selection were unpublished or sequences of unpublished novel species generated by colleagues at the Plant Protection Division of Agricultural Research Council (ARC) and the Plant Pathology Department of Stellenbosch University. Best-fit models of nucleotide substitution were estimated using jModelTest2 (Darriba et al. 2012), according to the Akaike information criterion (AIC) (Akaike, 1974). The suggested substitution models were used for Maximum likelihood and Bayesian analyses. If the suggested model was not available for selection in MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001), the next best-fit model of nucleotide substitution was selected. The alignments were subjected to maximum likelihood analyses at 100 bootstrap support using the PhyML plugin (Guindon *et al*., 2010) in Geneious® v9.1.7. Bayesian analyses were performed using MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001). For the Bayesian analysis, two independent chains were run for 1 000 000 generations and every 100th point sampled. Of the 10 000 samples, the first 2 500 were discarded as burn-in prior to assessing convergence.

Association between symptoms and fungi isolated

The association between symptoms and fungi was investigated. Disease symptoms such as discolouration of the pith and/or cortex and dieback were recorded. Where the frequency of a fungus × symptom combination occurred equal to or more than five times, a chi-square analysis was performed against the frequency of the no fungus × no symptom combination to determine an association of the fungus with a specific symptom.

RESULTS

Fungal species identifications

Subsets of the species identified as *P. richardsiae* by morphology and *P. parasiticum* by species-specific PCR primers (results not shown), as well as the remaining fungi resembling

trunk pathogens (in total 20 species with one to seven isolates/species), were subjected to phylogeny using the evolutionary models summarised in Table 2. The ungapped mean length of the sequence alignments and the percentage of bases/residuals that were identical to the representative species are given in Table 3. The species identified by phylogenetic analyses fell within species-level clades of either *Cadophora luteo-olivacea*, *Coniochaeta fasciculate*, *Coniochaeta lignicola*, *Dactylonectria macrodidyma*, *Dactylonectria novozelandica*, *Dactylonectria torresensis*, *Dactylonectria valentina*, *Didymosphaeria variabile*, *Ilyonectria capensis*, *Neofusicoccum australe*, *Neofusicoccum* sp. 8 (Du Plessis *et al*., unpublished), *Pleurostoma richardsiae*, *P. parasiticum*, *Pseudophaeomoniella* sp. and *Xenocylindrosporium* sp. CFJS-2015b, with high bootstrap support (72–100% maximum likelihood bootstrap and 0.87–1.00 Bayesian posterior probability) (Figs. 2–11). Furthermore, these species matched with >98.3% of the base pairs of all isolates in a clade (Table 3).

Not all the species could be distinguished by single locus phylogeny. One isolate fell within a *D. foeniculina*/*baccae* clade with high bootstrap support (100% maximum likelihood bootstrap and 1 Bayesian posterior probability) (Fig. 11), while another fell within a *Neofusicoccum stellenboschiana*/*cryptoaustrale* clade with high bootstrap support (71% maximum likelihood bootstrap and 0.88 Bayesian posterior probability) (Fig. 7). Furthermore, a *Coniochaeta* sp. (Fig. 3) and *Heterotruncatella* sp. (Fig. 12) did not always form well-supported species-level clades, but the base pairs matched with a high percentage with *C. lignicola* (99.6%; Table 3) and *Heterotruncatella acacigena*/*vinaceobubalina*/*grevilleae* (99.2–99.4%; Table 3), respectively. *Phaeomoniella* sp. and *Dactylonectria* sp. are potentially new species identified during this study. The *Phaeomoniella* sp. formed a clade with *Phaeomoniella* sp. CFJS-2015f but with weak bootstrap support (<70% maximum likelihood bootstrap and 0.82 Bayesian posterior probability) (Fig. 10) and matched with only 89.8% of the base pairs. The two isolates of *Dactylonectria* sp. formed what appeared to be a species-level clade with high bootstrap support (86% maximum likelihood bootstrap and 0.91 Bayesian posterior probability). *Dactylonectria* sp. is related to *Dactylonectria pauciseptata* with good bootstrap support (95% maximum likelihood bootstrap and 0.84 Bayesian posterior probability), but matched with only 96.6–97.9% of the base pairs.

Nursery status

In total, eight known olive trunk pathogens (*D. macrodidyma*, *D. torresensis*, *D. foeniculina*, *N. australe*, *Neofusicoccum* sp. 8, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp.) and 11 potential olive trunk pathogens (*C. luteo-olivacea*, *C. fasciculate*, *C. lignicola*, *D. novozelandica*, *D. valentina*, *Dactylonectria* sp., *Heterotruncatella* sp., *I. capensis*, *N. stellenboschiana/cryptoaustrale*, *D. variabile* and *Phaeomoniella* sp.) were detected and

identified from the olive tree nurseries in South Africa (Table 4). Only one known olive trunk pathogen (*N. australe*) was found in one olive tree nursery cutting during Stage 1. This was isolated from a cutting of Nursery A (Table 4). The frequency and diversity of potential and olive trunk disease fungi that were isolated was higher from plant material at the later nursery stages, with the most being isolated from Stage 3 material from both nurseries (Table 4). In total, 11 potential and known olive trunk pathogens (*C. lignicola*, *D. macrodidyma*, *D. novozelandica*, *D. valentina*, *D. foeniculina*, *Heterotruncatella* sp., *N. stellenboschiana/cryptoaustrale*, *P. parasiticum*, *Phaeomoniella* sp., *P. richardsiae* and *Pseudophaeomoniella* sp.) were detected in Stage 2 nursery material from either Nursery A or B with an incidence of 3.3–10.0% (Table 4). *Phaeoacremonium parasiticum* was the most frequently detected pathogen from Stage 2 material. It was detected in 10.0% of the rooted cuttings of Nursery B. Most of these fungi (*Coniochaeta lignicola*, *D. macrodidyma*, *D. novozelandica*, *N. stellenboschiana/cryptoaustrale*, *P. parasiticum*, *Xenocylindrosporium* sp. CFJS-2015b, *Phaeomoniella* sp., *P. richardsiae* and *Pseudophaeomoniella* sp.) were isolated from the plant parts that were submerged in perlite (Table 4). *Dactylonectria valentina*, *D. foeniculina* and *Heterotruncatella* sp. were the only species that was only found in the above ground parts of the Stage 2 nursery plants.

Most of the species identified in Stage 2 material was also found in Stage 3 material. In total, 14 potential and olive trunk pathogens were identified from Stage 3 material. These included *C. luteo-olivacea*, *C. fasciculata*, *D. macrodidyma*, *D. novozelandica*, *D. torresensis*, *Dactylonectria* sp., *D. foeniculina*, *D. variabile*, *I. capensis*, *N. australe*, *Neofusicoccum* sp. 8, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp. (Table 4). *Pleurostoma richardsiae* was detected the most frequently in both Nursery A and Nursery B during Stage 3. This fungus was detected in 82.2% and 36.7% of the trees from Nursery A and B, respectively. *Phaeoacremonium parasiticum* was isolated the second most frequently and was detected in 28.9% and 3.3% of the trees from Nursery A and B, respectively. *Ilyonectria capensis* and *D. novozelandica* were detected in 13.3% of the trees of Nursery A and B, respectively. *Pseudophaeomoniella* sp. was detected in 10.0% of the trees from Nursery B. The remaining olive trunk pathogens and potential olive trunk pathogens occurred in less than 7.0% of the trees of either Nursery A or Nursery B. Most of the fungi identified in Stage 3 plants were isolated from the underground plant part of the trees (Table 4). *Cadophora luteo-olivacea*, *C. fasciculata*, *D. macrodidyma*, *D. novozelandica*, *D. torresensis*, *Dactylonectria* sp., *D. variabile*, *I. capensis*, *N. australe*, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp. occurred in the underground parts of the plants. *Cadophora luteo-olivacea*, *D. variabile*, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp. was also detected in the above ground sections of the plant, while *D. foeniculina* and *Neofusicoccum*

sp. 8 was only detected in the above ground portions of the plants during Stage 3. The majority of the fungal species recorded in this study did not show a build-up as the material aged. Only *D. novozelandica* appeared to build up in Nursery B from Stage 2 to Stage 3, while *P. parasiticum* appeared abruptly in Nursery A during Stage 3 and *P. richardsiae* appeared in higher numbers in Nursery A and Nursery B during Stage 3 (Table 4).

Symptoms

The Stage 1 material did not display any symptoms. Symptoms observed in Stage 2 and 3 plants were dieback as well as discolouration of the pith and cortex in the plant material under perlite/soil (Table 5; Fig. 13). Necrotic roots were frequently observed, although the incidence of this symptom was not recorded. Dieback was observed in 6.0% or less of the plants in Stage 2 and Stage 3 (Table 5). The majority of the symptoms were in the foot of both Stage 2 and Stage 3 nursery plants (Table 5). Stage 2 material displayed discolouration of the pith and cortex in 18.0% and 30.0% of the rooted cuttings, respectively (Table 5). These symptoms occurred separately or in combination as seen in Figure 13. Stage 3 material displayed the discolouration of the pith and cortex in the foot of 50.7% and 14.7% of the plants, respectively (Table 5). Chi-square analysis indicated that the fungus × symptom association was significant for *P. richardsiae* infection and discolouration of the pith of the foot of Stage 3 plants, but not for *P. parasiticum* infection and the same symptom (Table 6). The P-value was determined from the chi-square statistic of 9.46 and 1.79, for *P. richardsiae* and *P. parasiticum* respectively, with 1 degrees of freedom, as $P < 0.05$ for *P. richardsiae* and $P > 0.05$ for *P. parasiticum*. *Pleurostoma richardsiae* could therefore be associated with discolouration of the pith of the foot of Stage 3 plants.

DISCUSSION

Nurseries harbour pathogens and can contribute to long distance dispersal of inoculum into orchards. In Greece, olive nurseries were suggested as the primary inoculum source of *V. dahlia* (Thanassoulopoulos, 1993). In South Africa, grapevine nurseries are regarded as a major means of long distance dispersal of grapevine trunk pathogens into vineyards (Fourie and Halleen, 2002, 2004a; Whiteman *et al*., 2007). *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., “*Cylindrocarpon*” spp., *Botryosphaeria* spp. and “*Phomopsis*” spp. have been detected in grapevine nurseries in either Italy (Carlucci *et al*., 2017), South Africa (Fourie and Halleen, 2004a) and/or in Spain (Aroca *et al*., 2006; Giménez-Jaime *et al.*, 2006). Long distance dispersal of trunk pathogens has been studied in a grapevine trunk disease context, but not for olive. In this study, trunk pathogens were identified in various parts of the plant tissues and during various stages of the nursery propagation process. The majority of the fungi identified in this study were isolated from the below surface/underground parts of the

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plant, suggesting that the majority of the infections developed from the basal end of the cuttings that were in direct contact with perlite/soil and water. Although, mother block cuttings were also infected. One olive trunk pathogen (*N. australe*) occurred in the plant material from a cutting from one of the olive nurseries. The frequency of *N. australe* detection was not higher during the subsequent propagation stages. This pathogen was also not present at a high incidence in olive trunk disease symptoms of established orchards (van Jaarsveld, 2015), but it is still of concern because it can cause disease (Triki *et al.*, 2015; Chapter 2), and Botryosphaeriaceae species are commonly associated with olive trunk disease symptoms in South Africa (van Jaarsveld, 2015) and in California (Úrbez-Torres *et al*., 2013).

The incidence and diversity of the trunk pathogens detected in the nurseries was higher in older nursery material. In total, 11 known trunk pathogens of olives or other woody hosts (*D. macrodidyma*, *D. novozelandica*, *D. valentina*, *D. foeniculina*, *N. stellenboschiana/cryptoaustrale*, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp.) were isolated from the rooted cuttings (Stage 2). Of these fungi, *D. foeniculina*, *P. parasiticum*, *P. richardsiae* and *Pseudophaeomoniella* sp. are known olive trunk pathogens occurring in South Africa (van Jaarsveld, 2015; Chapter 2). Furthermore, the '*Cylindrocarpon*' spp. complex are soilborne pathogens known to infect nursery material and cause root rot diseases of olives (Sánchez Hernández *et al*., 1998), avocado (Parkinson *et al*., 2017) and grapevine (Halleen *et al*., 2006; Cabral *et al*., 2012). *Dactylonectria macrodidyma* (=*Ilyonectria macrodidyma*) caused root rot symptoms in California on 'Koroneiki', 'Arbosana', and 'Arbequina' (Úrbez-Torres *et al*., 2012), while the pathogenicity of *D. novozelandica* and *D. valentina* has not yet been reported on olives. *Neofusicoccum stellenboschiana* and *N. cryptoaustrale* could not be distinguished during this study. *Neofusicoccum stellenboschiana* is an olive trunk pathogen occurring in established orchards in South Africa, while *N. cryptoaustrale* has not been isolated from olive trees in South Africa and has not been tested for pathogenicity on this host (van Jaarsveld, 2015; Chapter 2). However, *N. cryptoaustrale* is a pathogen of *Eucalyptus grandis* and *Pistacia lentiscus* trees and caused the largest lesions during pathogenicity studies when compared to *Neofusicoccum eucalypti*, *Neofusicoccum parvum, Neofusicoccum ursorum and Botryosphaeria dothidea* on *Eucalyptus grandis* (Maleme, 2008) and *Diplodia insularis, Diplodia olivarum* and *Neofusicoccum luteum* on *P. lentiscus* (Linaldeddu *et al*., 2016). Other potential olive trunk pathogens that were found in the rooted cuttings included, *C. lignicola*, *Phaeomoniella* sp. and *Heterotruncatella* sp. *Coniochaeta lignicola* (=*Lecythophora lignicola*) was isolated from trunk disease symptoms from olives in Italy (Carlucci *et al.*, 2013), but has not been tested for pathogenicity on woody crops, while species within *Phaeomoniella* and *Heterotruncatella* genera have been

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associated with grapevine trunk diseases (Sergeeva *et al*., 2005; Edwards *et al*., 2007; Úrbez-Torres *et al*., 2009; Arzanlou *et al*., 2013; Díaz and Latorre, 2014).

Most of the olive trunk pathogens detected in the rooted cuttings (Stage 2) were also detected in the 1–2-year-old trees grown in planting bags (Stage 3). Fourteen fungi known as trunk pathogens of olives or other woody hosts were detected from Stage 3 material. *Cadophora luteo-olivacea*, *C. fasciculata*, *D. torresensis*, *Dactylonectria* sp., *D. variabile* (=*Paraconiothyrium variabile*), *I. capensis* and *Neofusicoccum* sp. 8, were only detected in Stage 3 nursery material. Of these species, *Neofusicoccum* sp. 8 was the only known olive trunk pathogen and known to be associated with trunk disease symptoms of established orchards in South Africa (van Jaarsveld, 2015; Chapter 2). *Cadophora luteo-olivacea* and *D. variabile* are known as grapevine trunk pathogens (Halleen *et al*., 2007b; Cloete *et al*., 2011; Travadon *et al*., 2015), while *D. torresensis* is a black foot pathogen of grapevine (Carlucci *et al*., 2017) and has recently been determined as a root rot pathogen of olives in Apulia, Southern Italy (Nigro *et al*., 2019). *Ilyonectria capensis* is a black foot disease pathogen of *Protea* 'Sylvia' (Lombard *et al*., 2013). Furthermore, a possible undescribed *Dactylonectria* sp. was identified in the Stage 3 material. This species was closest related to *D. pauciseptata*, which is a black foot disease pathogen of grapevine (Schroers *et al*., 2008; Piperkova *et al*., 2017).

A higher diversity and frequency of trunk pathogens are expected to be detected in older nursery material (i.e. Stage 3). Trunk pathogens can occur in nursery material as endophytes (Schoeneweiss, 1981; Ferreira *et al*., 1999; Úrbez-Torres, 2011), and grow over an increased area within the nursery material (i.e. cuttings, rooted cuttings and trees) as the material ages, before symptoms develop, inevitably leading to an increased chance of detection. Furthermore, older plant material has been subjected to the environment and the risk of infection for longer. *Phaeoacremonium parasiticum* and *P. richardsiae* appeared abruptly in Stage 3 material, suggesting that the inoculum of these pathogens were mainly introduced to the nursery material during Stage 3. Furthermore, these fungi were typically in the underground parts of Stage 3 trees, indicating that infection with these trunk pathogens were most likely from the basal end of the cuttings, that was in direct contact with soil and water. Several fungal species causing trunk diseases of grapevines, including *P. parasiticum*, have been detected in grapevine nursery soils (Agustí-Brisach *et al*., 2013). Conjecturally, *P. parasiticum* and *P. richardsiae* could be residing in the potting mixture used for olive trees in the nurseries. Furthermore, although plants infected with *P. richardsiae* appeared healthy externally, internally, *P. richardsiae*-infection was associated with discolouration of the pith of the foot of the nursery olive trees.

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Pleurostoma richardsiae (=*Pleurostomophora richardsiae*) appears to be an important olive trunk pathogen occurring in Italy, and was suggested as the primary cause of a severe trunk disease outbreak in the Canosa di Puglia, Cerignola and Foggia areas of Italy (Carlucci *et al*., 2013). Most of the infected trees were 18–20-years-old, suggesting that *P. richardsiae* was introduced to the orchards during a specific time-period. The epidemiology of *P. richardsiae*-infection in Italy was not studied. It could be that asymptomatic olive nursery material was established in these orchards and that the symptoms only became apparent 18–20 years later, possibly after a stressful environmental condition. In South Africa, *P. richardsiae* was not commonly isolated during the olive trunk disease survey performed by van Jaarsveld (2015). However, the crown or underground portions of olive trees were never sampled during this study. Recently, *P. richardsiae* was isolated from the crown area of six out of seven dead or dying olive trees in South Africa (Bishop *et al*., unpublished). Latent infections of *P. richardisiae* and other trunk pathogens in olive nurseries could be responsible for the death of olive trees in established orchards and a disease management strategy in South African olive nurseries appears necessary.

The most efficient and sustainable control measures in an integrated disease management system are those that can be implemented before planting. Some of the current disease management practises used in olive nurseries, include 1) the collection of cuttings from well-maintained mother block trees that appear disease-free, 2) surface sterilisation of these cuttings with a chemical fungicide and 3) rooting these cuttings in sterile perlite growing medium in glasshouses. However, the majority of the fungi identified in this study were isolated from the below surface/underground parts of the plant, suggesting that the majority of the infections developed from the basal end of the cuttings that was in direct contact with perlite/soil and water. Further studies should be performed to establish the inoculum status of the perlite and other parts of the growing beds, as well as the soil and water used in the nurseries. Pathogen contaminated soils can be pasteurised to eradicate pathogens in the soil. Soil solarisation has been used during the summer months in Mediterranean climatic zones for the control of olive nematode pests (Stapleton et al. 1999; Nico *et al*., 2003). After soil sterilisation, the soil health can be improved by supplementing with beneficial micro-organisms such as mycorrhizal fungi (Estaún *et al*., 2003; Castillo *et al*., 2006; Porras-soriano *et al*., 2006; Dag *et al*., 2009) and/or *Pseudomonas fluorescens* (Mercado-Blanco *et al*., 2004). Furthermore, heat treatment of olive propagation material could be used to improve the olive trunk disease status of the nursery material, although the practical implications in South Africa should be explored first. Heat treatments have been used to eradicate internal trunk pathogen infestations from grapevine propagation material (Fourie and Halleen, 2004b; Halleen *et al*.,

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2007a; Bleach *et al*., 2013), while hot water treatment and hot air treatments of olive propagation material has been used to eradicate internal infestations of *V. dahlia*, although sometimes with adverse effects of the success of tree survival (Morello *et al*., 2016).

During this study, olive nurseries in South Africa were identified as a potential inoculum source for various trunk pathogens. *Neofusicoccum australe* was detected in a mother block cutting. Additional infections occurred during the propagation process, with the majority of infections occurring via the basal end of rooted cuttings and trees that was in direct contact with the perlite/soil suggesting that the inoculum sources were present in the perlite and soil. Furthermore, *P. parasiticum* and *P. richardsiae* was abruptly present at high incidences in the underground parts of Stage 3 plants. Considering that trunk pathogens can be soilborne, suggests that the soil may be a serious inoculum source of *P. parasiticum* and, more so, *P. richardsiae*. Further studies should be performed to discern this matter and establish whether this high incidence of *P. richardsiae* was an isolated event or chronic occurrence. Regardless, disease management strategies should focus on the prevention and elimination of infections in mother blocks, as well as during the propagation process to ensure pathogen-free material is delivered to producers. *Pseudophaeomoniella* sp. which was considered as one of the main olive trunk pathogens occurring in South Africa was also present in the nurseries. The nurseries were therefore identified as an inoculum source of this pathogen, although, the incidence of this pathogen in the nurseries was low, suggesting that more important inoculum sources of this pathogen exists elsewhere.

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TABLES AND FIGURES

Table 1. Numbers and types of plant material collected from two olive tree nurseries (A and B). Three types of plant material were collected: cuttings from mother blocks (Stage 1), rooted cuttings (Stage 2), and 1–2-year-old trees ready for sale (Stage 3).

Cultivar	MB[a]	Stage 1	Stage 2	Stage 3
Nursery A				
Arbequina	1	5	5	5
Coratina	1	5	5	5
Frantoio	1	5	5	5
FS17	1	5	5	5
Koroneiki	1	5	5	5
Leccino	1	5	n/a[b]	5
Mission	1	5	n/a	5
Nocellara	1	5	n/a	5
Picual	1	5	5	5
Total	9	45	30	45
Nursery B				
Coratina	1	5	5	5
Frantoio	1	5	5	5
Leccino	2	5	5	5
Mission	2	5	n/a	5
Total	6	30	20	30
Total (Nursery A and B)	15	75	50	75

[a] Number of mother blocks (MB) from which samples originated.

[b] Material not available.

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Table 2. Evolutionary models suggested by jModelTest2 according to the Akaike information criterion (AIC). The models chosen for the construction of the Maximum likelihood (ML) and Bayesian phylogenetic trees.

Taxon	AIC[a]	ML	Bayesian
Cadophora and *Graphium*	K80 + G	HKY85	HKY85 + G
Coniochaeta	SYM + G	GTR	GTR + G
Dactylonectria	GTR + I + G	GTR	GTR + I + G
Diaporthe	HKY85 + I + G	HKY85	HKY85 + I + G
Didymosphaeriaceae	GTR + I + G	GTR	GTR + I + G
Heterotruncatella	HKY85 + I + G	HKY85	HKY85 + I + G
Ilyonectria	GTR + I + G	GTR	GTR + I + G
Neofusicoccum	GTR + G	GTR	GTR + G
Phaeoacremonium	HKY85 + I + G	HKY85	HKY85 + I + G
Phaeomoniellales	GTR + I + G	GTR	GTR + I + G
Pleurostoma	K80 + G	HKY85	HKY85 + G

[a] I and G refers to "proportion of invariable sites estimated" and "gamma distribution paramater estimated," respectively

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Table 3. Strains of olive trunk pathogens and potential olive trunk pathogens isolated from two commercial olive tree nurseries located in the Western Cape Province, South Africa. The ungapped mean length and pairwise percentage identity is calculated against the reference strains (Appendix C).

Species	Isolates[a]	Ungapped mean length	% Identity[b]
Cadophora luteo-olivacea	MVB 133, 139	460	100.0
Coniochaeta fasciculata	MVB 107, 109, 179, 188	478	99.8
Coniochaeta lignicola	MVB 490	478	99.6
Dactylonectria macrodidyma	MVB 142, 643, 672, 673	382	99.0–99.2
Dactylonectria novozelandica	MVB 123, 642, 662	382	99.9–100.0
Dactylonectria torresensis	MVB 199	382	99.5
Dactylonectria valentina	MVB 102	382	98.5
Dactylonectria sp.	MVB 165, 471	382	96.6–97.1
Diaporthe foeniculina	MVB 240	638	99.5–99.6
Didymosphaeria variabile	MVB 135, 170	487	100.0
Heterotruncatella sp.	MVB 602	484	99.2–99.4
Ilyonectria capensis	MVB 157, 272, 287, 295, 385, 408, 416	492	99.1–99.3
Neofusicoccum australe	MVB 117, 620	219	99.6
Neofusicoccum stellenboschiana/cryptoaustrale	MVB 547	219	99.6–100.0
Neofusicoccum sp.	MVB 669	219	99.5–100.0
Phaeoacremonium parasiticum	MVB 146, 283	552	99.1–100.0
Phaeomoniella sp.	MVB 535	393	89.5
Pleurostoma richardsiae	MVB 105, 120	414	99.8
Pseudophaeomoniella sp.	MVB 88, 650	393	99.7
Xenocylindrosporium sp. CFJS-2015b	MVB 230	393	98.2–98.5

[a] Cultures of potential and olive trunk disease fungi isolated during the nursery survey.

[b] The pairwise percentage identity, including ambiguities, that matched against the representative species (Appendix C).

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Table 4. Percentage and diversity of olive trunk pathogens and potential olive trunk pathogens isolated from two commercial olive tree nurseries located in the Western Cape Province, South Africa.

Isolation zone[b]	Fungi[a]																		
	Cdl	Caf	Cal	Dcm*	Dcn	Dct*	*Dcv*	*Dcs*	*Def**	*Div*	*Het*	*Ica*	*Nma**	*Nmsc*	*Nms**	*Pmp**	*Pas*	*Plr**	*Pss**
Cuttings from mother blocks (Stage 1)[c]																			
Nursery A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**2.2**	0.0	0.0	0.0	0.0	0.0	0.0
Nursery B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nursery A & B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	0.0	0.0	0.0	0.0	0.0
Rooted cuttings (Stage 2)[d]																			
Nursery A	0.0	0.0	**3.3**	0.0	0.0	0.0	0.0	0.0	**3.3**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**3.3**	**3.3**
Nursery B	0.0	0.0	0.0	**5.0**	**5.0**	0.0	**5.0**	0.0	0.0	0.0	**5.0**	0.0	0.0	**5.0**	0.0	**10.0**	**5.0**	0.0	0.0
Nursery A & B	0.0	0.0	**2.0**	**2.0**	**2.0**	0.0	**2.0**	0.0	**2.0**	0.0	**2.0**	0.0	0.0	**2.0**	0.0	**4.0**	**2.0**	**2.0**	**2.0**
Top margin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**2.0**	0.0	**2.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Top	0.0	0.0	0.0	0.0	**2.0**	0.0	**2.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bottom	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**2.0**	0.0	0.0	0.0	0.0	0.0
Foot	0.0	0.0	**2.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**2.0**	0.0	**2.0**	**2.0**
Roots	0.0	0.0	0.0	**2.0**	**2.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**4.0**	0.0	0.0	0.0
Nursery trees (Stage 3)[e]																			
Nursery A	0.0	**10.0**	0.0	**2.2**	0.0	0.0	0.0	**2.2**	0.0	0.0	0.0	**13.3**	0.0	0.0	0.0	**28.9**	0.0	**82.2**	0.0
Nursery B	**3.3**	0.0	0.0	**6.7**	**13.3**	**3.3**	0.0	**3.3**	**3.3**	**6.7**	0.0	**3.3**	**3.3**	0.0	**3.3**	**3.3**	0.0	**36.7**	**10.0**
Nursery A & B	**1.3**	**6.0**	0.0	**4.0**	**5.3**	**1.3**	0.0	**2.7**	**1.3**	**2.7**	0.0	**9.3**	**1.3**	0.0	**1.3**	**18.7**	0.0	**64.0**	**4.0**

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Isolation zone[b]	Fungi[a]																		
	Cdl	**Caf**	**Cal**	**Dcm***	**Dcn**	**Dct***	***Dcv***	***Dcs***	***Def****	***Div***	***Het***	***Ica***	***Nma****	***Nmsc***	***Nms****	***Pmp****	***Pas***	***Plr****	***Pss****
Top margin	**1.3**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	0.0	0.0	0.0	**1.3**	0.0	0.0	0.0	**2.7**
Top	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	**5.3**	0.0
Bottom	0.0	**2.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**1.3**	0.0	**1.3**	**1.3**	0.0	0.0	**4.0**	0.0	**16.0**	0.0
Foot	0.0	**6.0**	0.0	**2.7**	**1.3**	0.0	0.0	**1.3**	0.0	0.0	0.0	**2.7**	0.0	0.0	0.0	**8.0**	0.0	**53.3**	**1.3**
Roots	**1.3**	**4.0**	0.0	**2.7**	**4.0**	**1.3**	0.0	**1.3**	0.0	0.0	0.0	**5.3**	0.0	0.0	0.0	**9.3**	0.0	**44.0**	0.0
Total (Stage 1 + Stage 2 + Stage 3)[f]																			
Nursery A & B	**0.5**	**1.5**	**0.5**	**2.0**	**2.5**	**0.5**	**0.5**	**2.0**	**2.0**	**2.0**	**0.5**	**3.5**	**1.0**	**0.5**	**0.5**	**16.0**	**0.5**	**49.0**	**4.0**

[a] Fungi: *Cdl*, *Cadophora luteo-olivacea*; *Caf*, *Coniochaeta fasciculate*; *Cal*, *Ca. lignicola*; *Dcm*, *Dactylonectria. macrodidyma*; *Dno*, *Dcn. novozelandica*; *Dct*, *Dc. torresensis*; *Dcv*, *Dc. valentina*; *Dcs*, *Dactylonectria* sp.; *Def*, *Diaporthe foeniculina*; *Div*, *Didymosphaeria variabile*; Het, *Heterotruncatella* sp.; *Ica*, *Ilyonectria capensis*; *Nma*, *Neofusicoccum australe*; *Nmsc*, *Nm. Stellenboschiana/cryptoaustrale*; *Nms*, *Neofusicoccum* sp.; *Pmp*, *Phaeoacremonium parasiticum*; *Pas*, *Phaeomoniella* sp., *Plr*, *Pleurostoma richardsiae*; *Psp*, *Pseudophaeomoniella* sp. Fungi denoted with an asterisks (*) are known olive trunk pathogens, while the remaining have not been tested for pathogenicity on olive trees but are trunk pathogens of other hosts.

[b] Isolations made from the “top margin” was at the margin of dead and living tissue of the plant stem growing above the perlite and soil of the Stage 2 and Stage 3 plants, respectively, whereas isolations made of the “top” was from the rest of the plant stem growing above the perlite or soil. Isolations made from the “bottom” were from portions of the stem growing under perlite or soil, while that made from the “foot” was specifically from the margin of dead and living tissue from the basal end of the Stage 2 and Stage 3 plants.

[c] Percentage of infected cuttings from Nursery A (sample size = 45), nursery B (sample size = 30) and Nursery A and B combined (sample size = 75)

[d] Percentage of infected rooted cuttings from Nursery A (sample size = 30), Nursery B (sample size = 20) and of Nursery A and B combined (sample size = 50), as well as from the separate isolation zones (top margin, top, bottom, foot and roots) of all Stage 2 plants (sample size = 50).

[e] Percentage of infected nursery trees from Nursery A (sample size = 45), Nursery B (sample size = 30) and of Nursery A and B combined (sample size = 75), as well as from the isolation zone of all Stage 3 plants (sample size = 75).

[f] Total percentage of infected nursery material across all isolation zones and nursery stages (sample size = 200).

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Table 5. Percentage of disease symptoms at the different propagation stages in commercial olive nurseries (Nursery A + B) located in the Western Cape Province, South Africa.

	Symptom[a]		
Isolation zone	**Discoloured pith**	**Discoloured cortex**	**Dieback**
Rooted cuttings (Stage 2)			
Top margin	-	-	6.0
Lowe parts	2.0	2.0	-
Foot	18.0	30.0	-
Nursery trees (Stage 3)			
Top margin	-	-	5.3
Lower parts	5.3	1.3	-
Foot	50.7	14.7	-

[a] Percentage of nursery plants with symptoms in the top margin, bottom and foot of the plants of Stage 2 (sample size = 50) and Stage 3 (sample size = 75).

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Table 6. The frequency of symptoms of rooted cuttings (Stage 2) and nursery trees (Stage 3) coinciding with olive trunk pathogens and potential olive trunk pathogens isolated from two commercial olive tree nurseries located in the Western Cape Province, South Africa.

	Fungi[a]																		
Symptom (zone)	***Cdl***	***Caf***	***Cal***	***Dcm****	***Dcn***	***Dct****	***Dcv***	***Dcs***	***Def****	***Div***	***Het***	***Ica***	***Nma****	***Nmsc***	***Nms****	***Pmp****	***Pas***	***Plr****	***Pss****
Rooted cuttings (Stage 2)																			
Dieback (Top Margin)	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0
Discoloured pith (Lower parts)	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0
Discoloured cortex (Lower parts)	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0
Discoloured pith (Foot)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Discoloured cortex (Foot)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**
None	0	0	**1**	**1**	**1**	0	**1**	0	**1**	0	0	0	0	0	0	**3**	**1**	**1**	0
Nursery trees (Stage 3)																			
Dieback (Top Margin)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**
Discoloured pith (Lower parts)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**	0	**1**	0
Discoloured cortex (Lower parts)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0
Discoloured pith (Foot)	0	**2**	0	**2**	0	0	0	**1**	0	0	0	0	0	0	0	**5**	0	**30**	**1**
Discoloured cortex (Foot)	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	0
None	**2**	**4**	0	**2**	**1**	**1**	0	**1**	**1**	**2**	0	**7**	**1**	0	**1**	**9**	0	**52**	0

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[a] Number of plants infected by the different fungi. Fungi: *Cdl*, *Cadophora luteo-olivacea*; *Caf*, *Coniochaeta fasciculata*; *Cal*, *Coniochaeta lignicola*; *Dcm*, *Dactylonectria macrodidyma*; *Dcn*, *Dactylonectria novozelandica*; *Dct*, *Dactylonectria torresensis*; *Dcv*, *Dactylonectria valentina*; *Dcs*, *Dactylonectria* sp.; *Def*, *Diaporthe foeniculina*; *Div*, *Didymosphaeria variabile*; Het, *Heterotruncatella* sp.; *Ica*, *Ilyonectria capensis*; *Nma*, *Neofusicoccum australe*; *Nmsc*, *Neofusicoccum stellenboschiana/cryptoaustrale*; *Nms*, *Neofusicoccum* sp.; *Pmp*, *Phaeoacremonium parasiticum*; *Pas*, *Phaeomoniella* sp., *Plr*, *Pleurostoma richardsiae*; *Psp*, *Pseudophaeomoniella* sp. Fungi denoted with an asterisks (*) are known olive trunk pathogens, while the remaining have not been tested for pathogenicity on olive trees but are trunk pathogens of other hosts.

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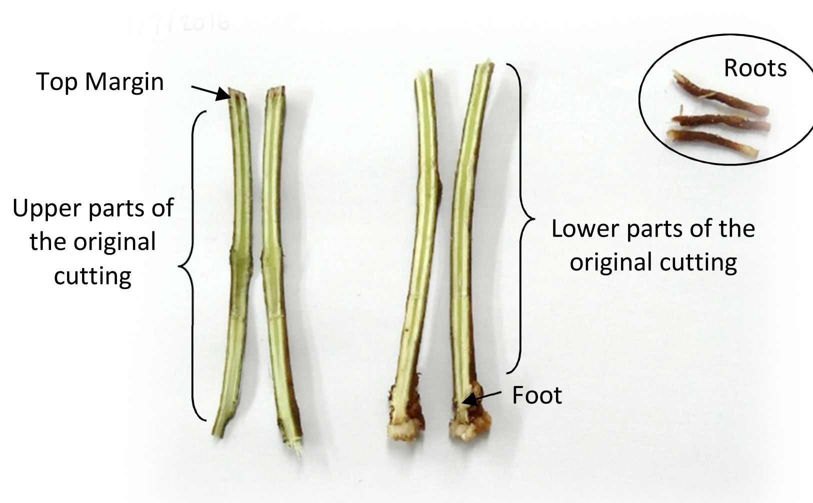

Figure 1. An example of Stage 2 rooted cuttings sectioned into the upper and lower parts of the original cutting. Isolations were made from the top margin, upper parts of the original cutting, below parts of the original cutting, foot and roots. The same approach was used for isolations made of the Stage 3 trees.

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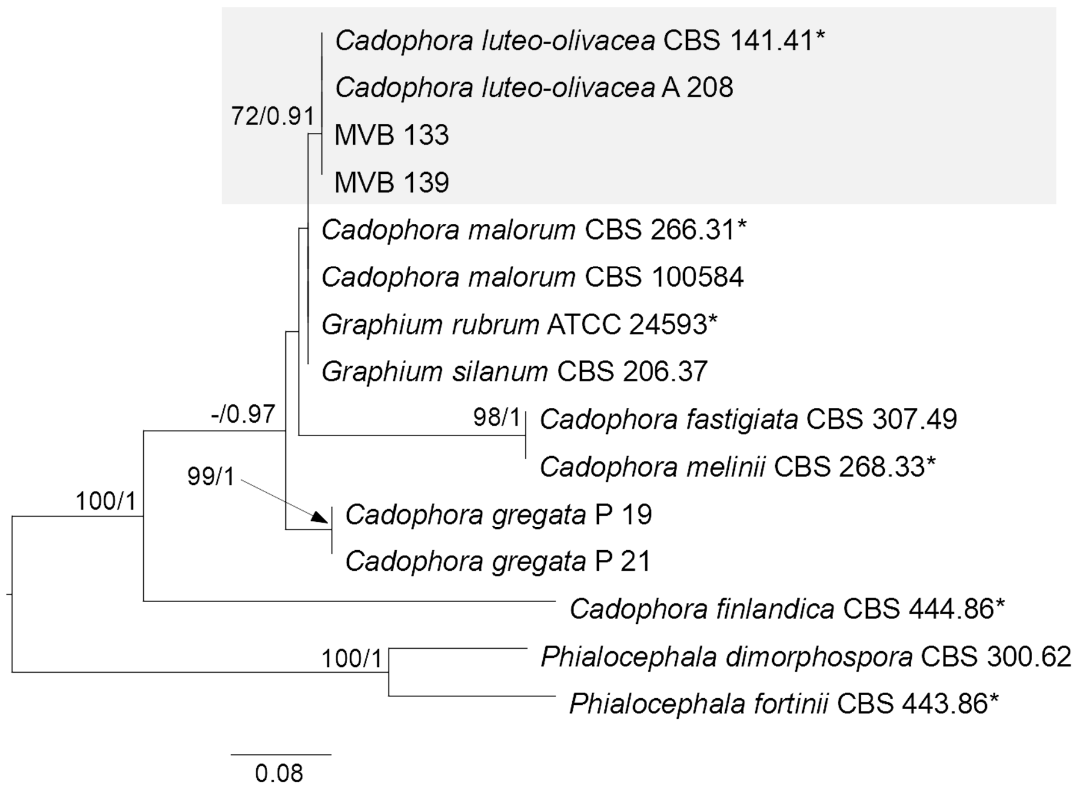

Figure 2. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of *Cadophora* and *Graphium*. The species-level clade comprising *Cadophora luteo-olivacea* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

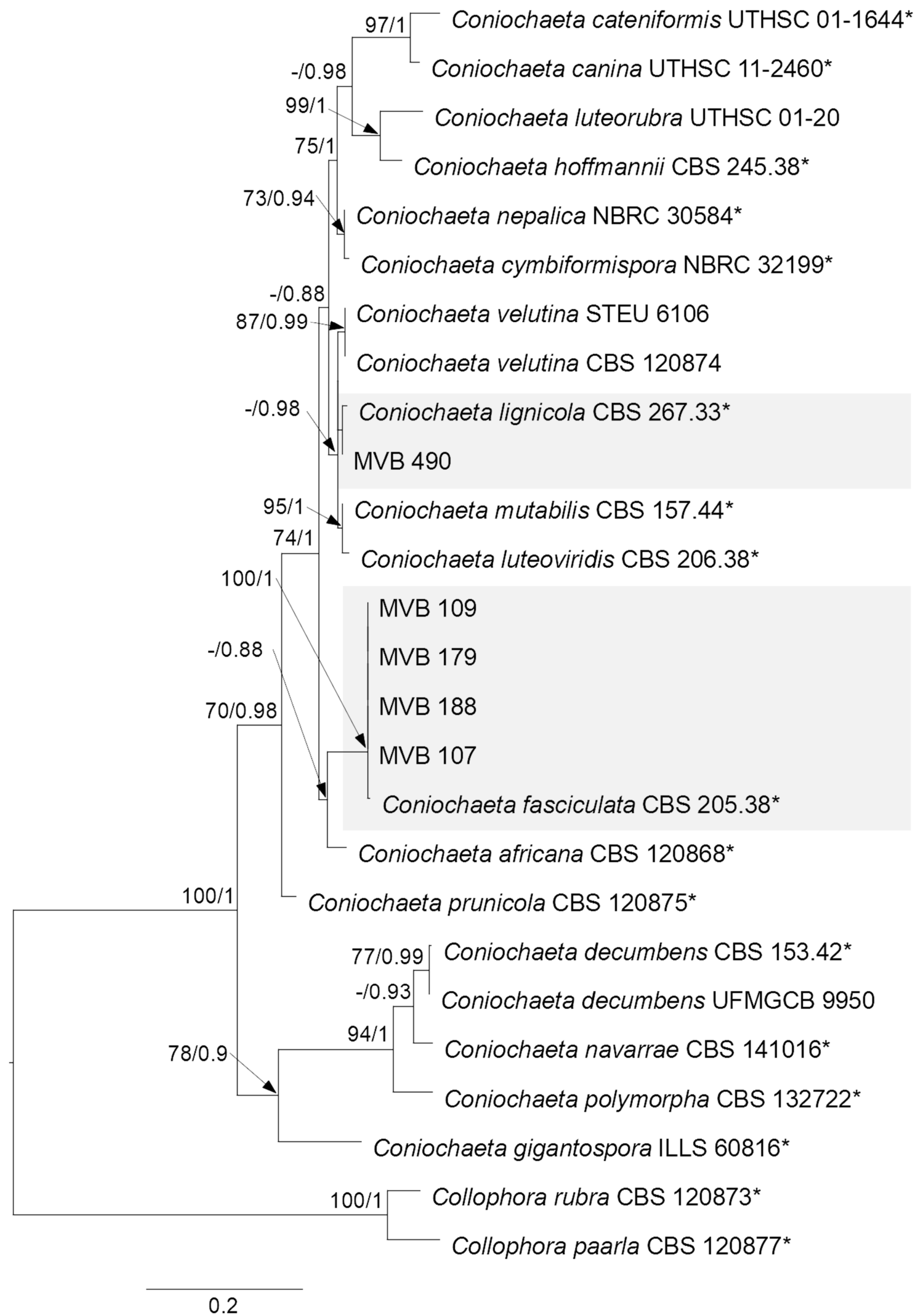

Figure 3. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of *Coniochaeta*. The species-level clades comprising *Coniochaeta lignicola* and *Coniochaeta fasciculata* are highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

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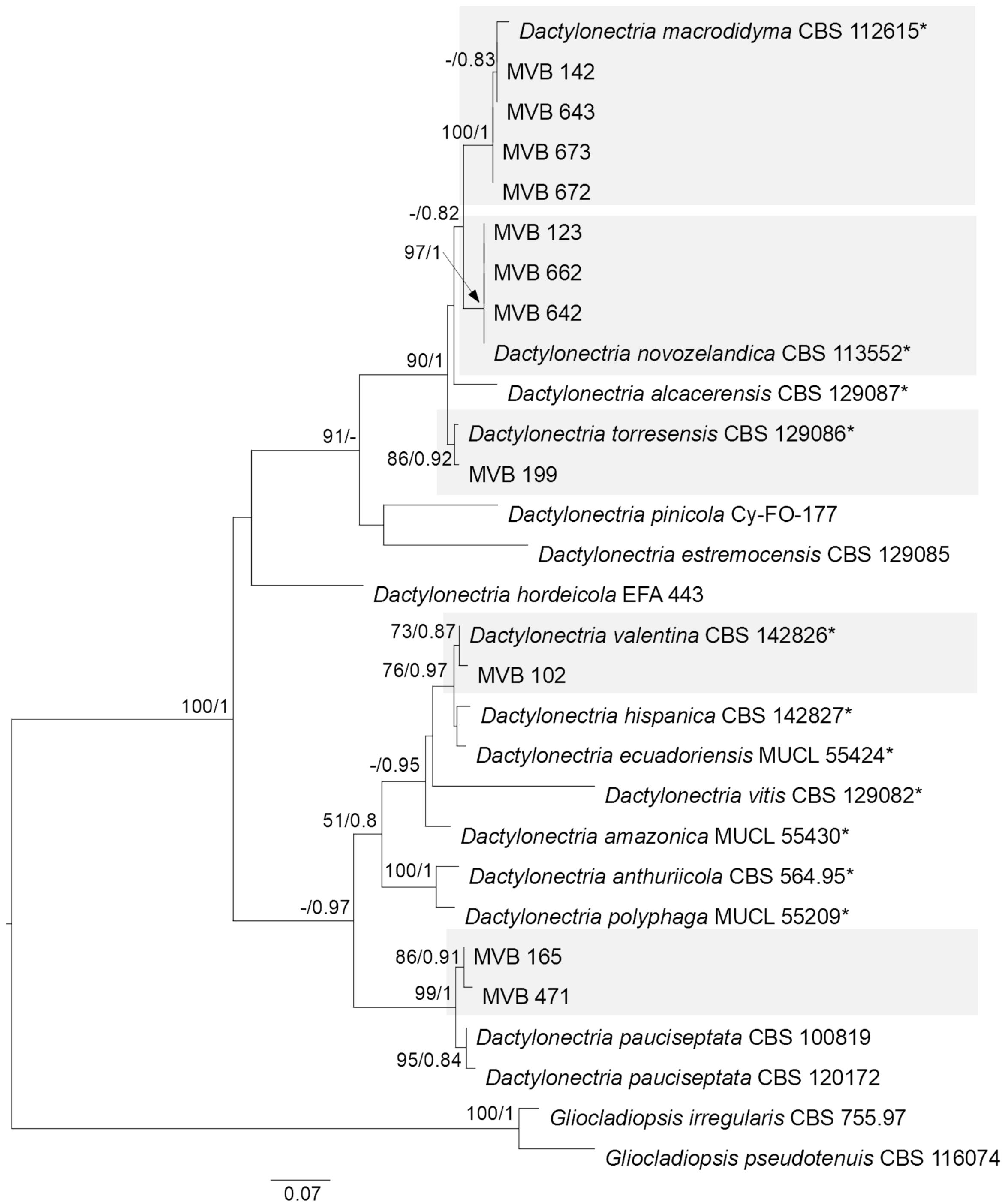

Figure 4. Maximum likelihood phylogeny of the histone (H3) region of *Dactylonectria*. The species-level clades comprising *D. macrodidyma*, *D. novozelandica*, *D. torresensis*, *D. valentina* and one potentially undescribed *Dactylonectria* sp. are highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

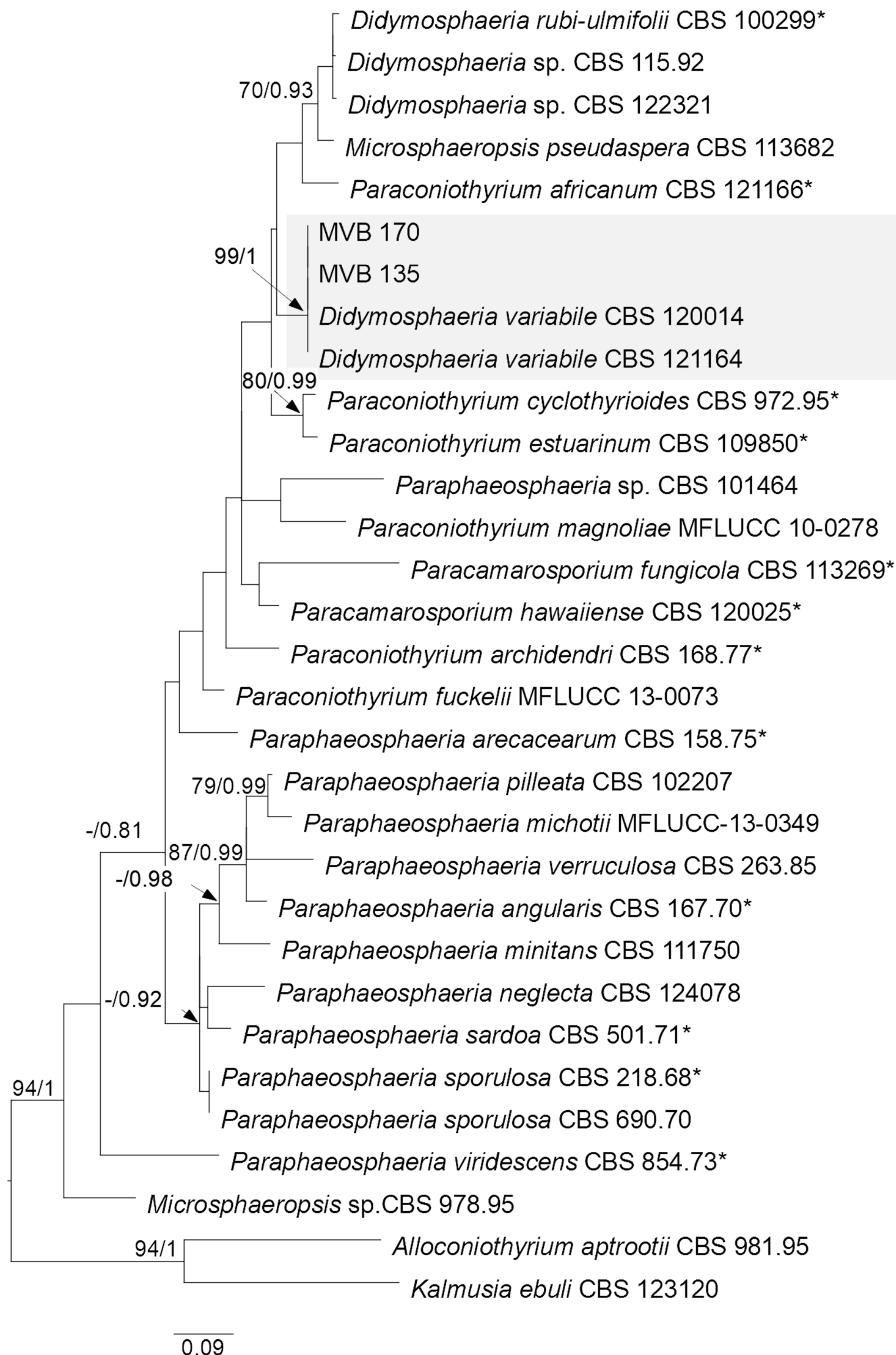

Figure 5. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of Didymosphaeriaceae. The species-level clade comprising *Didymosphaeria variabile* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

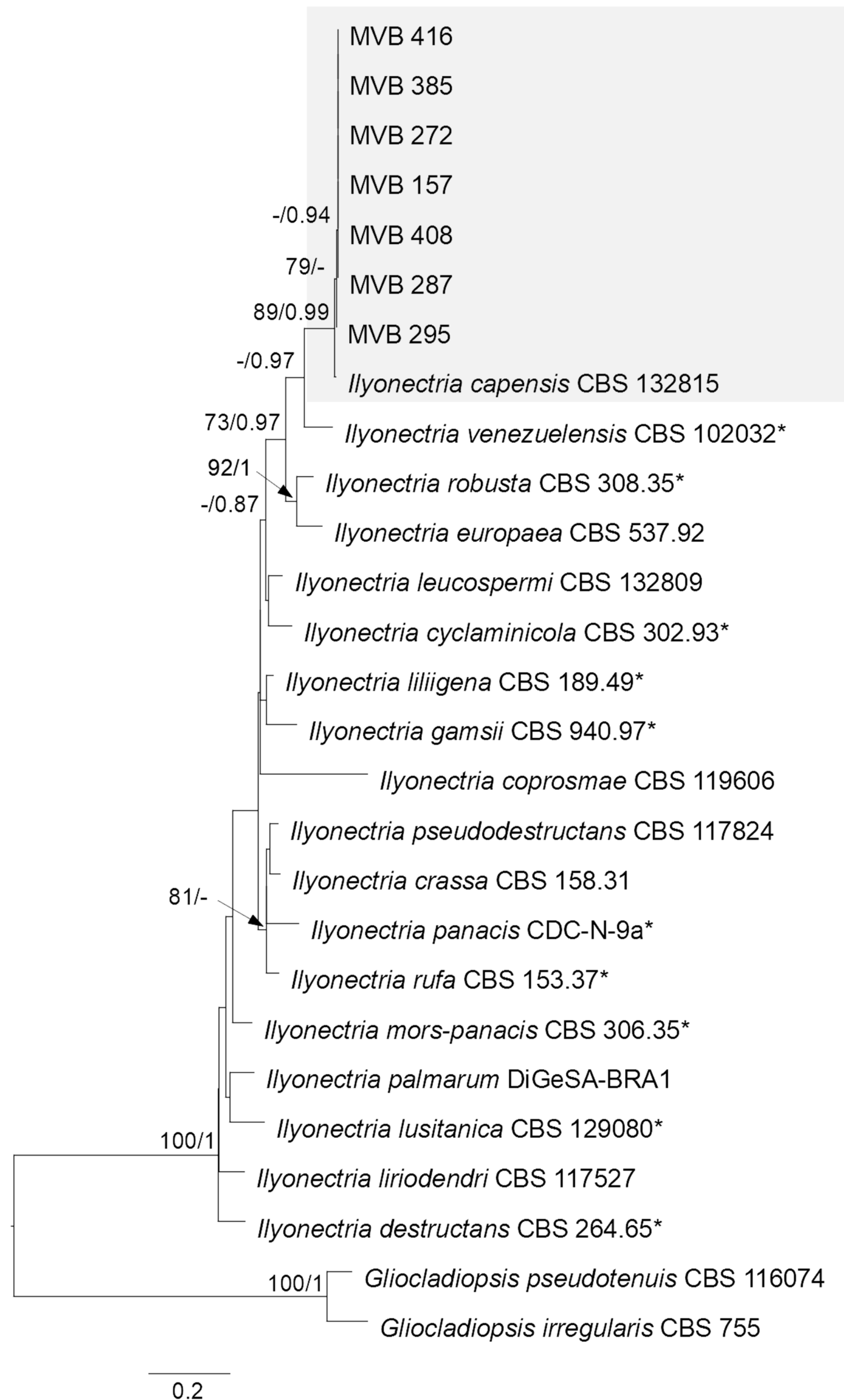

Figure 6. Maximum likelihood phylogeny of the histone (H3) region of *Ilyonectria*. The species-level clade comprising *I. capensis* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

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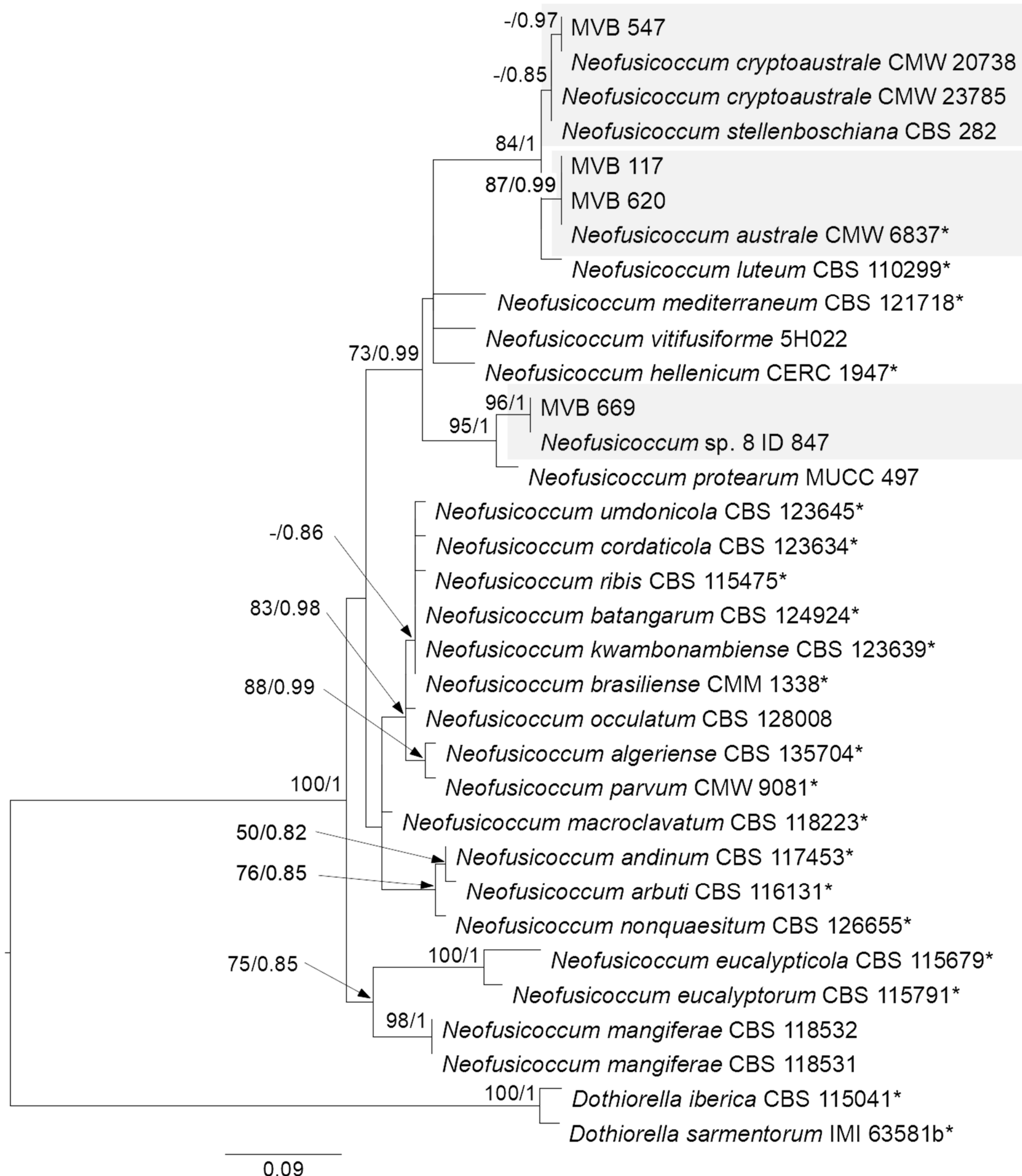

Figure 7. Maximum likelihood phylogeny inferred from sequences of the translation elongation factor 1 alpha (EF) region of *Neofusicoccum*. The species-level clades comprising *N. cryptoaustrale*/*stellenboschiana*, *N. australe* and *Neofusicoccum* sp. 8 are highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

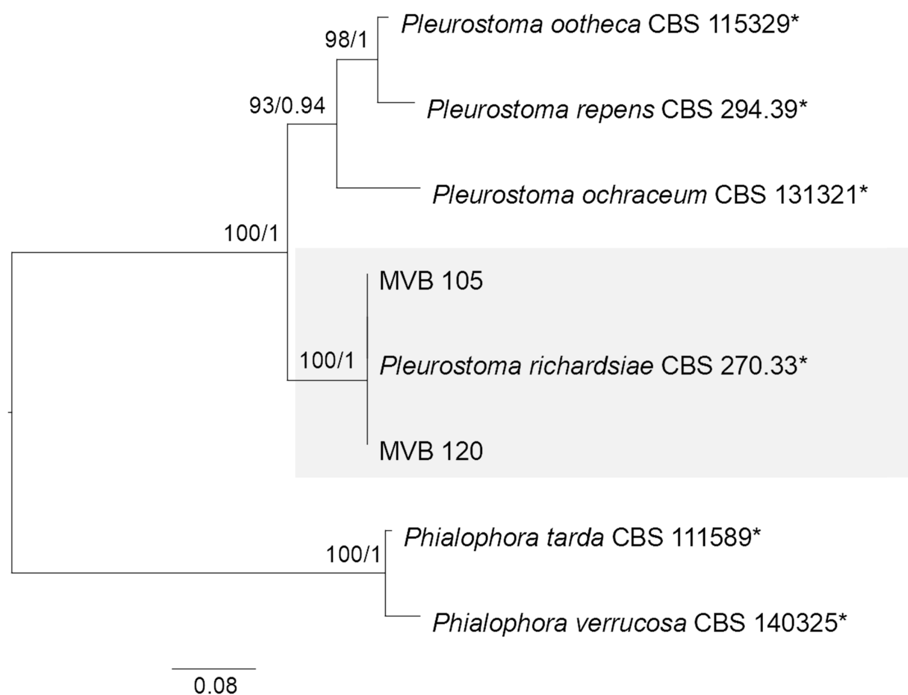

Figure 8. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of *Pleurostoma*. The species-level clade comprised of *P. richardsiae* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values less than 70% bootstrap or 0.80 posterior probability are not shown. Type and ex-type strains are notated with an asterisk (*).

Figure 9. Maximum likelihood phylogeny of the beta-tubulin (BTUB) region of *Phaeoacremonium*. The species-level clade comprising *P. parasiticum* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not included or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

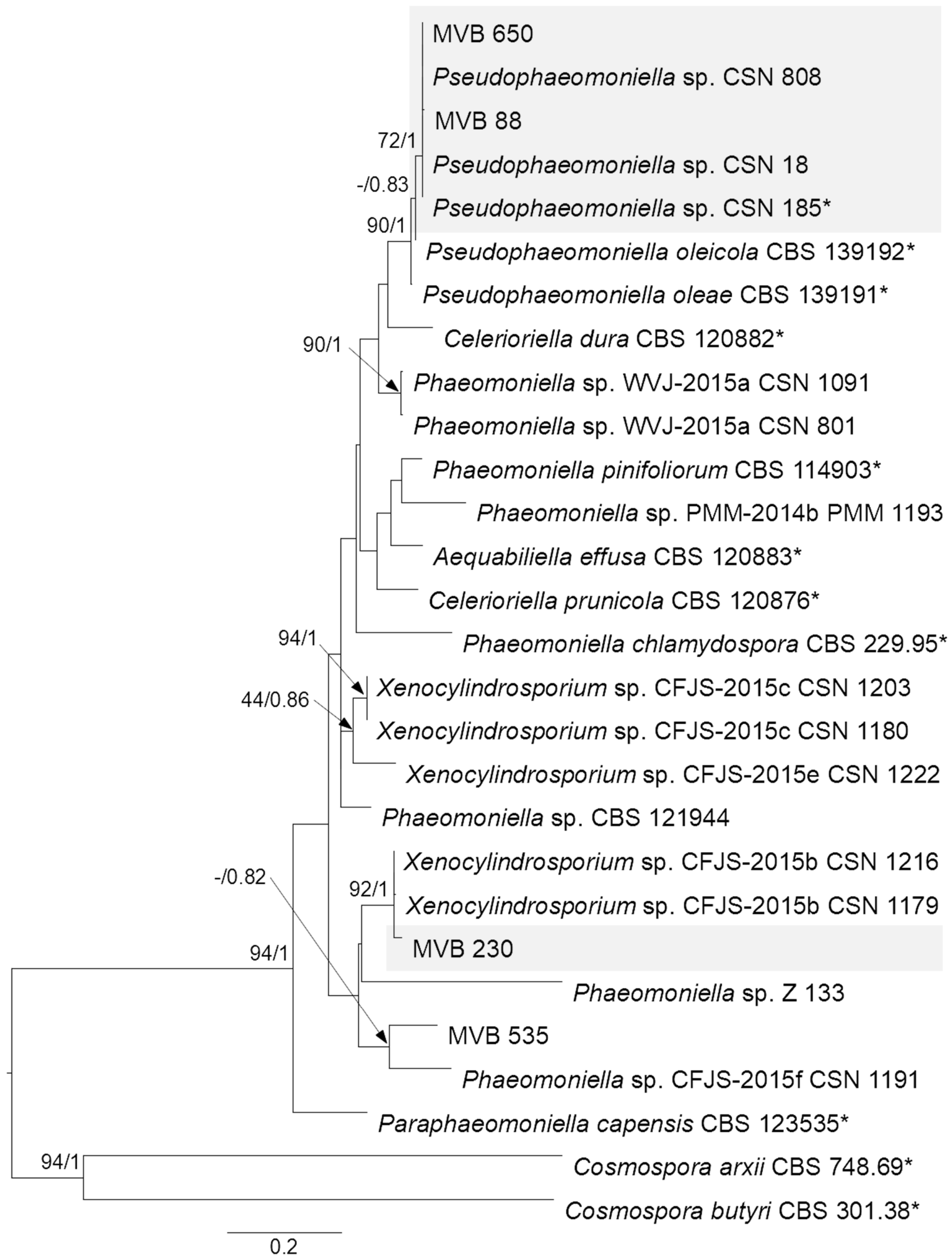

Figure 10. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of *Phaeomoniellales*. The species-level clades comprising *Pseudophaeomoniella* sp. and an undescribed *Phaeomoniella* sp. are highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

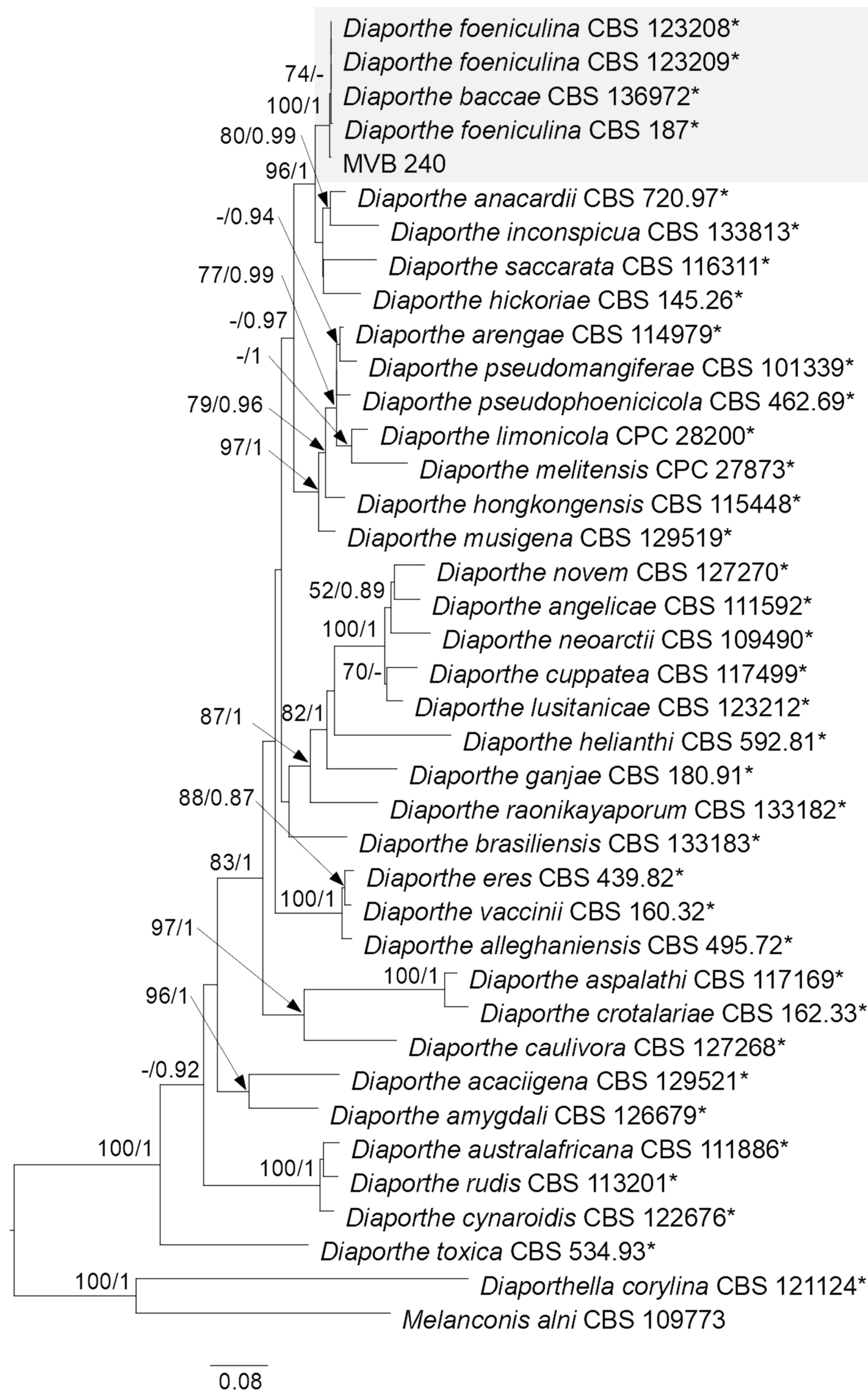

Figure 11. Maximum likelihood of the beta-tubulin (BTUB) region of *Diaporthe*. The species-level clade comprising *D. foeniculina*/*baccae* is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

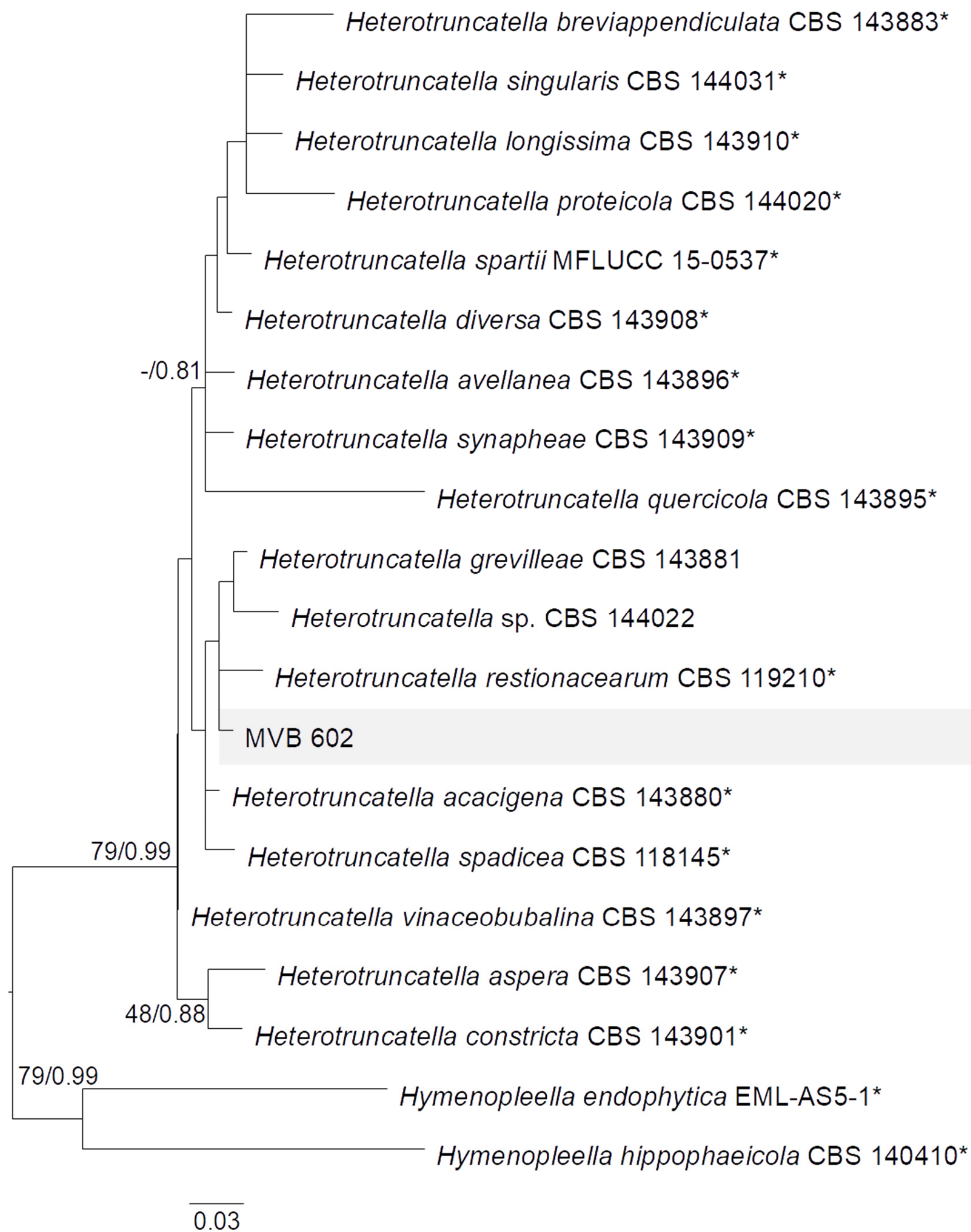

Figure 12. Maximum likelihood phylogeny inferred from sequences of the internal transcribed spacer (ITS) region of *Heterotruncatella*. The representative isolate of *Heterotruncatella* sp. found in the olive nursery material is highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

Figure 13. Symptoms of the internal wood of olive plants during Stage 2 and Stage 3. (A–B) The top margin of Corantina Stage 3 plants of Nursery B. (A) A healthy top margin. (B) Dieback of the top margin from which *Pseudophaeomoniella* was isolated. (C–E) The below soil level of Arbequina Stage 3 plants. (C) A healthy bottom margin. (D) Discolouration of the pith of the foot. (E) Discolouration of the pith as well as the cortex. Discolouration of the pith extends past the foot into the upper parts of the below soil level of the plant (indicated by arrows). *Pleurostoma richardsiae* was detected in the below ground parts of (D–E). (F) *Neofusicoccum crypto-australe* was detected in the below grounds parts of a Frantoio Stage 2 plant with extensive discolouration of the pith and cortex of the foot.

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Chapter 4

Detection of *Pseudophaeomoniella* sp., an olive trunk pathogen, on olive pruning debris

ABSTRACT

Pseudophaeomoniella sp. is an important olive trunk pathogen. However, little is known regarding the biology and epidemiology of this pathogen. The aim of this study was to investigate whether the pruning debris in established olive orchards sustain *Pseudophaeomoniella* sp. fruiting bodies and to determine whether the pruning debris can be considered inoculum sources of this pathogen within established orchards. A nested species-specific PCR was developed for the detection of this pathogen on 138 samples of pruning debris collected from Paarl (40 wood pieces), Stellenbosch (42 wood pieces) and Worcester (56 pieces), Western Cape Province, South Africa. Spore washes were made from the samples (5–10 cm in length), after which the nested species-specific primers were used to determine the presence of *Pseudophaeomoniella* sp. on the wood. *Pseudophaeomoniella* sp. was detected on 37.5% of the pruning debris collected from Paarl, 61.9% from Stellenbosch and 39.3% from Worcester. The pruning debris that tested positive for *Pseudophaeomoniella* sp., based on the species-specific PCRs, were evaluated visually by microscopic observations for *Pseudophaeomoniella* sp. pycnidia. Dark brown to black pycnidia were found. Conidia from these pycnidia were measured, cultured and confirmed as *Pseudophaeomoniella* sp. by sequencing the internal transcribed spacer (ITS) region. During this study, the pruning debris, in established olive orchards, were identified as inoculum sources of *Pseudophaeomoniella* sp. This emphasises the importance of orchard sanitation, to reduce the inoculum sources of this pathogen within the orchards, in order to control olive trunk diseases in these orchards.

INTRODUCTION

Olive trunk pathogens cause dieback of the twigs and branches, which can lead to a reduced fruit bearing capacity, lower fruit quality and a decrease in the lifespan of olive trees. These diseases can be managed within established orchards during pruning, by removing the dead branches and branches displaying dieback symptoms (Costa, 2019), after which pruning wound protectants can be applied to large pruning wounds directly after pruning to avoid new infections of the freshly made pruning wounds (Costa 1998). On some commercial olive farms in the Western Cape Province, large branches removed from the trees during pruning are used as firewood by the general farm workers, while the remaining pieces are shredded. Burning of pruning debris is expected to lower the inoculum source of trunk pathogens in the

orchards. However, shredding may not necessarily improve the inoculum status within the olive orchards (Moral *et al*., 2019). Shredded pruning debris can act as a substrate sustaining microbial communities, including olive trunk pathogens (Damm *et al.*, 2007; van Niekerk *et al*., 2010; Baloyi *et al*., 2016; Moral *et al*., 2019).

No studies regarding olive pruning debris as an inoculum source of olive trunk pathogens has been published. However, in vineyards, grapevine trunk pathogens are known to multiply on pruning debris and other dead wood (van Niekerk *et al*., 2010; Gramaje and Armengol, 2011). Pycnidia of *Phaeomoniella chlamydospora* was found in the cracks and crevices and on the surface of cordons, trunks and pruning wounds on 35-year-old 'Pinotage' grapevines in Stellenbosch (Baloyi *et al*., 2016). The release and the quantity of conidia released from fruiting bodies of trunk pathogens appears to be influenced by weather conditions prior to and during these events (Eskalen and Gubler, 2001; van Niekerk *et al*., 2010). Conidia of *P. chlamydospora* and *Phaeoacremonium inflatipes* were released during and after rainfall events during late-winter and early-spring in California (Eskalen and Gubler, 2001). During this study by Eskalen and Gubler (2001), only *Phaeoacremonium minimum*-conidia release was not associated with rainfall. Similarly, conidia of *Eutypa lata*, *Diaporthe* spp. and Botryosphaeriaceae spp. were also released during or after rainfall and/or high relative humidity in South Africa (van Niekerk *et al*., 2010). However, in South Africa, spore release by *P. chlamydospora* and *Phaeoacremonium* spp. was not correlated with rainfall or relative humidity in the study performed by Baloyi (2016). After release, the conidia of trunk pathogens can be dispersed via various means such as through the movement of water (e.g. rain) and wind (Ahimera *et al*., 2004; van Niekerk *et al*., 2010) and by internal or external dispersal of insects (Moyo *et al*., 2014). *Phaeomoniella chlamydospora*, *P. inflatipes* and *P. minimum* are considered airborne, but are also capable of being water-splashed onto wounds, including pruning wounds, where infection can take place (Eskalen and Gubler, 2001).

Pseudophaeomoniella sp. is the most prevalent olive trunk pathogen associated with olive trunk disease symptoms in the Western Cape Province, South Africa (van Jaarsveld, 2015; Chapter 2). Little is known regarding the epidemiology of *Pseudophaeomoniella* sp. This species was recently detected in olive nursery material at low incidences (Chapter 3). Additional inoculum sources of this pathogen is expected, considering its high incidence in olive trunk disease symptoms in the Western Cape Province. One of the sources of inoculum of *Pseudophaeomoniella* sp. in established olive orchards could be fruiting bodies on pruning debris. The aim of this study was therefore to establish whether pruning debris in established olive orchards can be considered as inoculum sources of this pathogen. A species-specific PCR screening technique was developed during this study for the detection of

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Pseudophaeomoniella sp. on olive pruning debris collected from olive orchards in the Western Cape. The samples that tested positive for the presence of *Pseudophaeomoniella* sp. by means of species-specific PCRs were evaluated further by microscopic observation.

MATERIALS AND METHODS

Molecular screening technique

Species-specific PCR primer development

The internal transcribed spacer (ITS) region of eight isolates of *Pseudophaeomoniella* sp. were aligned together with 23 closely related species (Table 1), using the MAFFT v7.2.2.2 (Katoh and Standley, 2013) plugin within Geneious® v9.1.7, to identify unique stretches of DNA that could be used as priming sites for *Pseudophaeomoniella* sp. The forward (ITS40; 5'-CCGACCTCCAACCCTTTGTT-3') and reverse (ITS525; 5'-GCATCTGGGGTCATTCGTGA-3') species-specific primers were identified using Geneious® v9.1.7. The species-specific PCR was optimised using a gradient PCR (at 55–65°C) with two isolates of the *Pseudophaeomoniella* sp. (STE-U 7947 = CSN 41 and STE-U 7950 = CSN 183) against isolates of *Pseudophaeomoniella oleae* (STE-U 7931), *Pseudophaeomoniella oleicola* (STE-U 7932) and *Pseudophaeomoniella* sp. AC (Carlucci *et al*., unpublished) (STE-U 7929). A 10 µL reaction was set up using 1 × Kapa, 0.08 µM of each primer pair (ITS40F-ITS525R) and 1 µL of DNA (10 ng/µl). The PCR conditions were at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55–65°C for 30 s and 72°C for 30 s, with a final step set at 72°C for 7 min.

The optimised species-specific PCR was validated by screening it against the DNA of 29 other fungal species associated with olive trees (Table 2). The integrity of the DNA of these species were evaluated first in a 10 µl PCR containing 1 × Kapa, 0.08 µM of each primer, ITS4 and ITS5 (White *et al*., 1990) and 1 µL of DNA (10 ng/µl). These reactions were placed under the following PCR conditions: 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final step set at 72°C for 7 min. The species-specific PCR primer pairs (ITS40F-ITS525R) were then validated using the optimal annealing temperature (64°C) against the 29 other species using the following PCR conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 64°C for 30 s and 72°C for 30 s, with a final step set at 72°C for 7 min. All PCR products were visualised together with Generuler™ 100bp plus DNA ladders on 1% agarose gels.

Sensitivity of the PCR for the detection of Pseudophaeomoniella *sp. conidia*

An isolate of *Pseudophaeomoniella* sp. (STE-U 7950 = CSN 183) was grown for 1–2 weeks at 25°C in Petri dishes containing Potato Dextrose Agar (PDA, Biolab, South Africa) amended

with chloromycetin (250 mg/L) (PDA-C). These PDA-C plates with fungal growth were flooded with 3 mL of autoclaved deionised water (dH_2O) and agitated lightly for approximately 30 s to suspend the conidia. Approximately 2 mL of the conidial spore suspension was transferred to clean 2-mL Eppendorf tubes. The concentration of this suspension was determined using a haemocytometer (Improved Neubauer, Germany). Both sides of the haemocytometer was loaded three times each with 250 000 $< x <$ 2500 000 conidia/mL, where x = the concentration of conidia. An overall average was calculated and the suspension diluted accordingly with dH_2O to obtain a dilution series of 20 000, 2000, 200 and 20 conidia/mL.

DNA was extracted from the conidial spore suspensions using a modified version of the protocol of Williams *et al.* (2001). The DNA extraction method was initiated by centrifuging 1 mL of the suspensions at 12 000 rpm. Most of the liquid of each tube was removed carefully by pipetting, while the remainder of the liquid in the tube evaporated at 50°C on a heating block. Glass beads were added to the tubes and were used to disrupt the conidia using a tissue lyser MM301 (Retsch, Germany) set at 30 mHz for 3 min. The tubes were then filled with 400 µL of sodium dodecyl sulfate (SDS) lysis buffer [50 mM Tris-HCl (pH 7.2), 50 mM EDTA, 3% SDS and 1% β-mecaptoethanol]. The samples were vortexed briefly, then incubated at 65°C for 1 hr. After the incubation period, 400 µL of chloroform:TE saturated [10 nM Tris-HCl (pH8.0), 1 mM EDTA] phenol (1:1, v:v) was added. The mixture was vortexed briefly before centrifuging at 12 000 rpm for 15 min at room temperature. Approximately 350 µL of the supernatant was transferred to clean 1.5 mL Eppendorf tubes, after which 35 µL of 3 M NaOAc, 1.44 µL of 20 mg/ml glycogen and 189 µL of isopropanol were added to the tubes. The mixture was inverted briefly before incubating for a second time at -20°C for 1 hr. The samples were centrifuged at 10 000 rpm for 15 min at room temperature and the supernatant discarded. The pellet was rinsed once with 189 µL cold 70% ethanol in a centrifugation step set at 10 000 rpm for 10 min. The pellets were air-dried and re-suspended in 20 µL of sterile dH_2O.

The species-specific primers were used in a nested PCR for improved detection of DNA extracted from *Pseudophaeomoniella* sp. conidial spore suspensions. The primary PCR was conducted using the universal ITS4-ITS5 primer pair (White *et al*., 1990). Taq DNA Polymerase Master Mix RED, 1.5 mM $MgCl_2$ (ampliqon) was used with 0.2 µM of both primers and 2 µL of the DNA in 20 µL reactions. The PCR conditions were set, according to Williams *et al.* (2001), at 95°C for 10 min, followed by 30 cycles of 94°C for 30 s, 42°C for 2 min and 72°C for 2 min, with a final step of 72°C for 7 min. Secondary PCRs were performed using the *Pseudophaeomoniella* sp. species-specific primer pair (ITS40F-ITS525R) with 1 µL of a 100× dilution of the primary PCR products as template in 10 µL reactions. Reagents and cycling

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conditions corresponded to those used during specificity testing. The PCR products were visualised together with a Generuler™ 100bp DNA ladder on 1% agarose gels. The entire process, from DNA extraction to primary and secondary PCRs, were repeated seven times.

Detection in olive orchards

Sampling and sample preparation

The olive pruning debris were collected from well-maintained olive orchards during October 2018 from Paarl, Stellenbosch and Worcester in the Western Cape Province (Table 3). The pruning debris were collected from the orchard floor of two to three orchards of four farms. The pruning debris of each orchard were placed into separate paper bags and transported to the laboratory. Within 48 hrs, the pruning debris were washed under tap water to remove excess dirt. The pruning debris were cut to 5–10 cm in length such that one to 20 pieces were available per orchard (Table 3). These sections were placed individually in 50-mL centrifugation tubes filled with sterile dH_2O for 30 min, after which the samples were removed from the water and allowed to dry before storing in brown paper bags. The dH_2O level of the tubes were adjusted to 50 mL, after removing the pruning debris, before centrifuging the tubes at 4000 rpm for 10 min at 3°C. The supernatant was discarded carefully. The remaining liquid suspension (or 'spore wash') was transferred to clean 1-mL Eppendorf tubes. These tubes were centrifuged at 12 000 rpm for 10 min and the remaining liquid discarded.

Molecular detection

The DNA was extracted from these spore washes using the same DNA extraction protocol described in the above section that was adapted from Williams *et al.* (2001). The DNA was subjected to nested PCRs using the species-specific primer pair (ITS40F-ITS525R) as described previously.

Microscopic detection

The pruning debris of spore washes that tested positive for *Pseudophaeomoniella* sp. were selected for microscopic observations. The samples were first viewed under a dissecting microscope (Leica MZ95) for pycnidia resembling those described by Spies *et al.* (unpublished). The pycnidia were transferred from the pruning debris to dH_2O on microscopic slides, using a sterile 21-gauge syringe needle (avacare™). The samples were squashed to release conidia embedded in the pycnidia. The conidia were viewed using a Nikon Eclipse E600 light microscope and photographed at 1000× magnification using a Nikon DS-Ri2 camera. The dimensions of 20–31 conidia were measured using NIS-Elements Viewer software (Nikon Instruments Inc.) at 1000x magnification.

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Verification

The conidial spore suspension of the microscopic slides (5–10 µL) resembling the *Pseudophaeomoniella* sp. were transferred to 2-mL Eppendorf tubes. A further 2 mL of sterile dH_2O was added to the suspension. The suspension was vortex before transferring 650 µL of the suspension to three Petri dishes containing PDA-C. The Petri dishes were incubated at 25°C and monitored for growth regularly. Hyphal tipping was performed to make pure cultures of the fungal isolates resembling the *Pseudophaeomoniella* sp. The DNA of these isolates were extracted using a simplified version of the protocol by Wang *et al*. (1993). In all tubes containing *Pseudophaeomoniella* mycelia, 200 µL of 0.5 M of NaOH was added. The rest of the protocol was followed as stated in Wang *et al*. (1993). The ITS region was then amplified and sequenced, and phylogenetic analyses were performed according to Chapter 3 for the Phaeomoniellales spp. using the GTR + I + G evolutionary model. The ungapped mean length of the sequence alignments were 354 bp.

RESULTS

Molecular screening technique

The optimal annealing temperature of the primer pair (ITS40F-ITS525R) for specificity to amplify DNA of *Pseudophaeomoniella* sp. was identified at 64°C. The *Pseudophaeomoniella* species-specific PCR formed a 406-bp product. The specificity of these reactions were tested further against the DNA of 29 fungal species, including the closely related species *P. oleae* and *P. oleicola*, of which the integrity of the DNA was also tested (Fig 1). The PCR gels indicated that the primer-pair was species-specific for *Pseudophaeomoniella* sp. DNA. The sensitivity of the species-specific PCR primer pair (ITS40F-ITS525R) was evaluated using a nested approach. The PCRs could detect 2000–20 000 conidia/mL in all reactions and 20–200 conidia/mL in 28.6% of the reactions (Table 4).

Detection in olive orchards

The species-specific primer (ITS40F-ITS525R) was able to detect *Pseudophaeomoniella* sp. on pruning debris collected in all geographic regions tested (Table 5). The *Pseudophaeomoniella* sp. was detected on 37.5% of the samples collected from Paarl, 61.9% of the samples collected from Stellenbosch and 39.3% of the samples collected from Worcester (Table 3). The pruning debris that gave positive PCR results were selected for microscopic observation. Fruiting bodies were observed on pruning debris from all locations and from all orchards that were tested positive with the species-specific assay, except for E(2), which had only four samples (Table 5). Dark brown to black pycnidia of the *Pseudophaeomoniella* sp. were found growing singularly and in clusters on the surface of wood where the bark was weathered off and under weathering bark, as well as in crevices of

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the wood (Table 5; Fig 2). The conidia of these pycnidia were smooth, hyaline, oblong and (2.5–)3.0–3.0(–3.5) × (1.0–)1.5–1.5(–2.0) µm in size (Table 6; Fig 2). The conidia of three of these pycnidia were cultured (Ep 1, Ep 2 and Ep 3) for ITS sequencing and phylogenetic analyses. The samples (Ep 1, Ep 2 and Ep 3) fell within the *Pseudophaeomoniella* sp. species-level clade with moderate to high support (78% maximum likelihood bootstrap and 0.86 Bayesian posterior probability) (Fig. 3). Furthermore, ITS sequences of these isolates matched with 99.7-100% of the base pairs of the representative *Pseudophaeomoniella* sp.

DISCUSSION

Pseudophaeomoniella sp. appears to be an important olive trunk pathogen occurring in South Africa (Chapter 2). It has only been reported in South Africa from disease symptoms in established olive orchards and wild olive trees (*Olea europaea* subsp. *cuspidata*) (Spies *et al*., unpublished). *Pseudophaeomoniella* sp. has been detected in olive nursery material (Chapter 3) at low incidences too, and it has now also been detected on pruning debris in established olive orchards. *Pseudophaeomoniella* sp. is known to form dark brown to black pycnidia on PDA, and on pine needles place on synthetic nutrient agar (Spies *et al*., unpublished). The current study, is the first report of pycnidia of this pathogen occurring in nature. The pycnidia occurred singly and in clusters on the surface of wood where the bark had been weathered off and under weathering bark, as well as in crevices of olive pruning debris collected from established olive orchards in Paarl, Stellenbosch and Worcester. Most of the pruning debris was collected from older orchards (17–65-year-old orchards). The younger orchards, such as the young mother blocks (4-years-old orchards), had only a few pieces of pruning debris available and *Pseudophaeomoniella* sp. was not detected on any of those samples. *Pseudophaeomoniella* sp. was detected the most frequently on pruning debris collected from Stellenbosch (61.9% of the samples), coinciding with the a generally higher humidity found in Stellenbosch compared to Paarl and Worcester.

The spore release and the dispersal mechanisms of *Pseudophaeomoniella* sp. within olive orchards are unknown, but it is expected to be similar to that of other Phaeomoniellales spp. The release of *P. chlamydospora* conidia, and of several other trunk pathogens, in grapevine vineyards typically increase during and after rainfall events (Eskalen and Gubler, 2001; van Niekerk *et al*., 2010). These spores can be dispersed by wind, rain and insects (Ahimera *et al*., 2004; van Niekerk *et al*., 2010; Moyo *et al*., 2014). The spores that settle on fresh pruning wounds can infect the susceptible woody tissues to cause trunk diseases (Ramos *et al*., 1975; Chapuis *et al*., 1998; Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011; Baloyi *et al*., 2016; Chen *et al*., 2016). Composting and chemical treatments of pruning debris can be used to manage inoculum sources of trunk

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pathogens within established plantations, although these methods have not been tested specifically for olive wood, and no studies have been performed specifically to manage *Pseudophaeomoniella* sp. populations. *Diplodia seriata* (=*Botryosphaeria obtusa*), *P. chlamydospora*, *P. minimum* and *E. lata* was eradicated from infected grapevine wood by a composting technique performed by Lecomte *et al*. (2006), while chemical treatments of the pruning debris with benomyl, fluazinam, mancozeb and 8-hydroxyquinoline sulphate was able to reduce the viability of *Diaporthe neoviticola* (=*Phomopsis viticola*) pycnidia (Castillo-Pando *et al*., 1997). These chemicals, including chemicals currently being used by olive producers, such as mancozeb to control anthracnose, can be tested in South Africa to determine the effect against the viability of *Pseudophaeomoniella* sp. pycnidia.

Olive trunk diseases in established orchards are managed in South Africa by removing the diseased branches and dead wood from infected trees and by applying pruning wound protectants to large freshly made pruning wounds (Costa 1998). The large branches removed during pruning are burned, inevitably also reducing the inoculum sources of trunk pathogens, including *Pseudophaeomoniella* sp., while the smaller branches are shredded and used as mulch in olive orchards. However, during this study it was shown that shredded branches can sustain *Pseudophaeomoniella* sp. populations and therefore act as an inoculum source of this pathogen within established orchards. These findings emphasise the importance of orchard sanitation for the management of olive trunk diseases. Composting and chemical treatment of pruning debris, while improving the soil microbiome, can be studied further, to reduce the inoculum sources of trunk pathogens in established orchards. Little is known regarding the spore release and dispersal mechanisms of *Pseudophaeomoniella* sp. However, it is expected that spore release is higher during and after rainfall and that these conidia can be dispersed by various mechanisms onto pruning wounds, where infection of freshly made pruning wounds can occur.

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TABLES AND FIGURES

Table 1. Fungal species aligned with *Pseudophaeomoniella* sp. for the development of the species-specific primer pair.

Species	Strain[a]	Accession	Host	Origin
Aequabiliella effusa	CBS 120883 = STE-U 6121*	GQ154598	*Prunus salicina*	South Africa
Celerioriella dura	CBS 120882 = STE-U 6122*	GQ154597	*P. salicina*	South Africa
Celerioriella prunicola	CBS 120876 = STE-U 6118*	GQ154590	*P. salicina*	South Africa
	CSN 1089	n/a[b]	*Prunus domestica*	South Africa
Celerioriella sp. CFJS-2015a	CSN 171 = STE-U 7949	n/a	*Schinus molle*	South Africa
Dolabra nepheliae	AR4872 = BPI 882442	JQ004281	*Nephelium lappaceum*	Honduras
	AR4873 = BPI 882443	JQ004280	*Nephelium mutabile*	Honduras
	CBS123297 = BPI 878188 = AR 4421	GU345749	*Litchi chinensis*	Puerto Rico
	P11-1-1	JX566449	*Lansium domesticum*	Hawaii
Minutiella tardicola	CBS 121757 = STE-U 6123*	GQ154599	*Prunus armeniaca*	South Africa
Moristroma quercinum	BN 1678	AY254051	*Quercus. robur*	Sweden
Moristroma sp.	CSN 1124 = STE-U 7967	n/a	*P. domestica*	South Africa
Neophaeomoniella eucalypti	CBS 139919 = CPC 25161	KR476749	*Eucalyptus globulus*	USA
Neophaeomoniella niveniae	CBS 131316 = CPC 18231*	JQ044435	*Nivenia stokoei*	South Africa
	CSN 742	n/a	*Olea europaea* subsp. *cuspidata*	South Africa
Neophaeomoniella zymoides	CBS 114905 = SFC AW203	DQ270241	*Pinus densiflora*	Korea
Neophaeomoniella zymoides (cont.)	CBS 114904 = SFC AW304*	DQ270242	*P. densiflora*	Korea
	CBS 121168 = STE-U 6120	GQ154600	*P. salicina*	South Africa
	SFC CW 302	DQ270247	*P. densiflora*	Korea
	CSN 743	n/a	*O. europaea* subsp. *cuspidata*	South Africa
Paraphaeomoniella capensis	CBS 123535 = CPC 15416*	FJ372391	*Encephalartos altensteinii*	South Africa
Phaeomoniella chlamydospora	CBS 239.74	AB278179	*Vitis vinifera*	USA
	CBS 117179	KF764544	*V. vinifera*	South Africa
Phaeomoniella pinifoliorum	CBS 114903 = SFC CW 202*	DQ270240	*P. densiflora*	Korea
Phaeomoniella sp. PMM-2014b	PMM 1193 = STE-U 7969	n/a	*O. europaea* subsp. *europaea*	South Africa
Phaeomoniella sp. PMM-2014c	CSN 157 = STE-U 7948	n/a	*Q. suber*	South Africa
	PMM 2666 = STE-U 7970	n/a	*Q. suber*	South Africa
Phaeomoniella sp. WVJ-2015a	CSN 801	n/a	*O. europaea* subsp. *cuspidata*	South Africa

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Species	Strain[a]	Accession	Host	Origin
	CSN 1091	n/a	*O. europaea* subsp. *europaea*	South Africa
Pseudophaeomoniella oleae	CBS 139191 = FV 84*	KP635972	*O. europaea* subsp. *europaea*	Italy
Pseudophaeomoniella oleicola	CBS 139192 = M 24*	KP411807	*O. europaea* subsp. *europaea*	Italy
Pseudophaeomoniella sp.	CSN 18 = STE-U 7946	n/a	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 386 = STE-U 7956	n/a	*O. europaea* subsp. *europaea*	South Africa
	CSN 435 = STE-U 7957	n/a	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 451 = STE-U 7958	n/a	*O. europaea* subsp. *europaea*	South Africa
Pseudophaeomoniella sp. (cont.)	CSN 806 = STE-U 7962	n/a	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 808 = STE-U 7963	n/a	*O. europaea* subsp. *europaea*	South Africa
	CSN 824 = STE-U 7964	n/a	*O. europaea* subsp. *europaea*	South Africa
	PMM 1192 = STE-U 7968	n/a	*O. europaea* subsp. *europaea*	South Africa
Rhynchostroma proteae	CBS 112051*	NR132824	*Protea laurifolia*	South Africa
Strelitziana cliviae	CPC 19822*	NR111823	*Clivia miniata*	South Africa
Strelitziana malaysiana	CPC 24874*	KR476731	*Acacia mangium*	Malaysia
Xenocylindrosporium kirstenboschense	CBS 125545 = CPC 16311, 16312*	GU229890	*Encephalartos friderici-guilielmi*	South Africa

[a] BN, Botanical Institute, Göteborg University, Göteborg, Sweden; BPI, U.S. National Fungus Collections, USA; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CPC: Culture collection of Pedro Crous housed at CBS; CSN, collection of Chris Spies at ARC-Nietvoorbij, Stellenbosch, South Africa; PMM, collection of Providence Moyo at Stellenbosch University, Department of Plant Pathology, Stellenbosch; SFC, Seoul National University Fungus Collection, Seoul National University, Seoul, Korea; STE-U, fungal collection of Stellenbosch University, Department of Plant Pathology, Stellenbosch. Fungi denoted with an asterisks (*) are “Type” material.

[b] n/a = not available

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Table 2. Fungi commonly found in olive wood in South Africa used to validate the specificity of the *Pseudophaeomoniella* sp. species-specific PCR primers.

Species	Strains[a]	Host	Origin
Aureobasidium	CSN 295	*Olea europaea* subsp. *europaea*	South Africa
Biscogniauxia mediterranea	CSN 1052	*O. europaea* subsp. *europaea*	South Africa
Calosphaeria africana	PMM 2075	*O. europaea* subsp. *europaea*	South Africa
Coniochaeta velutina	CSN 615	*O. europaea* subsp. *europaea*	South Africa
Cytospora pruinosa	PMM 2025	*O. europaea* subsp. *europaea*	South Africa
Cytospora sp. WvJ-2015a	CSN 625	*O. europaea* subsp. *europaea*	South Africa
Diaporthe ambigua	PMM 2078	*O. europaea* subsp. *europaea*	South Africa
Diaporthe foeniculina	CSN 297	*O. europaea* subsp. *europaea*	South Africa
Diplodia seriata	ID 370	*O. europaea* subsp. *cuspidata*	South Africa
	PMM 2093	*O. europaea* subsp. *europaea*	South Africa
Eutypa lata	ID 318	*O. europaea* subsp. *cuspidata*	South Africa
Fomitiporella sp.	CSN 155	*Schinus molle*	South Africa
Neofusicoccum capensis	ID 396	*O. europaea* subsp. *europaea*	South Africa
	PMM 2091	*O. europaea* subsp. *europaea*	South Africa
	PMM 2090	*O. europaea* subsp. *europaea*	South Africa
Neofusicoccum stellenboschiana	CSN 179	*O. europaea* subsp. *cuspidata*	South Africa
Neofusicoccum vitifusiforme	ID 827	*O. europaea* subsp. *europaea*	South Africa
Neofusicoccum sp. 8	ID 847	*O. europaea* subsp. *europaea*	South Africa
Neophaeomoniella niveniae	CSN 742	*O. europaea* subsp. *cuspidata*	South Africa
Neophaeomoniella zymoides	CSN 743	*O. europaea* subsp. *cuspidata*	South Africa
Peniophora sp.	CSN 235	*Quercus suber*	South Africa
Phaeoacremonium africanum	CSN 946	*O. europaea* subsp. *europaea*	South Africa
Phaeoacremonium minimum	PMM 2073	*O. europaea* subsp. *europaea*	South Africa
Phaeoacremonium oleae	CBS 142701 = STE-U 8381 = CSN 403	*O. europaea* subsp. *cuspidata*	South Africa
Phaeoacremonium parasiticum	CSN 476	*O. europaea* subsp. *europaea*	South Africa
Phaeomoniella sp. PMM2014b	PMM 1193 = STE-U 7969	*O. europaea* subsp. *europaea*	South Africa
Phaeomoniella sp. WVJ-2015a	CSN 801	*O. europaea* subsp. *cuspidata*	South Africa
Phoma caloplacae	CSN 588	*O. europaea* subsp. *europaea*	South Africa
Pleurostoma richardsiae	CSN 144	*O. europaea* subsp. *europaea*	South Africa
Pseudophaeomoniella oleae	STE-U 7931	*O. europaea* subsp. *europaea*	South Africa
Pseudophaeomoniella oleicola	STE-U 7932	*O. europaea* subsp. *europaea*	South Africa

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Species	Strains[a]	Host	Origin
Pseudophaeomoniella sp. AC	STE-U 7929	*O. europaea* subsp. *europaea*	South Africa
Pseudophaeomoniella sp.	CSN 18 = STE-U 7946	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 41	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 183	*O. europaea* subsp. *cuspidata*	South Africa
	CSN 185	*O. europaea* subsp. *europaea*	South Africa
	CSN 186	*O. europaea* subsp. *europaea*	South Africa
	CSN 314	*O. europaea* subsp. *europaea*	South Africa
Punctularia sp.	CSN 1060	*O. europaea* subsp. *cuspidata*	South Africa
Schizophyllum commune	PMM 2087	*O. europaea* subsp. *europaea*	South Africa
Trametes versicolor	CSN 1058	*O. europaea* subsp. *europaea*	South Africa

[a] CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CSN, collection of Chris Spies at ARC-Nietvoorbij, Stellenbosch, South Africa; ID, collection of Ihan du Plessis at ARC- Nietvoorbij, Stellenbosch; PMM, collection of Providence Moyo at Stellenbosch University, Department of Plant Pathology, Stellenbosch; STE-U, fungal collection of Stellenbosch University, Department of Plant Pathology, Stellenbosch.

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Table 3. The olive orchards from which olive pruning debris were collected in the Western Cape Province, South Africa.

Area	Farm (block)	Type of orchard	Cultivar(s)[a]	Age	Sampling date
Paarl	A (1)	Production	Mission, Frantoio and Manzanella	65 years	05-Oct-18
Paarl	A (2)	Production	Mission, Frantoio and Manzanella	65 years	09-Oct-18
Stellenbosch	C (1)	Production	Mission and Frantoio	18 years	12-Oct-18
Stellenbosch	C (2)	Production	Leccino and Frantoio	18 years	12-Oct-18
Stellenbosch	D (1)	Mother block	Frantoio	4 years	11-Oct-18
Stellenbosch	D (2)	Mother block	Corantina	4 years	11-Oct-18
Worcester	E (1)	Mother block and production	Frantoio	17 years	08-Oct-18
Worcester	E (2)	Mother block and production	FS17 and Mission	17 years	08-Oct-18
Worcester	E (3)	Mother block and production	Norcellara	17 years	08-Oct-18

[a] Single blocks were planted with one or more cultivar(s).

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Table 4. The sensitivity of the nested species-specific PCR of the ITS40F-ITS525R primer pair for the detection of *Pseudophaeomoniella* sp.

Conidial suspension (conidia/mL)	PCR (conidia/20µL)[a]	Positive PCRs (%)[b]
2×10^4	2×10^3	100.0 (7/7)
2×10^3	2×10^2	100.0 (7/7)
2×10^2	2×10^1	28.6 (2/7)
2×10^1	2×10^0	28.6 (2/7)
0	0	0.0 (0/7)

[a] Amount of DNA used in the PCR detection assay is expressed as the estimated number of conidia DNA added to the reaction.

[b] The percentage of positive PCRs out of a total of seven replicates. The set of values in parentheses are the number of experiments with a positive result per total number of experiments. Each replication was of the entire experiment, including the DNA extractions.

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Table 5. The incidence of *Pseudophaeomoniella* sp. on olive pruning debris collected from established olive orchards in the Western Cape Province, South Africa.

				Number of pycnidia				Position of pycnidia (Number)[c]		
Area [Farm (block)]	Number of samples	Positive PCR[a]	Samples with pycnida[b]	Single	In clusters (cluster size)	Total	Number of cluster	Surface	Under Bark	Crevices
Paarl	40	15 (37.5%)								
A (1)	20	7 (35.0%)	3	34	37 (2–7)	71	11	50	0	21
A (2)	20	8 (40.0%)	3	2	68 (2–26)	70	6	70	0	0
Stellenbosch	42	26 (61.9%)								
C (1)	20	15 (75.0%)	7	49	13 (2–4)	62	4	6	56	0
C (2)	17	11 (64.7%)	9	49	12 (2–8)	61	8	34	8	19
D (1)	4	0 (0.0%)	-	-	-	-	-	-	-	-
D (2)	1	0 (0.0%)	-	-	-	-	-	-	-	-
Worcester	56	22 (39.3%)								
E (1)	20	11 (55.0%)	4	11	-	11	0	9	0	2
E (2)	20	4 (20.0%)	0	0	-	0	0	0	0	0
E (3)	16	7 (43.8%)	4	19	-	19	0	0	10	9

[a] Number of samples tested positive by nested-PCR and the percentage thereof in parenthesis.

[b] Number of samples tested positive by nested-PCR on which pycnidia were observed.

[c] Pycnidia were found growing on either the surface of wood where the bark had been weathered off, under the weathered bark or in crevices in the wood.

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Table 6. *Pseudophaeomoniella* sp. conidia dimensions from pycnidia sampled from olive pruning debris collected from established orchards in the Western Cape Province, South Africa.

Pycnidia	**Nr. of conidia**[a]	**conidia dimensions (µm)**[b]
Ep 1	20	(2.5–)3.0–3.0(–3.5) × (1.0–)1.5–1.5(–1.5)
Ep 2	21	(3.0–)3.0–3.0(–3.5) × (1.5–)1.5–2.0(–2.0)
Ep 3	31	(2.5–)3.0–3.0(–3.0) × (1.0–)1.5–1.5(–2.0)
Average	72	(3.0–)3.0–3.0(–3.5) × (1.0–)1.5–1.5(–2.0)

[a] Number of conidia measured.

[b] Conidial dimensions falling within the 95% confidence range. Values in parenthesis are the minimum and maximum extremities.

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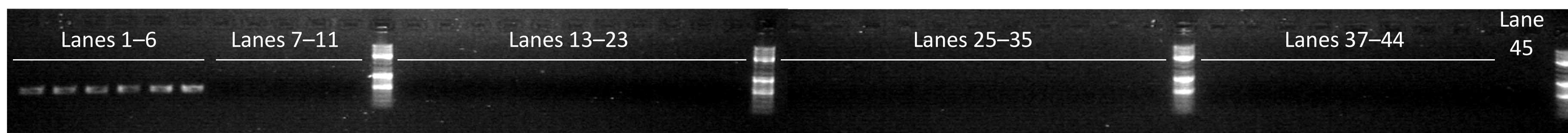

Figure 1. Validation of the optimised *Pseudophaeomoniella* sp. species-specific PCR primers. Lanes 1-6: DNA amplification of the *Pseudophaeomoniella* sp. (± 406 bp); Lanes 7–11, 13–23, 25–35, 37–44: No PCR products found for the remaining species tested; Lanes 12, 24, 36 and 46: DNA ladders; Lane 45: Non-template control.

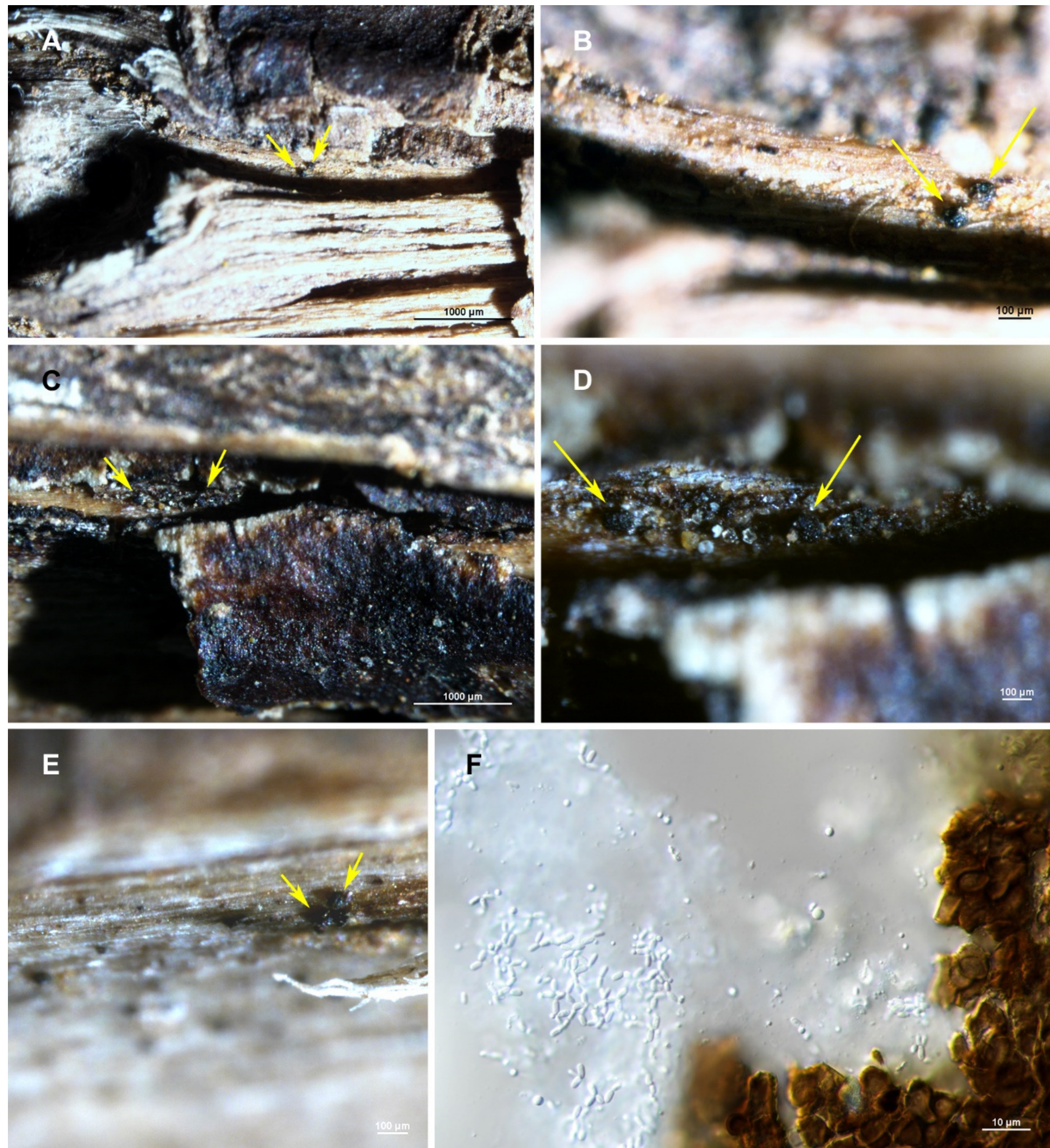

Figure 2. *Pseudophaeomoniella* sp. found growing on partially shredded olive pruning debris collected from established olive orchards in the Western Cape Province, South Africa. *Pseudophaeomoniella* sp. pycnidia (indicated by the arrows) growing (A-D) under the bark of olive pruning debris that has naturally lifted by weathering. (B and D) Close up images of “A” and “B”. (E) *Pseudophaeomoniella* sp. pycnidia on the surface of pruning debris, where the bark was naturally weathered off. (F) Pycnidia crushed open to expel conidia.

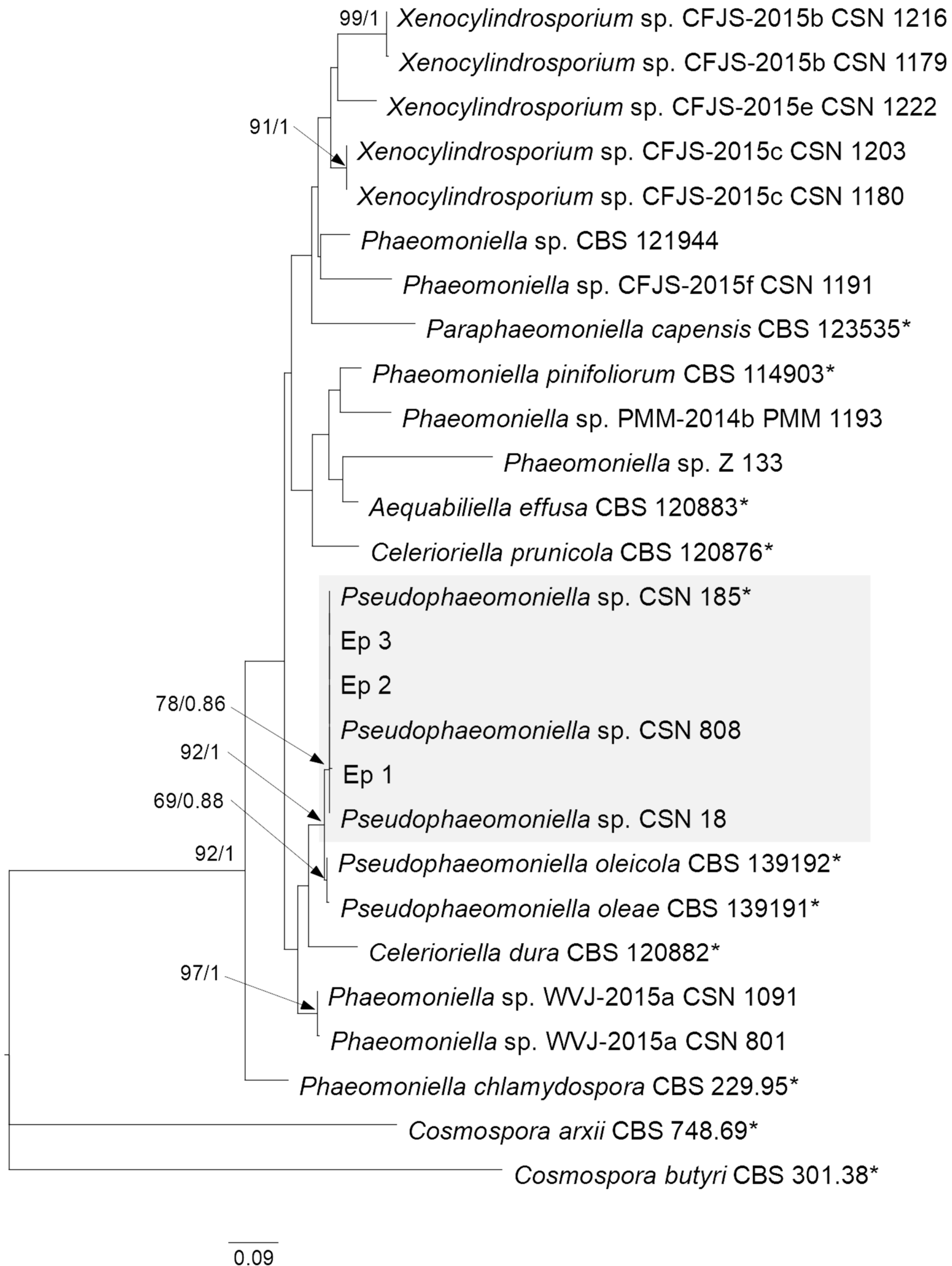

Figure 3. Maximum likelihood phylogeny of the internal transcribed spacer (ITS) region of the Phaeomoniellales. The species-level clades comprising *Pseudophaeomoniella* sp. are highlighted in grey. Maximum likelihood bootstrap percentages and Bayesian posterior probability values are indicated at the nodes. Support values of less than 70% bootstrap or 0.80 posterior probability are not shown or indicated with a dash (-). Type and ex-type strains are notated with an asterisk (*).

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Chapter 5

Olive pruning wound susceptibility to *Pseudophaeomoniella* sp. infections and the efficacy of potential pruning wound protectants

ABSTRACT

Olive pruning wound susceptibility to *Pseudophaeomoniella* sp., an important olive trunk pathogen, was tested, after which the efficacy of potential pruning wound protectants was evaluated. The pruning wound susceptibility trials were performed during winter and spring to determine the seasonal effects. Two-year-old olive branches of 16-year old olive trees were pruned and inoculated with conidial spore suspensions of *Pseudophaeomoniella* sp. 0, 1, 7, 21 and 42 days after pruning. A non-inoculated control was included to record natural infection. After 6 months, the trials were evaluated and the pruning wounds were found to be susceptible for up to 42 days, with no difference in susceptibility between seasons (winter vs. spring). The wounds were the most susceptible within the first week, thus the application of pruning wound protectant is critical during this time. The efficacy of pruning wound protectants were tested under low (80 conidia) and high (10 000 conidia) inoculum pressure in 2017/2018 and 2018/2019, respectively. Eleven pruning wound protectants [Tree Seal, Kemseel, Neocil Plus, Garrison Rapid, Merpan/Dithane, Coprox Super (in paint), Coprox Super/Bendazid (in paint), Bendazid (in water), MT1 (*Trichoderma atroviride*), MT1/Bendazid and Eco77 (*T. atroviride*)] were applied directly after pruning 2-year-old branches. These treated wounds and positive (non-treated) controls were challenged with *Pseudophaeomoniella* sp. at two time points after applying the pruning wound protectants either 1 day after pruning or 7 days after pruning. A final set of treated wounds were used as non-inoculated controls. Under low inoculum pressure (2017/2018 trial), Garrison Rapid, MT1 (water), Neocil Plus and Tree Seal, consistently reduced *Pseudophaeomoniella* sp. infections. Under higher inoculum pressure (2018/2019 trial), Tree Seal and Coprox Super/Bendazid consistently performed the best. Tree Seal can be considered an effective olive pruning wound protectant since it was consistently effective under high and low inoculum pressure. Under low inoculum pressure, the *Trichoderma*-based protectant, MT1, can be considered an effective water-based pruning wound protectant.

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INTRODUCTION

The pruning of olive trees is an essential part of maintaining olive production (Costa, 1998). It stimulates new growth from which fruit can develop, and improves the aeration through an orchard, which lowers the risk of disease. Skirting of the trees (i.e. pruning of the lower branches of the tree) can be done to avoid branches being exposed to weeds, shade and dew, which further reduce the risk of disease (Costa, 2019). Furthermore, pruning can be performed to create a tree and orchard structure that facilitates general farming practises such as harvesting, mulching and spraying as well as improving the spray distribution. It has been recommended that large branches are pruned during mid-winter to remove large diseased branches and branches or parts of branches obstructing sunlight, as well as branches that have grown too tall (Costa, 2019). Skirting can also be performed during this time. During spring after the buds have differentiated into flower buds, regrowth from the winter pruning can be managed by removing the most upright suckers, while some suckers are cut back and headed, and the canopy thinned by secateurs cuts (Costa, 2019).

Recently, a new olive trunk pathogen, *Pseudophaeomoniella* sp., was identified in the Western Cape Province, South Africa (van Jaarsveld, 2015; Chapter 2). Other highly virulent olive trunk pathogens occurring in the Western Cape include *Neofusicoccum capensis*, *Neofusicoccum vitifusiforme*, *Neofusicoccum* sp. 4 and *Phaeoacremonium africanum* (Chapter 2). Trunk pathogens are known to infect via pruning wounds (Ramos *et al*., 1975; ; Chapuis *et al*., 1998; Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011; Chen *et al*., 2016). These infections can lead to dieback of twigs and branches, which can reduce the fruit bearing capacity and lifespan of the olive trees (Carlucci *et al*., 2013, 2015; Úrbez-Torres *et al*., 2013). Apricot (Ramos *et al*., 1975), apple (Chen *et al*., 2016) and grapevine (Chapuis *et al*., 1998; Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011) pruning wound susceptibility decreases as the pruning wounds age but often remained susceptible at a low level for prolonged periods (more than 30 days) (Eskalen *et al*., 2007). The susceptibility of the pruning wounds can be influenced by climatic conditions with the majority of pruning wound susceptibility studies indicating higher susceptibility earlier during the pruning season (autumn to mid-winter) opposed to later (late-winter to spring) (Ramos *et al*., 1975; Munkvold and Marois, 1995; Chapuis *et al*., 1998; Úrbez-Torres and Gubler, 2011). Munkvold and Marois (1995) and Úrbez-Torres and Gubler (2011) suggested that the increased resistance of grapevine pruning wounds during later pruning was due to an accelerated production of suberin and lignin, since increased suberin and lignin production of grapevine has been positively correlated with an increase in degree-days (Munkvold and Marois, 1995). However, other studies showed grapevine pruning wounds to be less

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susceptible earlier during the pruning season (early winter) in Stellenbosch, Western Cape Province, South Africa (van Niekerk *et al*., 2011), while in Spain the pruning wounds made earlier in the season by Elena and Luque (2016) were less susceptible to *Diplodia seriata* with no difference in susceptibility towards *Phaeomoniella chlamydospora* infection between seasons. To date, no information has been published regarding the susceptibility of olive pruning wounds to trunk pathogens.

Currently, no chemical or biological pruning wound protectants are registered for olive pruning wounds in South Africa and no information has been published previously regarding the most effective olive pruning wound protectants. Benomyl, carbendazim and thiophanate-methyl are highly effective for the management of trunk diseases of grapevine and have been used in trials to identify additional pruning wound protectants with similar efficacy sometimes with the intention of providing safer and/or more environmentally friendly alternatives (Munkvold and Marois, 1993; Halleen *et al*., 2010; Kotze *et al*., 2011; Latorre *et al*., 2013). Water-based pruning wound protectants providing protection comparable to carbendazim against *Neofusicoccum luteum* on ‘Chardonnay’ grapevine pruning wounds in field trials include flusilazole, tebuconazole and mancozeb (Amponsah *et al*., 2012). However, flusilazole, among some other demethylation inhibitor fungicide compounds, did not provide the same duration of protection as benomyl and carbendazim (Sosnowski *et al*., 2008). Furthermore, although tebuconazole and mancozeb provided some protection of almonds against various *Botryosphaeria* trunk pathogens, but these were not always as effective as the thiophanate-methyl treatments (Olmo *et al*., 2017).

Pruning wound sealants applied onto large pruning wounds have been recommended for the control of olive trunk diseases in South Africa (Costa, 1998). Sealants provide a protective barrier hindering the entry of fungal propagules (Spiers and Brewster, 1997). Cracking of the sealants after drying can reduce the efficacy of the pruning wound protectants (Spiers and Brewster, 1997). According to Sosnowski *et al*. (2008), sealants amended with fungicides can be used to prevent infection through these cracks. Furthermore, the efficacy of benomyl, thiophanate-methyl, pyraclostrobin and tebuconazole applied in paste form (vinyl acrylic paint) were more effective on grapevine pruning wounds in Chile compared with water-based applications (Díaz and Latorre, 2013). In paste form, iprodione and tebuconazole were comparable to a benomyl-amended paste against *Neofusicoccum parvum* on the blue berry cultivar ‘Duke’ in Chile (Latorre *et al*., 2013). The product Garrison is a paste amended with cyproconazole and iodocarb that has been comparable to a carbendazim spray application for the protection of ‘Shiraz’ against *Diplodia mutila* and *D. seriata* (Pitt *et al*., 2012) and ‘Cabernet Sauvignon’ against *Eutypa lata* (Sosnowski *et al*., 2008) in Australia. Garrison was also

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comparable to thiophanate-methyl in protecting 'Chardonnay' pruning wounds against various trunk pathogens in California, except for *Phaeoacremonium parasiticum* (Rolshausen *et al*., 2010). Biological control has shown potential towards long-term protection of pruning wounds regardless of rainfall events unlike some chemical control agents (Munkvold and Marois, 1993). Various *Trichoderma* products (Eco77, Vinevax, Biotricho, USPP T1 and USPP T2) and *Bacillus subtilis* were in some cases comparable to benomyl on 'Merlot' and 'Chenin Blanc' grapevine cultivars. This was after spray inoculating with a spore suspension containing either *Diaporthe neoviticola* (=*Phomopsis viticola*), *E. lata*, *P. chlamydospora*, *Neofusicoccum australe*, *N. parvum*, *D. seriata* and *Lasiodiplodia theobromae*, in field trials 7 days after applying the pruning wound protectants (Kotze *et al*., 2011). Vinevax and Eco77, did not provide as much control as benomyl in field trials when treated pruning wounds were challenged with *E. lata* 24 hours after application (Halleen *et al*., 2010). However, these products did significantly reduce *E. lata*, *D. mutila* and *D. seriata* infections and could potentially provide adequate protection under natural infection levels (Halleen *et al*., 2010). On the contrary, some of the commercial biological control agents such as Serenade Max (*B. subtilis*) and Trichonativa (*Trichoderma harzianum*, *Trichoderma parceramosum* and *Trichoderma virens*) were not effective at reducing *N. parvum* infection of blue berry plants in Chile (Latorre *et al*., 2013).

An abundance of research is available regarding the pruning wound susceptibility and the efficacy of pruning wound protectants, mostly on grapevine. No such studies have been published regarding olive tree pruning wounds. In this chapter, the duration of olive tree pruning wound susceptibility to *Pseudophaeomoniella* sp. and the seasonal effects thereof were evaluated. Furthermore, the efficacy of various potential olive pruning wound protectants were evaluated.

MATERIALS AND METHODS

Inoculum

Pseudophaeomoniella sp. (STE-U 7950) was used as inoculum for the pruning wound susceptibility and pruning wound protectant trials. This isolate is stored in the culture collection at the Plant Pathology department of Stellenbosch University and at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij, Plant Protection division (as CSN 183). The fungus was grown for 1–2 weeks at 25ºC in Petri dishes containing Potato Dextrose Agar (PDA, Biolab, South Africa) amended with chloromycetin (250 mg/L) (PDA-C). The Petri dishes were flooded with 4 mL of autoclaved deionised water (dH_2O) and agitated lightly before transferring the

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liquid into 15 mL centrifugation tubes. The concentration of the conidial spore suspension was determined using a haemocytometer and adjusted for .

Pruning wound susceptibility trials

Inoculations were performed in two locations on 16-year-old 'Frantoio' olive trees that were drip irrigated on a commercial farm in Paarl, Western Cape Province, South Africa. The two locations served as trial replicates. Pruning and subsequent inoculations were performed on 2-year-old shoots in July (winter) and September (spring) during 2017. Upright shoots were selected as far as possible of which pruning wounds were made using clean pruning shears, while leaving at least 30 cm of the shoot on the tree for experimental use.

Individual pruning wounds were challenged by inoculating the wounds with 100 µL of 1×10^6 conidia/mL of *Pseudophaeomoniella* sp. 0, 1, 7, 21 and 42 days after pruning. A non-inoculated control treatment served to record the natural level of infection of olive trees during winter and spring. For each trial, the experimental layout was a randomised block design with six treatments (challenge times) replicated in three blocks. Each block comprised of six olive trees with all treatments applied on each tree, i.e. an experimental unit consisted of six wounds of each treatment per block replicate. The pruning wounds were evaluated 6 months after the trial was initiated (during April and June 2018, for the winter and spring trials, respectively).

Pruning wound protectant trials

2017/2018 trials

The first pruning wound protectant trials were established on 16-year-old 'Frantoio' olive trees in July and September 2017 and evaluated in April and June 2018, respectively. The July and September trials served as trial repeats and were approximately 1 km apart. Eleven pruning wound protectants were selected based on current usage by olive producers in the Western Cape and a selection of untested treatments were specifically included for this project. Eleven pruning wound protectants [Tree Seal, Kemseel, Neocil Plus, Garrison Rapid, Merpan/Dithane, Coprox Super (in paint), Coprox Super/Bendazid (in paint), Bendazid (in water), MT1 (*Trichoderma atroviride*), MT1/Bendazid and Eco77 (*T. atroviride*)] (Table 1) were applied directly after pruning the 2-year-old olive shoots. Non-treated, inoculated treatments were included in the experiments as positive controls. Tree Seal, Kemseel, Neocil Plus and Garrison Rapid were used as directed by the supplier's information. The remaining protectants were amended with either paint or water, according to the recommended or registered dosage used for olive trees or other similar crops (Table 2). Water-based chemicals were applied by spray application of approximately 1 mL of the product per pruning wound using a spray bottle. Of the treated wounds one set served as non-inoculated controls (no challenge), while the rest

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were challenged with 80 µL of 1 × 10^3 conidia/ml of *Pseudophaeomoniella* sp. at either 1 or 7 days after pruning. The 11 pruning wound protectants and three challenge period combinations resulted in a total of 42 treatment combinations. The experimental design was a randomised block design with the 42 treatment combinations replicated in three blocks. Each block consisted of 18 olive trees with each treatment applied six times according to a balanced incomplete block design over 14 branches per tree, i.e. an experimental unit consisted of six wounds of each treatment combination per block replicate.

2018/2019 trials

The inoculum pressure was increased to determine the efficacy of pruning wound protectants under higher inoculum pressure. These trials were established on 17-year-old 'Frantoio' trees during August 2018 in two locations, approximately 1 km apart, and evaluated in April 2019. The two locations served as trial repeats. The shoots were pruned using the same procedure as before with minor adjustments to the concentration of Merpan, Dithane and Coprox Super (Table 2). A 100 µL of 1 × 10^5 conidia/mL were applied per wound 1 and 7 days after pruning wounds using the same approach as performed in the previous trial.

Trial evaluations

The shoots of the pruning wound susceptibility and pruning wound protectant trials were removed in batches, transported in a cooler box, and stored in a refrigerator at 3°C for 1–3 days. The shoots were split longitudinally using an electric band saw and processed on the same day. The internal wood was inspected for symptoms before sterilising in a series of 70% ethanol for 30 s, 3.5% sodium hypochlorite for 1 min and 70% ethanol for 30 s. Isolations were made from both sides of each sample at the margin of dead and living tissue by aseptically removing 12 wood chips (~1 × 1 × 1 mm) per shoot. These wood chips were plated out onto three Petri dishes containing PDA-C. The plates were incubated on a lab bench at 23–25°C for 4 weeks. The plates were monitored daily for fungal growth. Subsets of cultures resembling the inoculated fungal species, *Pseudophaeomoniella* sp., were hyphal tipped and plated onto clean PDA-C dishes. These cultures were identified based on morphology. A subset of these isolates were selected to confirm the identity molecularly. DNA was extracted from the representative isolates as well as from a known isolate of *Pseudophaeomoniella* sp. (STE-U 7950) using a standard SDS DNA extraction protocol (Lee et al., 1988). The DNA was subjected to PCR using the *Pseudophaeomoniella* sp. species-specific primers as described in Chapter 4 without nesting. The PCR products were visualised on 1% agarose gels together with a DNA ladder (GeneRuler 100bp; Thermo Scientific).

Statistical analyses

The incidence of *Pseudophaeomoniella* sp. was calculated for the pruning wound susceptibility and protectant trials for each treatment and block as the number of wounds within an experimental unit (six wounds) that *Pseudophaeomoniella* sp. was isolated from. The incidence of *Trichoderma* sp. was also calculated for the *Trichoderma*-based pruning wound protectants in the same manner as above. The incidence data were subjected to randomised block analysis of variance (ANOVA), for each trial repeat separately using GLM (General Linear Models) Procedure of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Observations for trials were also combined in one ANOVA after confirming trial homogeneity of variance using Levene's test (Levene, 1960). Shapiro-Wilk test was performed to test for deviation from normality (Shapiro, 1965). Fisher's least significant difference (LSD) was calculated at the 5% level to compare means for significant effects (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

Weather data

A daily weather report consisting of the maximum and minimum temperatures, humidity and total rainfall was obtained from the Nederburg weather station (within 2 km of the trial sites) by the Agricultural Research Council (ARC) - Institute for Soil, Climate and Water (ARC-ISCW). The weather data was overlaid with the pruning periods of the pruning wound susceptibility trials.

RESULTS

Pseudophaeomoniella sp. was able to form long streaky lesions in 2-year-old olive shoots during the pruning wound susceptibility and pruning wound protectant trials (Fig. 1). The pathogen was isolated from the inoculated wounds and a representative subset of these isolates were confirmed as *Pseudophaeomoniella* sp. based on the PCR results of the species-specific primers (Fig. 2).

Pruning wound susceptibility

The trial variances were equal and the data was normally distributed according to Levene's ($P > 0.05$) and Shapiro-Wilk's ($P > 0.05$) tests, respectively. There was no trial × treatment interaction ($P > 0.05$) for both the winter and spring experiments (Appendix D: Tables 1–2). The trial repeats of the winter and spring experiments were thus combined and the treatment effect evaluated for winter and spring (Figs. 3–4). The mean re-isolation incidence was highest from wounds that were inoculated immediately after pruning during both the winter (94.44%) and spring (88.89%) trials (Figs. 3–4). The incidence of *Pseudophaeomoniella* sp. from the pruning wounds tended to decreased as the interval between the pruning and the inoculation

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time point increased for both the winter and spring trials (Figs. 3–4). The lowest re-isolation incidences were found from wounds that were not inoculated (13.89% for winter and 8.89% for spring; Figs. 3–4), which was also significantly lower than all inoculated wounds.

During winter, the incidence of *Pseudophaeomoniella* sp. was not significantly different when inoculations were performed on the day of pruning and 1 day after pruning (Fig. 3). The incidence was significantly less 7 days after pruning. The incidence of *Pseudophaeomoniella* sp. inoculated 21 and 42 days after pruning was significantly lower than the earlier treatments with no significant difference between these two treatments. The spring trial had a similar trend except that at 1 day after pruning, there was a significantly lower incidence of *Pseudophaeomoniella* sp. (Fig. 4). Shortly after this treatment (29 September 2017), the minimum and maximum temperatures increased, while the minimum and maximum relative humidity (RH) decreased drastically. The maximum RH continued to drop until 1 October 2017 (RH = 50.31%) before rising again (Fig. 4). Due to this outlier in spring (1 day after pruning), a significant season × treatment interaction existed (Appendix D: Table 3). With the exception of this outlier, there were no significant differences in the incidences found for the different treatments between seasons (Table 3).

Pruning wound protection

2017/2018 trials

The trial variances were close to equal ($P = 0.068$), while the normality of the data was acceptable. There was a protectant × challenge × trial interaction ($P < 0.05$; Appendix D: Table 4). The protectant × challenge × trial interaction was thus evaluated (Table 4). The inoculated water controls of Trial 1 (36.67%) and Trial 2 (37.78%) had a significantly higher incidence of *Pseudophaeomoniella* sp. compared to the non-inoculated controls (0.00–16.67%; $P < 0.05$; Table 4). During the winter trial, when challenged 1 day after pruning, Coprox Super Coprox Super/Bendazid, Eco77, MT1/Bendazid, Kemseel, Merpan/Dithane, Bendazid (water) (22.22–31.11%), did not reduce the incidence of *Pseudophaeomoniella* sp. compared to the control challenged 1 day after pruning (36.67%; Table 4). However, except for Coprox Super and Eco77, there were significant differences between these treatments between the two trials. During Trial 2, these treatments reduced the incidence of *Pseudophaeomoniella* sp. to a level significantly lower (0.00–11.11%) than the control challenged 1 days after pruning (37.78%; Table 4), while Eco77 (31.11%) was not able to reduce the incidence of *Pseudophaeomoniella* to a level significantly different from the inoculated control (37.78%; Table 4).

During Trial 1, when pruning wounds were challenged 7 days after pruning, Eco77 did not reduce the incidence of *Pseudophaeomoniella* sp. (33.33%; Table 4). The incidence was

significantly higher than the control when inoculated after 7 days (6.67%; Table 4). However, there was a trial interaction between these treatments and Eco77 was able to reduce the incidence of *Pseudophaeomoniella* sp. (0.00%) during the spring trial, although not significantly different from the control (6.67%; Table 4). The incidence of *Pseudophaeomoniella* sp. in the remaining pruning wounds inoculated 7 days after pruning during winter and spring (0.00–18.89%) were not significantly different from the positive controls inoculated 7 days after pruning (6.67%) and the non-inoculated controls (0.00–16.67%; Table 4).

Trichoderma was isolated from the pruning wounds that were treated with the *Trichoderma*-based pruning wound protectants. The trial variances were equal and the data was normally distributed according to Levene's ($P > 0.05$) and Shapiro-Wilk's ($P > 0.05$) tests, respectively. There was a challenge × trial interaction ($P < 0.05$; Appendix D: Table 5). The protectant × challenge × trial interaction was thus evaluated (Table 6). No significant differences in the incidence of *Trichoderma* was found between the challenged (when *Pseudophaeomoniella* sp. was inoculated 1 and 7 days after pruning) and non-challenged groups for MT1 (Trial 1: 22.22–38.89%; Trial 2: 50.00–55.56%) and Eco77 (Trial 1: 11.11–33.33%; Trial 2: 22.22–50.00%) during Trial 1 and Trial 2, respectively. No significant differences in the incidence of *Trichoderma* was found between the challenged (when *Pseudophaeomoniella* sp. was inoculated 1 and 7 days after pruning) and non-challenged groups for MT1/Bendazid during winter (Trial 1: 22.22–33.33%). During Trial 2, the incidence of *Trichoderma* isolated from the MT1/Bendazid-treated wounds challenged after 1 day was significantly lower (11.11%) compared to when MT1/Bendazid treated wounds were not challenged with *Pseudophaeomomiella* sp. (38.89%)

2018/2019 trials

The results of the 2018/2019 trials challenged with a high inoculum pressure showed equal trial variances according to Levene's test ($P > 0.05$) and the normality of the data was acceptable. There was no trial interaction ($P > 0.05$; Appendix D: Table 6). The trial repeats were thus combined and protectant × challenge effect evaluated (Table 5). Of the pruning wound protectants challenged 1 day after pruning, Tree Seal (22.22%) and Coprox Super/Bendazid (26.11%) had the lowest incidence of *Pseudophaeomoniella* sp. and this did not differ significantly from the non-inoculated control (5.56%; $P > 0.05$; Table 5). This is a reduction of 60 and 66% of *Pseudophaeomoniella* sp. incidence, respectively. The highest mean incidence of *Pseudophaeomoniella* sp. from pruning wounds challenged 1 day after pruning was the inoculated control treated. MT1, Eco77, Neocil Plus and MT1/Bendazid (47.22–52.78%) did not differ significantly ($P > 0.05$) from the inoculated control (65.56%;

Table 5). The remaining treatments offered an intermediate level of protection against *Pseudophaeomoniella* sp. infections (Table 5).

Of the pruning wound protectants challenged 7 days after pruning during the 2018/2019 experiment, Tree Seal, Coprox Super/Bendazid, Kemseel, Coprox Super and Bendazid had the lowest incidence of *Pseudophaeomoniella* sp. (8.89–25.00%) and this did not differ significantly from the non-inoculated control (5.56%; Table 5). This represents a reduction of 54.9–84.3% in *Pseudophaeomoniella* sp. incidence, respectively. The highest mean incidence of *Pseudophaeomoniella* sp. from pruning wounds challenged 7 days after pruning was the inoculated control treated. Garrison Rapid (38.89%) and Merpan/Dithane M-45 (45.00%) were not significantly different from the positive control treatment inoculated 7 days after pruning (56.67%; Table 5). The remaining treatments offered an intermediate level of protection against *Pseudophaeomoniella* sp. infections (Table 5). Furthermore, there were no significant differences of the incidence of *Pseudophaeomoniella* sp. between the pruning wound protectants that were not challenged during the 2018/2019 experiment (0.00–15.28%; Table 5).

Trichoderma was isolated from the pruning wounds that were treated with the *Trichoderma*-based pruning wound protectants. The trial variances were equal and the data was normally distributed according to Levene's ($P > 0.05$) and Shapiro-Wilk's ($P > 0.05$) tests, respectively. There was protectant × trial interaction ($P < 0.05$; Appendix D: Table 7). The protectant × challenge × trial interaction was thus evaluated (Table 6). No significant differences in the incidence of *Trichoderma* was found between the challenged (when *Pseudophaeomoniella* sp. was inoculated 1 and 7 days after pruning) and non-challenged groups for MT1 (Trial 1: 16.67–44.44%; Trial 2: 50.00–55.56%), MT1/Bendazid (Trial 1: 22.22–27.78%; Trial 2: 44.44–61.11%) and Eco77 (Trial 1: 44.44–50.00; Trial 2: 27.78–50.00) during Trial 1 and Trial 2, respectively.

DISCUSSION

The pruning wounds of woody crops such as apricot (Ramos *et al*., 1975), apple (Chen *et al*., 2016) and grapevine (Chapuis *et al*., 1998; Eskalen *et al*., 2007; Úrbez-Torres and Gubler, 2011; van Niekerk *et al*., 2011) decreases in susceptibility as the wounds age, with the wounds often remaining susceptible for prolonged periods of up to 4 months or longer (Eskalen *et al*., 2007). The length of time that pruning wounds remain susceptible has further been associated with environmental conditions/season during which the pruning wounds are made (Ramos *et al*., 1975). Pruning wound susceptibility was shown by most studies to be higher earlier during the pruning season (autumn to mid-winter) and less susceptible later (late-winter to spring)

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(Ramos *et al*., 1975; Munkvold and Marois, 1995; Chapuis *et al*., 1998; Úrbez-Torres and Gubler, 2011). During this study, the susceptibility of olive pruning wounds to *Pseudophaeomoniella* sp. was highest within the first week after pruning and then decreased over time but remained susceptible for at least 42 days after pruning, while the susceptibility of the olive pruning wounds were generally not different between winter and spring pruning. Rather, the incidence of *Pseudophaeomoniella* sp. from pruning wounds challenged 1 day after pruning was significantly lower in spring compared to winter. Soon after this treatment in spring, the temperatures started to rise and the RH decreased rapidly. Although increased temperature has been linked to a decrease in the susceptibility of pruning wounds (Munkvold and Marois, 1995; Úrbez-Torres and Gubler, 2011), this does not appear to be the cause of the lower infection rate in this study, since the recovery of *Pseudophaeomoniella* sp. from the pruning wounds challenged thereafter (at 7 days after pruning) was higher and similar to the winter trial. During fungal spore germination, the spores are particularly sensitive to moisture levels and may die during low RH of below 75%, depending on the fungal species (Gottlieb, 1950). Therefore, the low RH that occurred shortly after inoculation could be responsible for desiccation and death of germinating *Pseudophaeomoniella* sp. conidia; hence, the lower incidence of *Pseudophaeomoniella* sp. found from pruning wounds inoculated 1 day after pruning during spring.

No difference in the pruning wound susceptibility during seasons is contradictory to similar studies on other hosts (Ramos *et al*., 1975; Munkvold and Marois, 1995; Chapuis *et al*., 1998; Úrbez-Torres and Gubler, 2011). However, during these studies, the pruning wound susceptibility was tested against Botryosphaeriaceae and *Eutypa* spp. No significant difference of susceptibility was found between early and late pruning against *P. chlamydospora* in California (Eskalen *et al*., 2007; Elena and Luque, 2016) or in Italy (Serra *et al*., 2008). It was only in the study by van Niekerk *et al*. (2011) where a significant difference of pruning wound susceptibility to *P. chlamydospora* was suggested, with higher susceptibility observed during late pruning in South Africa. The pruning wound susceptibility to species of Phaeomoniellaceae could be influenced less by environmental conditions. One could further argue that the lack of difference found in *Pseudophaeomoniella* sp. infection of olive pruning wounds between seasons was a result of a considerably high inoculum dosage (1×10^5 conidia/wound), which was well above the recommended dosage for 50–70% recovery of *P. chlamydospora* during grapevine pruning wound susceptibility studies (Elena *et al*. 2015). However, the difference of the incidence of *Pseudophaeomoniella* sp. in the control treatments inoculated at 1 and 7 days after pruning during the winter and spring trial of the pruning wound protectant study was also not significantly different between trials, which further suggests that

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the pruning wound susceptibility against *Pseudophaeomoniella* sp. infections is not influenced greatly by season.

There is no evidence of complete resistance of pruning wounds against trunk pathogens, regardless of the pruning season and host evaluated. Furthermore, due to the prolonged pruning wound susceptibility observed in this study, and the presence of pycnidia of *Pseudophaeomoniella* sp. in olive orchards, serving as an inoculum source within established orchards (Chapter 3), pruning wound protectants offering a long duration of protection against trunk pathogens (or at least against *Pseudophaeomoniella* sp.) appears necessary to manage trunk diseases. Under high inoculum pressure, Coprox Super, Coprox Super/Bendazid, Garrison Rapid, Kemseel, Merpan/Dithane M-45 and Tree Seal were comparable to what can be considered the industry standard used for grapevine pruning wounds, carbendazim (Bendazid). Tree Seal and Coprox Super/Bendazid consistently reduced *Pseudophaeomoniella* sp. infections the most. Tree Seal was also able to significantly reduce the incidence of *Pseudophaeomoniella* sp. during low inoculum pressure when challenged 1 day after pruning during the winter and spring trials, while Coprox Super/Bendazid was able to reduce the incidence of *Pseudophaeomoniella* sp. considerably during spring but not during the winter trial. Generally, at low inoculum pressure, the incidence of *Pseudophaeomoniella* sp. from pruning wounds treated with pruning wound protectants was higher during winter compared to spring. During winter, Coprox Super/Bendazid as well as the other PVP-based pruning wound protectants and Kemseel were cracked which resembled water damage. These pruning wound protectants were applied shortly after a rainfall event (weather data not shown), which may have caused the cracking of the pruning wound sealants and contributed to trial interaction between the winter and spring trials. Garrison Rapid has been highly effective in grapevine pruning wound protection studies of 'Shiraz' against *D. mutila* and *D. seriata* in Australia (Pitt *et al*., 2012) and 'Chardonnay' against various trunk pathogens in California (Rolshausen *et al*., 2010), but long-term protection was not evaluated during these studies. Under high inoculum pressure, Garrison Rapid and Merpan/Dithane M-45 did not provide adequate long-term protection against *Pseudophaeomoniella* sp., although this was not as prevalent under low inoculum pressure.

Biological control is thought of as an environmentally friendly approach for long-term protection of pruning wounds (Munkvold and Marois, 1993). However, under high inoculum pressure, the biological control agents included in this study were not able to reduce the incidence of *Pseudophaeomoniella* sp. considerably when fresh pruning wounds were challenged with *Pseudophaeomoniella* sp. 1 day after pruning. These pruning wound protectants were able to inhibit *Pseudophaeomoniella* sp. when inoculum was introduced to

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the wounds later (7 days after pruning). By this time, the *Trichoderma* were expected to have been able to colonise the pruning wounds sufficiently (Mutawila *et al*., 2015). Similarly, in a study by Halleen *et al*. (2010), Vinevax and Eco77, did not provide as much control as benomyl in field trials where treated pruning wounds were challenged with *E. lata* 1 days after pruning. Halleen *et al*. (2010) further stated that the products significantly reduce *E. lata*, *D. mutila* and *D. seriata* infections and could potentially provide adequate protection under natural infection levels (Halleen *et al*., 2010). Under low inoculum pressure (or more natural level of inoculum), MT1 was able to reduce the incidence of *Pseudophaeomoniella* sp. during both the winter and spring trials, when the pruning wounds were challenged 1 day after pruning. Furthermore, when MT1 treated pruning wounds were challenged 7 days after pruning under low inoculum pressure, MT1 was able to reduce the incidence of *Pseudophaeomoniella* sp. to zero for both the winter and spring trials, which was considerably lower than that found in pruning wounds treated with Tree Seal during the winter trial. MT1/Bendazid did not perform better than MT1 when used alone, which was contradictory to the results of the study by Mutawila *et al*. (2015) for the protection of grapevine pruning wounds. The incidence of *Pseudophaeomoniella* sp. from Eco77 treated pruning wounds was variable and inconclusive. The performance of Eco77 may be better and more consistent on a different olive cultivar. For example, specific *Trichoderma* species were able to colonise specific grapevine cultivars better, while holding its ability to protect pruning wounds against trunk pathogens in the study by Mutawila *et al*. (2011).

In conclusion, the olive pruning wounds were susceptible to *Pseudophaeomoniella* sp. for up to 42 days or longer. The susceptibility of the pruning wounds made during winter and spring declined over time, generally with no difference in susceptibility between seasons (winter vs. spring). This indicates that the season during which pruning wounds are made cannot currently be used as a measure to manage olive trunk diseases caused by *Pseudophaeomoniella* sp. However, the successful germination of *Pseudophaeomoniella* sp. conidia and infection appears to be influenced by RH. Pruning when RH is low can potentially be used to manage olive trunk diseases caused by *Pseudophaeomoniella* sp., however, further studies should be performed to test the practicality and to confirm this phenomenon. The wounds were the most susceptible within the first week, thus application of a pruning wound protect is critical during this time. Under high inoculum pressure, Tree Seal and Coprox Super/Bendazid consistently provided the best protection of pruning wounds against *Pseudophaeomoniella* sp. infections. Tree Seal also provided protection of the pruning wounds during low inoculum pressure. Under low inoculum pressure MT1 was also able to reduce the incidence of *Pseudophaeomoniella* sp. during both the winter and spring trials when challenged 1 day after pruning. When MT1 was challenged 7 days after pruning under

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low inoculum pressure, the incidence of *Pseudophaeomoniella* sp. was reduced to zero for both the winter and spring trials, which was considerably lower than that of Tree Seal pruning wounds during the winter trial. These results suggest the potential use of MT1 to effectively manage olive trunk diseases of the pruning wounds of 'Frantoio' olive trees in well-maintained orchards that are expected to have low inoculum pressure. These products should be evaluated further on more olive cultivars in different climatic and geographic areas and against other olive trunk pathogens (such as the Botryosphaeriaceae and *Phaeoacremonium* spp.) to establish its full potential as effective pruning wound protectants.

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TABLES AND FIGURES

Table 1. Pruning wound protectants selected for the evaluation of the efficacy against *Pseudophaeomoniella* sp. infections of olive tree pruning wounds.

Trade name	Active ingredient(s)	Formulation[a]	Manufacturer (Distributer)
Garrison Rapid	cyproconazole	2.5 g/L	Grochem Australia (Australia)
	iodocarb	1.0 g/L	
Kemseel	None	n/a	chempac [South Africa (SA)]
Neocil Plus	8-hydroxyquinoline sulfate	5 g/kg	Algro-chem (SA)
	octhilinone	5 g/kg	
Tree Seal (pruning grade)	None	n/a	a.b.e.® Construction Chemicals (SA)
Coprox Super + PVA[b]	copper oxychloride	850 g/kg WP	Arysta LifeScience (SA)
Coprox Super/Bendazid + PVA	copper oxychloride	850 g/kg WP	Arysta LifeScience (SA)
	carbendazim	500 g/L SC	AECI (SA)
Merpan/Dithane M-45	captan	500 g/L SC	Adama SA (SA)
	mancozeb	800 g/kg WP	Dow Agrosciences Southern Africa (SA)
Bendazid	carbendazim	500 g/L SC	AECI (SA)
MT1	*Trichoderma atroviride*	2×10^8 spores/g	BioSolutions (SA)
MT1/Bendazid	*T. atroviride*	2×10^8 spores/g	BioSolutions (SA)
	carbendazim	500 g/L SC	AECI (SA)
Eco77®	*T. atroviride*	2×10^9 spores/g	Madumbi Sustainable Agriculture (SA)

[a] SC = suspension concentrate; WP = wettable powder. Spores denoted by the manufacturers as CFU (colony forming units)

[b] PVA = Polyvinyl alcohol. Manufactured by Bergermaster (SA).

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Table 2. Application rate of the pruning wound protectants selected to study the efficacy against the *Pseudophaeomoniella* sp. infection of olive tree pruning wounds during the 2017/2018 and 2018/2019 field trials.

		Dosage (a.i.)[a]	
Trade name	**Active ingredient(s)**	**2017/2018**	**2018/2019**
Garrison Rapid	cyproconazole	n/a	n/a
	iodocarb		
Kemseel	None	n/a	n/a
Neocil Plus	8-hydroxyquinoline sulfate	n/a	n/a
	octhilinone		
Tree Seal	None	n/a	n/a
Coprox Super + PVA[b]	copper oxychloride	4.25 g/L[c]	3.72 g/L[c]
Coprox Super/Bendazid + PVA	copper oxychloride	4.25 g/L	3.72 g/L
	carbendazim	0.50 g/L[d]	0.50 g/L
Merpan/Dithane M-45	captan	0.875 g/L[e]	2.00 g/L[f]
	mancozeb	1.60 g/L[g]	2.50 g/L[f]
Bendazid	carbendazim	0.50 g/L	0.50 g/L
MT1	*Trichoderma atroviride*	1×10^9 spores/g[h]	1×10^9 spores/g
MT1/Bendazid	*T. atroviride*	1×10^9 spores/g	1×10^9 spores/g
	carbendazim	0.50 g/L	0.50 g/L
Eco77®	*T. atroviride*	1×10^9 spores/g[h]	1×10^9 spores/g

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[a] a.i. = active ingredient

[b] PVA = Polyvinyl alcohol

[c] Dosage selected based on range applied to avocado pear after extensive hail damage (personal communication with colleagues)

[d] Dosage Mutawila *et al*. (2015) used on grapevine pruning wound studies

[e] Registered dosage for Leafspot of olives in South Africa

[f] Highest rate registered for almonds in Spain (Olmo *et al*., 2017)

[g] Registered dosage for anthracnose of olives in South Africa

[h] Dosage according to products label instructions

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Table 3. The mean incidence of *Pseudophaeomoniella* sp. isolated from olive pruning wounds during the winter and spring pruning wound susceptibility trials.

	Mean incidence (%) ± SD[a,b]	
Treatment	**Winter**	**Spring**
Challenged (days after pruning)		
0	94.44 ± 8.61 a	88.89 ± 8.61 a
1	80.56 ± 19.48 ab	47.22 ± 12.55 d
7	63.89 ± 19.48 c	66.67 ± 10.54 bc
21	36.11 ± 12.55 d	44.44 ± 17.21 d
42	37.22 ± 12.55 d	33.33 ± 21.08 d
Non-challenged	13.89 ± 6.80 e	8.89 ± 9.81 e

[a] SD = Standard deviation from the mean. Means with the same letter are not significantly different ($P < 0.05$; LSD = 15.68);

[b] Mean incidences were calculated from complete block design as the number of trees that *Pseudophaeomoniella* sp. was isolated from within an experimental unit (six wounds) for three experimental units.

Table 4. Mean incidence of *Pseudophaeomoniella* sp. isolated from olive pruning wounds during the first and second trial of the 2017/2018 pruning wound protectant trials.

	Mean incidence (%) ± SD[a,b,c]					
	Winter (Trial 1)			Spring (Trial 2)		
Protectant	**Challenged after 1 day**	**Challenged after 7 days**	**Natural infection**	**Challenged after 1 day**	**Challenged after 7 days**	**Natural infection**
Neocil Plus	**5.56 ± 9.62 fg**	**5.56 ± 9.62 fg**	**12.22 ± 10.72 defg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**	**11.11 ± 19.25 efg**
Tree Seal	**11.11 ± 9.62 efg**	**19.44 ± 17.35 abcdefg**	**15.00 ± 13.23 cdefg**	**16.67 ± 16.67 cdefg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**
Garrison Rapid	**11.11 ± 9.62 efg**	**17.78 ± 1.92 bcdefg**	**12.22 ± 10.72 defg**	**12.22 ± 10.72 defg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**
MT1	**16.67 ± 16.67 cdefg**	**0.00 ± 0.00 g**	**6.67 ± 11.55 fg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**
Coprox Super	**22.22 ± 19.25 abcdef**	**5.56 ± 9.62 fg**	**0.00 ± 0.00 g**	**11.11 ± 9.62 efg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**
Coprox Super/ Bendazid	**22.22 ± 25.46 abcdef**	**16.67 ± 16.67 cdefg**	**6.67 ± 11.55 fg**	**0.00 ± 0.00 g**	**11.11 ± 9.62 efg**	**0.00 ± 0.00 g**
Eco77	**22.22 ± 25.46 abcdef**	**33.33 ± 16.67 abc**	**0.00 ± 0.00 g**	31.11 ± 30.06 abcd	**0.00 ± 0.00 g**	**5.56 ± 9.62 fg**
MT1/Bendazid	**27.78 ± 9.62 abcde**	**11.11 ± 19.25 efg**	**13.33 ± 23.09 defg**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**
Kemseel	**27.78 ± 25.46 abcde**	**0.00 ± 0.00 g**	**17.78 ± 16.78 bcdefg**	**11.11 ± 19.25 efg**	**20.00 ± 20.00 abcdef**	**0.00 ± 0.00 g**
Merpan/Dithane	**30.56 ± 17.35 abcde**	**17.78 ± 16.78 bcdefg**	**5.56 ± 9.62 fg**	**11.11 ± 9.62 efg**	**16.67 ± 0 cdefg**	**0.00 ± 0.00 g**
Bendazid (water)	**31.11 ± 25.02 abcd**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**	**0.00 ± 0.00 g**	**5.56 ± 9.62 fg**	**0.00 ± 0.00 g**
Control (water)	36.67 ± 15.28 ab	**6.67 ± 11.55 fg**	**16.67 ± 0.00 cdefg**	37.78 ± 20.37 a	**6.67 ± 11.55 fg**	**0.00 ± 0.00 g**

[a] SD = Standard deviation from the mean. Means with the same letter are not significantly different ($P < 0.05$; LSD = 19.71).

[b] Mean incidences were calculated from an incomplete block design as the number of trees that *Pseudophaeomoniella* sp. was isolated from within an experimental unit (six wounds) replicated in three experimental blocks.

[c] Bold values do not differ significantly from the non-challenged water control for winter and spring respectively. Writing in "red" show values not significantly different from the positive control inoculated 1 day after pruning. Writing in "purple" show values not significantly different from the positive control inoculated 7 days after pruning. Writing in "green" show values of non-inoculated pruning wounds with the pruning wound protectant that are not significantly different from the non-challenged control. Values highlighted in Grey show trial interaction between the winter and spring trial.

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Table 5. Mean incidence of *Pseudophaeomoniella* sp. isolated from olive pruning wounds during the 2018/2019 pruning wound protectant trials.

	Mean incidence (%) ± SD[a,b]		
Protectant	**Challenged after 1 day**	**Challenged after 7 days**	**Natural infection**
Tree Seal	**22.22 ± 13.61 fghijk**	**8.89 ± 14.40 ijkl**	**0.00 ± 0.00 l**
CoproxSuper/Bendazid	**26.11 ± 14.97 efghij**	**25.56 ± 8.61 efghij**	**0.00 ± 0.00 l**
Kemseel	26.67 ± 18.01 defghi	**17.78 ± 16.56 ghijkl**	**2.78 ± 6.80 kl**
Coprox Super	37.22 ± 12.55 bcdefg	**22.22 ± 29.19 fghijk**	**5.56 ± 8.61 jkl**
Garrison Rapid	37.78 ± 24.01 bcdefg	38.89 ± 17.21 bcdef	**3.33 ± 8.16 kl**
Bendazid	38.89 ± 22.77 bcdef	**25.00 ± 17.48 efghij**	**2.78 ± 6.80 kl**
Merpan/Dithane M-45	38.89 ± 17.21 bcdef	45.00 ± 21.16 abcde	**0.00 ± 0.00 l**
MT1/Bendazid	47.22 ± 17.56 abcd	33.89 ± 29.47 cdefgh	**2.78 ± 6.80 kl**
Neocil Plus	47.22 ± 24.53 abcd	26.67 ± 25.39 defghi	**5.56 ± 8.61 jkl**
Eco77	52.78 ± 16.39 abc	31.11 ± 28.41 defgh	**10.28 ± 11.57 ijkl**
MT1	52.78 ± 24.53 abc	33.89 ± 17.69 cdefgh	**15.28 ± 13.35 hijkl**
Control (water)	65.56 ± 18.46 a	56.67 ± 24.04 ab	**5.56 ± 13.61 jkl**

[a] SD = Standard deviation from the mean. Means with the same letter are not significantly different ($P < 0.05$; LSD = 20.63). Bold values do not differ from the non-challenged water control.

[b] Mean incidences were calculated from an incomplete block design as the number of trees that *Pseudophaeomoniella* sp. was isolated from within an experimental unit (six wounds) replicated in three experimental blocks.

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Table 6. Mean incidence of *Trichoderma* spp. in pruning wounds treated with *Trichoderma*-based pruning wound protectants during the 2017/2018 and 2018/2019 trials.

Protectant	Mean incidence (%) ± SD[a,b]					
	Trial 1			Trial 2		
	Challenged after 1 day	Challenged after 7 days	Natural infection	Challenged after 1 day	Challenged after 7 days	Natural infection
2017/2018						
MT1	33.33 ± 28.87 abcd	22.22 ± 19.25 cd	38.89 ± 9.62 abcd	55.56 ± 9.62 ab	55.56 ± 9.62 ab	50.00 ± 0.00 abc
MT1/Bendazid	22.22 ± 25.46 cd	27.78 ± 25.46 bcd	33.33 ± 44.10 abcd	11.11 ± 9.62 d	61.11 ± 9.62 a	38.89 ± 19.25 abcd
Eco77	27.78 ± 25.46 bcd	11.11 ± 9.62 d	33.33 ± 16.67 abcd	33.33 ± 0.00 abcd	50.00 ± 16.67 abc	22.22 ± 9.62 cd
2018/2019						
MT1	38.89 ± 19.25 abcd	16.67 ± 16.67 d	44.44 ± 25.46 abcd	50.00 ± 16.67 abc	50.00 ± 16.67 abc	55.56 ± 19.25 ab
MT1/Bendazid	22.22 ± 19.25 cd	22.22 ± 19.25 cd	27.78 ± 9.62 bcd	44.44 ± 25.46 abcd	61.11 ± 25.46 a	61.11 ± 9.62 a
Eco77	44.44 ± 19.25 abcd	44.44 ± 9.62 abcd	50.00 ± 16.67 abc	27.78 ± 25.46 bcd	50.00 ± 16.67 abc	33.33 ± 16.67 abcd

[a] SD = Standard deviation from the mean. Means with the same letter are not significantly different ($P < 0.05$), separately for the 2017/2018 (LSD = 27.78) and 2018/2019 (LSD = 27.88) trials.

[b] Mean incidences were calculated from an incomplete block design as the number of trees that *Trichoderma* spp. was isolated from within an experimental unit (six wounds) replicated in three experimental blocks.

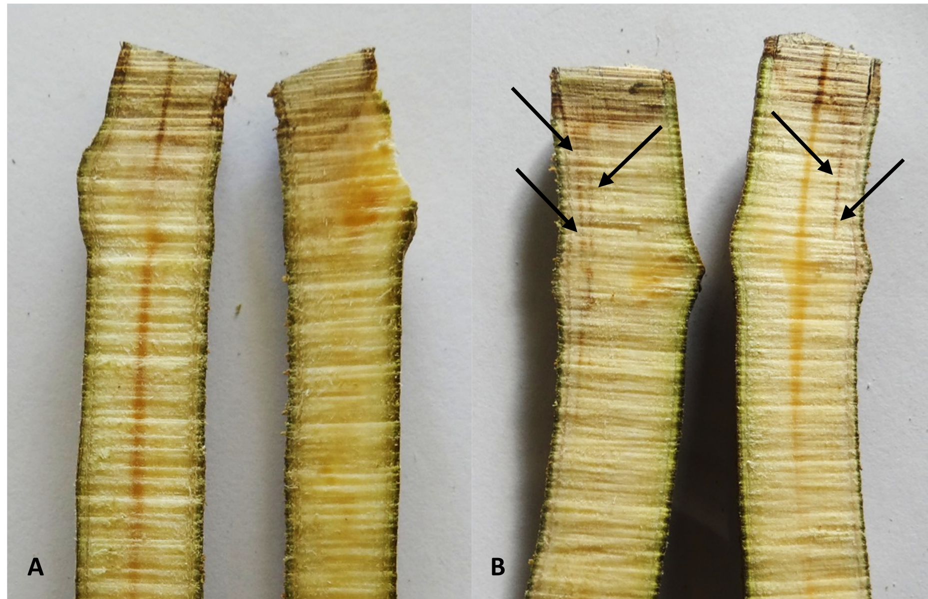

Figure 1. An example of (A) a non-inoculated healthy olive pruning wound and (B) an infected olive pruning wound that was inoculated with *Pseudophaeomoniella* sp. 7 days after pruning. Arrows in (B) indicate streaky lesions formed in the olive shoot 7 months after inoculation.

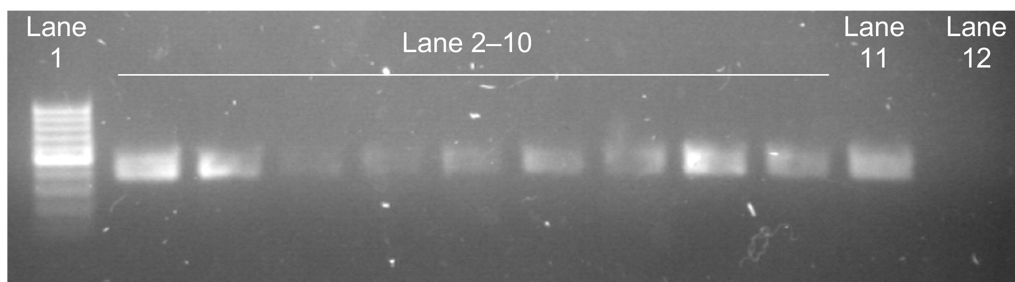

Figure 2. PCR products (400–500bp) of the species-specific primer (ITS40F/ITS525R) tested on representative isolates of *Pseudophaeomoniella* sp. isolated from the pruning wound susceptibility and pruning wound protectant trials. Lane 1 = ladder. Lane 2–10 = representative isolates. Lane 11 = positive control (STE-U 7950) and Lane 12 = non-template control.

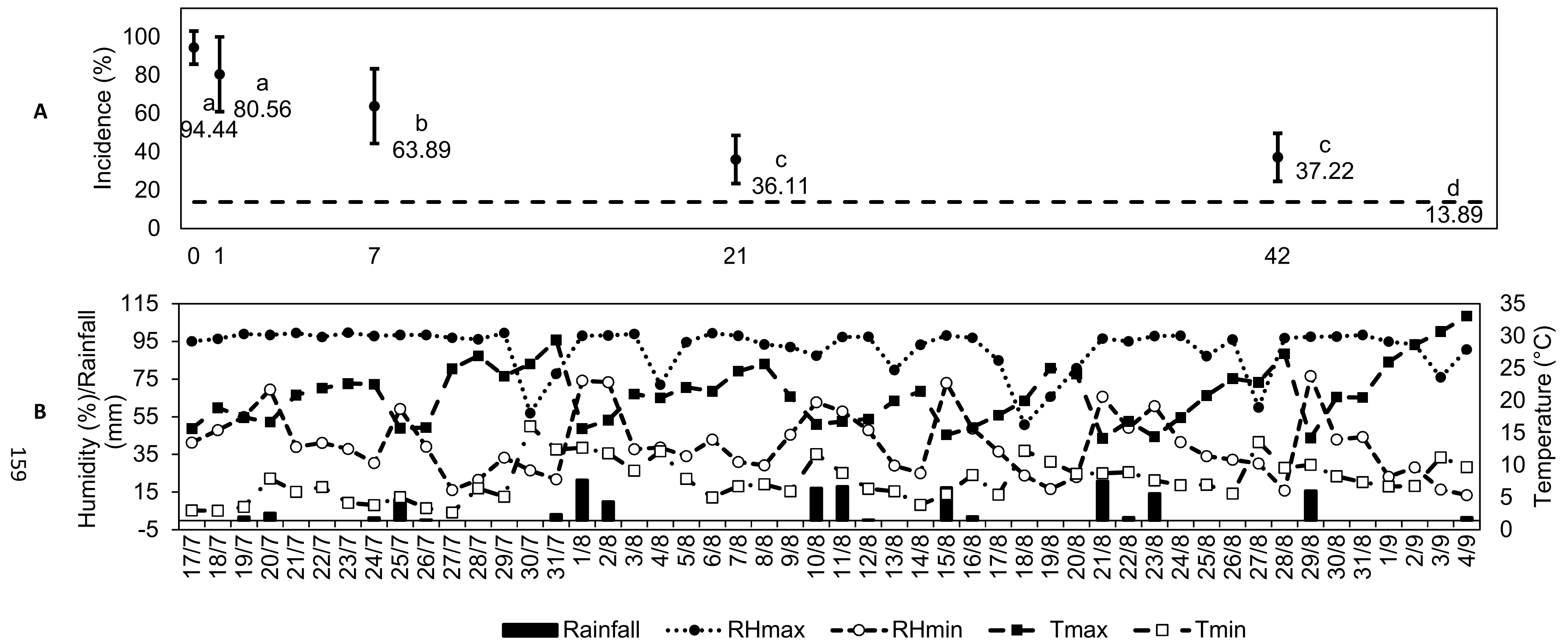

Figure 3. The mean incidence of *Pseudophaeomoniella* sp. isolated from olive pruning wounds and the complimentary weather data during which the inoculations were performed for the winter trial. (A) The incidence of *Pseudophaeomoniella* sp. isolated from wounds inoculated at 0, 1, 7, 21 and 42 days after pruning during winter. The dotted line represents the mean incidence of the natural level of infection by *Pseudophaeomoniella* sp. in the non-inoculated controls (13.89%). Means with the same letter are not significantly different ($P < 0.05$; LSD = 14.90). The vertical bars represent the standard deviation from the mean incidences. (B) The maximum and minimum humidity (RHmax and RHmin), rainfall and the maximum and minimum temperatures (Tmax and Tmin).

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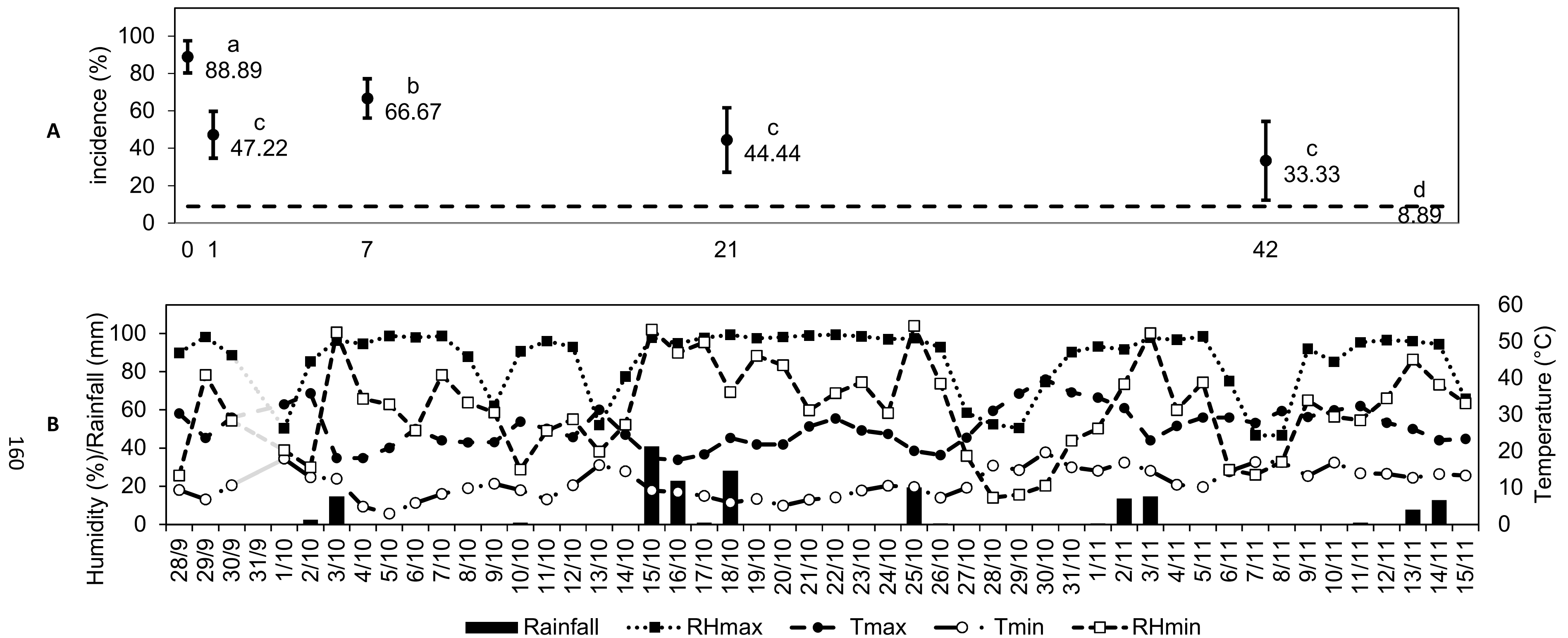

Figure 4. The mean incidence of *Pseudophaeomoniella* sp. isolated from olive pruning wounds and the complimentary weather data for the spring trial. (A) The incidence of *Pseudophaeomoniella* sp. isolated from wounds inoculated at 0, 1, 7, 21 and 42 days after pruning. The dotted line represents the mean incidence of the natural level of infection by *Pseudophaeomoniella* sp. in the non-inoculated controls (8.89%). Means with the same letter are not significantly different ($P < 0.05$; LSD = 17.37). The vertical bars represent the standard deviation from the mean incidences. (B) The maximum and minimum humidity (RHmax and RHmin), rainfall and the maximum and minimum temperatures (Tmax and Tmin). The grey line is extrapolated due to missing data points of 31 September 2017.

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Chapter 6

Conclusions and future prospects

The olive industry in South Africa produces an excellent quality olive oil. However, the Olive Sector Development Plan of the Department of Trade and Industry identified low production and the lack of local research as weaknesses of the olive industry in South Africa. The effective management of pests and diseases, including olive trunk diseases, forms an integral part in improving the lifespan and yield of olive trees. In South Africa, a high diversity of trunk pathogens contributes to the olive trunk disease symptoms found in the Western Cape Province, South Africa (Chapter 2). *Pseudophaeomoniella* sp. was identified as what can be considered as one of the most important olive trunk pathogens in the Western Cape Province, due to its high occurrence in trunk disease symptoms of established orchards and due to its confirmed pathogenicity towards olive trees (van Jaarsveld, 2015; Chapter 2). Currently, the only olive trunk disease management strategies implemented by olive producers in the Western Cape Province include the removal of trunk disease symptoms from the infected olive trees (Costa, 2019) and the application of pruning wound protectants such as Kemseel, Neocil Plus, Tree Seal and copper oxychloride (amended in paint). Other general disease management practises, including those performed in olive nurseries, can further reduce the olive trunk disease incidence in established olive orchards.

In olive nurseries, healthy-looking cuttings are selected from well-maintained olive orchards. These are surface sterilised before dipping the basal end of the cutting into a rooting hormone and placing the cuttings into heated beds, containing sterile perlite, in glasshouses (Costa, 1998; Fabbri *et al*., 2004). The cuttings are rooted and treated with fertilisers and fungicide sprays (Costa, 1998). However, olive nursery material has been identified as inoculum sources of various trunk pathogens (Chapter 3), emphasising the importance of additional disease management strategies within the nurseries, to ensure that pathogen-free material is delivered to producers. The majority of the olive trunk pathogens were detected from the below perlite/soil parts of the plants (Chapter 3), suggesting that the majority of the infections developed from the basal end of the cuttings, that was in direct contact with perlite/soil and water. Further investigation should be performed to determine the components (i.e. perlite, soil and/or water) contaminated by the olive trunk pathogens to develop strategies to reduce infections by these pathogens in olive nurseries. *Phaeoacremonium parasiticum* and *Pleurostoma richardsiae*, two commonly known olive trunk pathogens, were present at high incidences, specifically in the below soil surface parts of 1–2-year old potted olive trees

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(Chapter 3). This suggested strongly that the soil used for the 1–2-year old olive trees were contaminated by *P. parasiticum* and *P. richardsiae*. Further investigations of these pathogens occurring in the soil and 1–2-year old trees should be performed to establish whether this was an isolated event or if it is a chronic occurrence. Pathogen contaminated soils can be pasteurised to eradicate pathogens in the soil. Soil solarisation has been used during the summer months in Mediterranean climatic zones for the control of olive nematode pests (Stapleton *et al*., 1999; Nico *et al*., 2003). After soil solarisation, the soil health can be improved further by supplementing with beneficial micro-organisms, such as mycorrhizal fungi (Estaún *et al*., 2003; Castillo *et al*., 2006; Dag *et al*., 2009; Porras-soriano *et al*., 2006) and/or *Pseudomonas fluorescens* (Mercado-Blanco *et al*., 2004). Heat treatments of the olive nursery propagation material can be studied as an additional disease management strategy. In grapevine nurseries, hot water treatments of the propagation material were used to eradicate trunk pathogens growing inside the wood (Gramaje *et al*., 2009; Bleach *et al*., 2013; Halleen and Fourie, 2016; Bruez *et al*., 2017). Furthermore, hot water and hot air treatments of olive propagation material has been used to eradicate internal infestations of *Verticillium dahliae*, sometimes with adverse effects of the success of tree survival (Morello *et al*., 2016). The practical implications of these control methods in olive nurseries should be evaluated followed by the establishment of the optimal temperature (tree survival vs. olive trunk pathogen eradication) for the stage during which the heat treatment will be applied (fresh cuttings vs. rooted cuttings).

Pseudophaeomoniella sp. was also present in the nurseries, suggesting that the olive nursery trees were also an inoculum source of this pathogen (Chapter 3). However, the incidence of this pathogen in the nurseries was low, suggesting that additional inoculum sources of this pathogen exists elsewhere. Dead wood infected with trunk pathogens in vineyards serve as inoculum sources for short distance dispersal of these pathogens (van Niekerk *et al*., 2010). Olive trunk diseases are currently managed by removing diseased branches from infected trees and orchards, thereby also reducing the inoculum sources of these pathogens within the established orchards (Costa, 2019). The large branches are burned and the smaller branches shredded, and pruning wound protectants are applied to large pruning wounds to avoid new infections from occurring. However, the shredded branches can sustain microbial communities, including *Pseudophaeomoniella* sp. (Chapter 4). Pycnidia of *Pseudophaeomoniella* sp. were detected on the shredded olive pruning debris in established olive orchards (Chapter 4). Little is known regarding the spore release and dispersal mechanisms of this pathogen and this should be explored. Although, it is generally understood that an increase in trunk pathogens spores are released during and after rainfall

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and that these spores can be dispersed by various mechanisms onto susceptible pruning wounds.

The pruning of olive trees is an essential part of maintaining olive production (Costa, 2019). The general disease status of orchards can be improved by pruning for increased light distribution and aeration, which inevitably also improves the uniformity of fungicide spray applications (Costa, 2019). However, pruning wounds are known to be susceptible to trunk pathogens. Furthermore, olive pruning wounds were susceptible to *Pseudophaeomoniella* sp. for up to 42 days during this study (Chapter 5). The susceptibility of the pruning wounds made during winter and spring declined over time, generally with no difference in susceptibility between seasons (winter vs. spring). However, the successful germination of *Pseudophaeomoniella* sp. spores and infection appeared to be influenced by the relative humidity (RH). This indicates that the season during which pruning wounds are made cannot currently be used as a measure to manage olive trunk diseases caused by *Pseudophaeomoniella* sp., rather pruning during low RH can potentially be used as a management strategy against olive trunk diseases in established orchards. Further studies should be performed to test the practical implications of this practise and the degree to which trunk pathogen infections can be reduced when pruning during low RH.

Olive pruning wounds were found to be most susceptible within the first week after pruning (Chapter 5), thus application of a pruning wound protect appears to be critical during this time. Under high inoculum pressure, Tree Seal and Coprox Super/Bendazid consistently provided the best protection against *Pseudophaeomoniella* sp. infections. Tree Seal also provided protection of the pruning wounds during low inoculum pressure. Under low inoculum pressure MT1, a *Trichoderma*-based product, was also able to reduce the incidence of *Pseudophaeomoniella* sp. during both the winter and spring trials when challenged 1 and 7 days after pruning. These results suggest the potential use of MT1 to effectively manage olive trunk diseases of the pruning wounds of 'Frantoio' olive trees in well-maintained orchards that are expected to have a low inoculum pressure. These products, as well as other *Trichoderma*-based pruning wound protectants, should be evaluated further on other olive cultivars in different climatic and geographic areas and against more olive trunk pathogens (such as Botryosphaeriaceae and *Phaeoacremonium* spp.) to establish its full potential in effectively managing olive trunk disease infections from pruning wounds.

In summary, this study identified a high diversity of trunk pathogens contributing to the olive trunk diseases found in the Western Cape Province, with *Pseudophaeomoniella* sp. being identified as one of the main olive trunk pathogens occurring in olive trunk disease

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symptoms (Chapter 2). Several of the olive trunk pathogens were detected in commercial olive tree nurseries (Chapter 3). Further studies should be performed to identify the specific inoculum sources of the individual components such as perlite, soil and/or water. It is of particular interest to determine the pathogen status of the potting soil of the olive tree nurseries, since *P. parasiticum* and *P. richardsiae* were present at high incidences, specifically in the below soil surface parts of 1–2-year old potted olive trees (Chapter 3). The olive nurseries and established orchards were also an inoculum source of *Pseudophaeomoniella* sp. The presence of *Pseudophaeomoniella* sp. pycnidia on shredded olive pruning debris in established orchards (Chapter 4), suggests the potential dispersal of *Pseudophaeomoniella* sp. conidia onto susceptible olive pruning wounds where infection can take place. Olive pruning wounds were susceptible to *Pseudophaeomoniella* sp. for up to 42 days during this study (Chapter 5). The season during which pruning wounds are made cannot currently be used as a measure to manage olive trunk diseases caused by *Pseudophaeomoniella* sp. However, pruning during low RH can potentially be used as a management strategy against olive trunk diseases in established orchards and should be evaluated. The olive pruning wounds were most susceptible within the first week after pruning (Chapter 5), thus application of a pruning wound protect appears to be critical during this time. Tree Seal can be used to reduce inoculum pressure under low and high inoculum pressure, while MT1, a *Trichoderma*-based product, can be used to effectively manage olive trunk diseases of the pruning wounds of 'Frantoio' olive trees in well-maintained orchards that are expected to have a low inoculum pressure (Chapter 5). These pruning wound protectants, as well as other *Trichoderma*-based pruning wound protectants should be evaluated further to establish its full potential in effectively managing olive trunk disease infections. These new findings can be used to aid in the development of management strategies to reduce the olive trunk disease infections occurring in established orchards. This should in turn result in improved longevity and productivity of olive trees in South Africa.

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Appendix A: *Phaeoacremonium minimum* and *Phaeoacremonium parasiticum* species-specific primers

The *Phaeoacremonium minimum* and *Phaeoacremonium parasiticum* species-specific primers were developed by Mostert *et al*. (2006) and further adapted to be used with KAPA Taq ReadyMix (Kapa Biosystems). The DNA of several *Phaeoacremonium* spp. (Table 1) were extracted using a general CTAB DNA extraction method. The integrity of the DNA was first tested using the beta-tubulin (BTUB) primer pairs, T1F (O'Donnell and Gigelnik, 1997) and Bt2bR (Glass and Donaldson, 1995). A PCR of the BTUB region was set up in 10 µl reactions using 1 × KAPA Taq ReadyMix (Kapa Biosystems), 0.2 µM of both T1F and Bt2bR and 1 µL DNA (10 ng/µL). The PCR conditions for BTUB region was 94°C for 5 min, followed with 36 cycles of 94°C for 45 sec, 55°C for 30 sec and 72°C for 1 min 30 sec with a final single step of 72°C for 6 min. The PCR products of these reactions were visualised together with the Generuler™ 100 bp Plus DNA ladders on 1% agarose gels (Fig. 1)

The specificity of several *Phaeoacremonium* spp. were tested in 10 µl reactions containing 1 × KAPA Taq ReadyMix (Kapa Biosystems), 0.16 µM of T1F and 0.16 µM of Pbr6_1 (*P. minimum*) or Pbr2_2 (*P. parasiticum*) and 1 µl DNA (10 ng/µl). The PCR conditions for both *P. minimum* and *P. parasiticum* was a touchdown procedure initiated with 94°C for 5 min followed with 5 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 30 sec, 5 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 30 sec, 25 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec. The final single step was set at 72°C for 7 min. The PCR products of these reactions were visualised together with the Generuler™ 100bp Plus DNA ladders on 1% agarose gels (Fig. 2). The *P. minimum* and *P. parasiticum* species-specific primers were specific when tested against 26 *Phaeoacremonium* species.

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Table 1. South African *Phaeoacremonium* spp. used to test the specificity of the *Phaeoacremonium minimum* and *Phaeoacremonium parasiticum* species-specific primers.

Species	**Strain**[a]	**Host**
Phaeoacremonium album	STE-U 8377 = CSN 660	*Cydonia oblonga*
Phaeoacremonium alvesii	CSN 1239	*Prunus persica*
Phaeoacremonium aureum	STE-U 8371 = CSN 20	*Melia azedarach*
Phaeoacremonium australiense	CSN 490	*Psidium guajava*
Phaeoacremonium bibendum	CBS 142694 = STE-U 8365 = CSN 894*	*Schinus molle*
Phaeoacremonium fraxinopennsylvanicum	CSN 66	*Malus domestica*
Phaeoacremonium globosum	CSN 471	*Cydonia oblonga*
Phaeoacremonium griseo-olivaceum	PMM 1829	*Vitis vinifera*
Phaeoacremonium griseorubrum	PMM 1828	*Vitis vinifera*
Phaeoacremonium inflatipes	CSN 47	*Morus* sp.
Phaeoacremonium iranianum	CSN 170	*Prunus persica* var. *nucipersica*
Phaeoacremonium italicum	CSN 59	*Melia azedarach*
Phaeoacremonium junior	CBS 142695 = STE-U 8398 = CSN 13	*Vitis vinifera*
Phaeoacremonium longicollarum	CBS 142699 = STE-U 8393 = CSN 84*	*Prunus armeniaca*
Phaeoacremonium meliae	CBS 142709 = STE-U 8391 = CSN 256	*Melia azedarach*
Phaeoacremonium minimum	PMM 2073	*Olea europaea* subsp. *europaea*
Phaeoacremonium oleae	CBS 142701 = STE-U 8381 = CSN 403	*Olea europaea* subsp. *cuspidata*

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Species	Strain[a]	Host
Phaeoacremonium parasiticum	CSN 912	*Melia azedarach*
Phaeoacremonium paululum	CBS 142705 = STE-U 8389 = PMM 1914*	*Psidium guajava*
Phaeoacremonium proliferatum	CBS 142706 = STE-U 8368 = PMM 2231*	*Malus domestica*
Phaeoacremonium prunicola	CSN 398	*Cydonia oblonga*
Phaeoacremonium rosicola	CBS 142708 = STE-U 8390 = PMM 1002*	*Rosa* sp.
Phaeoacremonium scolyti	CSN 27	*Melia azedarach*
Phaeoacremonium sicilianum	CSN 930	*Juglans* sp.
Phaeoacremonium spadicum	CBS 142714 = STE-U 8388 = CSN 49	*Rhoicissus tomentosa*
Phaeoacremonium subulatum	CSN 42	*Pyrus communis*
Phaeoacremonium viticola	CSN 678	*Cydonia oblonga*

[a] CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CSN, collection of Chris Spies at ARC-Nietvoorbij, Stellenbosch, South Africa; PMM, collection of Providence Moyo at Stellenbosch University, Department of Plant Pathology, Stellenbosch; STE-U, fungal collection of Stellenbosch University, Department of Plant Pathology, Stellenbosch.

* Type strains

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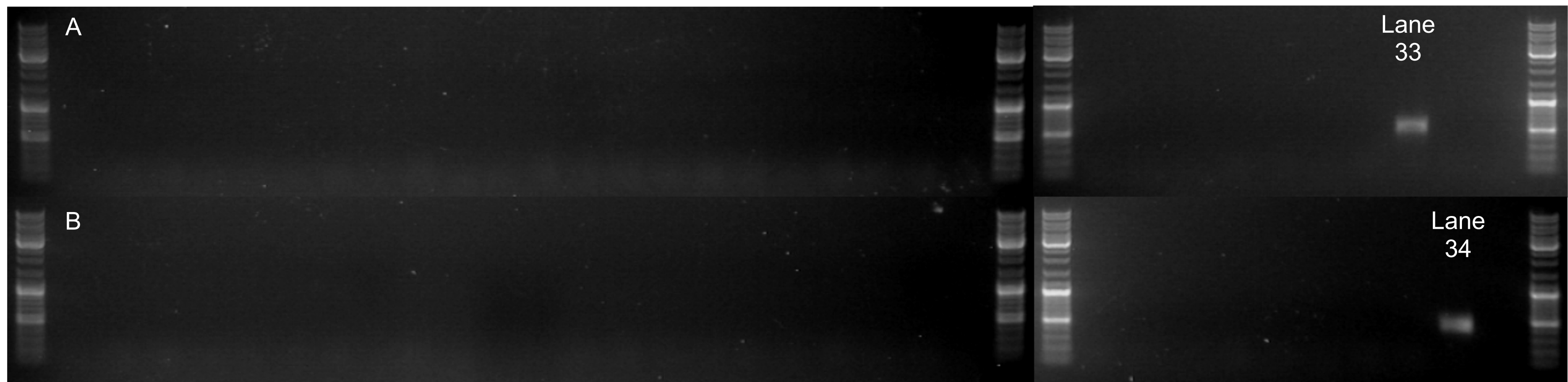

Figure 1. PCR products of the species-specific primers used for the identification of A) *Phaeoacremonium minimum* and B) *Phaeoacremonium parasiticum.* Ladders and PCR products were loaded in the same order for 'A' and 'B'. Lanes 1, 24, 25 and 36 were the DNA ladders. Lanes 2-23 and 26-34 are the PCR products of the *Phaeoacremonium* spp. with Lane 33 being *P. minimum* and Lane 34 being *P. parasiticum*. Lane 35 is the non-template control.

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APPENDIX B: Supplementary statistical analyses output of Chapter 2

Table 1. Analysis of variance of the lesion lengths (mm) formed by the inoculated fungal species on detached 2-year-old shoots of the 'Frantoio' olive cultivar during the detached shoot virulence screening.

Source	Degrees of Freedom	Sum of Squares (Type I)	Mean Square	F-Value	Pr > F
Trial	1	0.07	0.07	3.06	0.08
Trial (container)	88	5.28	0.06	2.66	0.00
Species x Isolate	98	6.59	0.07	2.98	0.00
Species x Isolate x Trial	98	2.72	0.03	1.23	0.08

Table 2. Analysis of variance of the lesion lengths (mm) formed by the inoculated fungal species on 2-year-old shoots of the 'Frantoio' olive cultivar in field trials.

Source	Degrees of Freedom	Sum of Squares (Type I)	Mean Square	F-value	Pr > F
Trial	1	70.10	70.10	96.78	0.00
Trial (tree)	90	240.37	2.67	3.69	0.00
Species x Isolate	65	161.64	2.49	3.43	0.00
Species x Isolate x Trial	65	59.47	0.91	1.26	0.08

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APPENDIX C: Reference DNA sequences used for the phylogenetic analyses in Chapter 3.

Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Cadophora* and**	*Cadophora fastigiata*	CBS 307.49 = A 168	AY249073	Harrington and Mcnew, 2003
Graphium	*Cadophora finlandica*	CBS 444.86 = P 60*	AY249074	Harrington and Mcnew, 2003
	Cadophora gregata	P 21	AY249071	Harrington and Mcnew, 2003
		P 19	AY249070	Harrington and Mcnew, 2003
	Cadophora luteo-olivacea	A 208	AY249067	Harrington and Mcnew, 2003
		CBS 141.41 = A 175*	AY249066	Harrington and Mcnew, 2003
	Cadophora malorum	CBS 100584 = A 173	AY249062	Harrington and Mcnew, 2003
		CBS 266.31 = A 163*	AY249057	Harrington and Mcnew, 2003
	Cadophora melinii	CBS 268.33 = A 164*	AY249072	Harrington and Mcnew, 2003
	Graphium rubrum	ATCC 24593 = C 1223*	AF198245	Harrington and Mcnew, 2003
	Graphium silanum	CBS 206.37 = C 1221	AY249065	Harrington and Mcnew, 2003
Coniochaeta	*Coniochaeta africana*	CBS 120868 = STE-U 5952*	NR137725	Reviewed
	Coniochaeta canina	UTHSC 11-2460*	NR120211	Reviewed
	Coniochaeta cateniformis	UTHSC 01-1644*	NR111517	Reviewed
	Coniochaeta cymbiformispora	NBRC 32199*	LC146726	Ban *et al.*, unpublished

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Coniochaeta **(cont.)**	*Coniochaeta decumbens*	CBS 153.42*	NR144912	Reviewed
	Coniochaeta decumbens	UFMGCB 9950	KU727747	Ferreira *et al*., unpublished
	Coniochaeta fasciculata	CBS 205.38*	NR154770	Reviewed
	Coniochaeta gigantospora	ILLS 60816*	NR121521	Reviewed
	Coniochaeta hoffmannii	CBS 245.38*	NR111518	Reviewed
	Coniochaeta lignicola	CBS 267.33*	NR111520	Reviewed
	Coniochaeta luteorubra	UTHSC 01-20	HE610330	Perdomo *et al*., unpublished
	Coniochaeta luteoviridis	CBS 206.38*	NR154769	Reviewed
	Coniochaeta mutabilis	CBS 157.44*	NR111519	Reviewed
	Coniochaeta navarrae	CBS 141016*	NR154808	Reviewed
	Coniochaeta nepalica	NBRC 30584*	LC146727	Ban *et al*., unpublished
	Coniochaeta polymorpha	CBS 132722*	NR121473	Reviewed
	Coniochaeta prunicola	CBS 120875 = STE-U 6107*	NR137037	Reviewed
	Coniochaeta velutina	CBS 120874 = STE-U 5950	GQ154542	Damm *et al*., 2010
		STE-U 6106	GQ154545	Damm *et al*., 2010

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Dactylonectria	*Dactylonectria alcacerensis*	CBS 129087 = Cy 159*	JF735630	Lombard *et al*., 2015
	Dactylonectria amazonica	MUCL 55433*	MF683686	Gordillo and Decock, 2017
	Dactylonectria amazonica	MUCL 55430	MF683685	Gordillo and Decock, 2017
	Dactylonectria anthuriicola	CBS 564.95 = PD 95/1577*	JF735579	Lombard *et al*., 2014
	Dactylonectria ecuadoriensis	MUCL55432	MF683681	Gordillo and Decock, 2017
		MUCL52226	MF683682	Gordillo and Decock, 2017
	Dactylonectria estremocensis	CBS 129085 = Cy 145	JF735617	Lombard *et al*., 2015
	Dactylonectria hispanica	CBS 142827 = Cy-FO-45	KY676864	Mora-Sala *et al*., unpublished
	Dactylonectria hordeicola	EFA 443	MF471471	Costas *et al*., unpublished
	Dactylonectria macrodidyma	CBS 112615 = STE-U 3976 = C 98*	JF735647	Lombard *et al*., 2015
	Dactylonectria novozelandica	CBS 113552 = STE-U 5713 = HJS 1306 = NZ C 41*	JF735633	Lombard *et al*., 2015
	Dactylonectria pauciseptata	CBS 120172	JF735588	-
		CBS 100819 = LYN 16202/2	JF735582	Lombard *et al*., 2014
	Dactylonectria pinicola	Cy-FO-177	KX709555	Mora-Sala *et al*., unpublished
	Dactylonectria torresensis	CBS 129086 = Cy 218*	JF735681	Lombard *et al*., 2015
	Dactylonectria valentina	CBS 142826 = Cy-FO-133	KY676863	Mora-Sala *et al*., unpublished

Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Dactylonectria* (cont.)**	*Dactylonectria vitis*	CBS 129082 = Cy 233*	JF735580	Lombard *et al*., 2014
Diaporthe	*Diaporthe acaciigena*	CBS 129521 = CPC 17622*	KC343973	Gomes *et al*., 2013
	Diaporthe alleghaniensis	CBS 495.72 = ATCC 24097*	KC343975	Gomes *et al*., 2013
	Diaporthe amygdali	CBS 126679*	KC343990	Gomes *et al*., 2013
	Diaporthe anacardii	CBS 720.97*	KC343992	Gomes *et al*., 2013
	Diaporthe angelicae	CBS 111592 = AR 3776*	KC343995	Gomes *et al*., 2013
	Diaporthe arengae	CBS 114979 = HKUCC 5527*	KC344002	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe aspalathi	CBS 117169 = STE-U 5428 = CPC 5428*	KC344004	Gomes *et al*, 2013
	Diaporthe australafricana	CBS 111886 = STE-U 2676 = CPC 2676*	KC344006	Gomes *et al*., 2013
	Diaporthe baccae	CBS 136972*	MF418509	Guarnaccia and Crous, 2017
	Diaporthe brasiliensis	CBS 133183 = LGMF 924 = CPC 20300*	KC344010	Gomes *et al*., 2013
	Diaporthe caulivora	CBS 127268 = Dpc 1*	KC344013	Gomes *et al*., 2013
	Diaporthe crotalariae	CBS 162.33*	KC344024	Gomes *et al*., 2013
	Diaporthe cuppatea	CBS 117499 = STE-U 5431 = CPC5431*	KC344025	Gomes *et al*., 2013

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Diaporthe* (cont.)**	*Diaporthe cynaroidis*	CBS 122676 = CMW 22190 = CPC 13180*	KC344026	Gomes *et al*., 2013
	Diaporthe eres	CBS 439.82 = BBA P-407 = IMI 162181a*	KC344058	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe foeniculina	CBS 123208 = Di-C004/5*	KC344072	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
		CBS 123209 = Di-C004/4*	KC344073	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
		CBS 187*	KC344075	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe ganjae	CBS 180.91 = ILLS 43621*	KC344080	Gomes *et al*., 2013
	Diaporthe helianthi	CBS 592.81*	KC344083	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe hickoriae	CBS 145.26*	KC344086	Gomes *et al*., 2013
	Diaporthe hongkongensis	CBS 115448 = HKUCC 9104 = AT 646 DF 24*	KC344087	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe inconspicua	CBS 133813 = LGMF 930 = CPC 20306*	KC344091	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe limonicola	CBS 142549 = CPC 28200*	MF418582	Guarnaccia and Crous, 2017
	Diaporthe lusitanicae	CBS 123212 = Di-C001/5*	KC344104	Gomes *et al*., 2013
	Diaporthe musigena	CBS 129519 = CPC 17026*	KC344111	Gomes *et al*., 2013

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Diaporthe* (cont.)**	*Diaporthe melitensis*	CBS 142551 = CPC 27873*	MF418584	Guarnaccia and Crous, 2017
	Diaporthe neoarctii	CBS 109490 = GB 6421 = AR 3450*	KC344113	Gomes *et al*., 2013
	Diaporthe novem	CBS 127270*	KC344124	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe pseudomangiferae	CBS 101339*	KC344149	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe pseudophoenicicola	CBS 462.69*	KC344152	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe raonikayaporum	CBS 133182 = LGMF 923 = CPC 20299*	KC344156	Gomes *et al*., 2013
	Diaporthe rudis	CBS 113201*	KC344202	Guarnaccia and Crous, 2017
	Diaporthe saccarata	CBS 116311 = STE-U 3743 = CPC 3743*	KC344158	Gomes *et al*., 2013; Guarnaccia and Crous, 2017
	Diaporthe toxica	CBS 534.93 = ATCC 96741*	KC344188	Gomes *et al*., 2013
	Diaporthe vaccinii	CBS 160.32 = IFO 32646*	KC344196	Gomes *et al*., 2013
Heterotruncatella	*Heterotruncatella acacigena*	CBS 143880 = CPC 15130*	NR161094	Reviewed
	Heterotruncatella aspera	CBS 143907 = CPC 28992*	MH554159	Liu *et al*., 2019
	Heterotruncatella avellanea	CBS 143896 = CPC 25377*	NR161104	Reviewed
	Heterotruncatella breviappendiculata	CBS 143883 = CPC 17239*	NR161096	Reviewed

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Heterotruncatella* (cont.)**	*Heterotruncatella constricta*	CBS 143901 = CPC 27578*	NR161106	Reviewed
	Heterotruncatella diversa	CBS 143908 = CPC29040*	NR161107	Reviewed
	Heterotruncatella grevilleae	CBS 143881 = CPC 16997*	NR161095	Reviewed
	Heterotruncatella longissima	CBS 143910 = CPC 29114*	NR161109	Reviewed
	Heterotruncatella proteicola	CBS 144020 = CPC 13700*	MH554077	Liu *et al*., 2019
	Heterotruncatella quercicola	CBS 143895 = CPC 25365*	MH554135	Liu *et al*., 2019
	Heterotruncatella restionacearum	CBS 119210 = CMW 18755*	NR160986	Reviewed
	Heterotruncatella singularis	CBS 144031 = CPC 29042*	MH554161	Liu *et al*., 2019
	Heterotruncatella sp.	CBS 144022 = CMW 22230 = CPC 17913	MH554099	Liu *et al*., 2019
	Heterotruncatella spadicea	CBS 118145 = CMW 17958 = SL 0762*	DQ278912	Liu *et al*., 2019
	Heterotruncatella spartii	MFLUCC 15-0537*	NR154504	Reviewed
	Heterotruncatella synapheae	CBS 143909 = CPC 29096*	MH554164	Liu *et al*., 2019
	Heterotruncatella vinaceobubalina	CBS 143897 = CPC 26201*	MH554139	Liu *et al*., 2019
Ilyonectria	*Ilyonectria capensis*	CBS 132815	JX231135	Lombard *et al*., 2015
	Ilyonectria coprosmae	CBS 119606	JF735505	Lombard *et al*., 2015

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Ilyonectria* (cont.)**	*Ilyonectria crassa*	CBS 158.31 = IMI 061536 = NRRL 6149	JF735535	Cabral *et al.*, 2012
	Ilyonectria cyclaminicola	CBS 302.93*	JF735581	Lombard *et al.*, 2014
	Ilyonectria destructans	CBS 264.65*	JF735506	Lombard *et al.*, 2015
	Ilyonectria europaea	CBS 537.92	JF735568	Lombard *et al.*, 2014
	Ilyonectria gamsii	CBS 940.97*	JF735577	Lombard *et al.*, 2014
	Ilyonectria leucospermi	CBS 132809	JX231145	Lombard *et al.*, 2015
	Ilyonectria liliigena	CBS 189.49 = IMI 113882*	JF735573	Lombard *et al.*, 2014
	Ilyonectria liriodendri	CBS 117527 = Cy 76	JF735509	Lombard *et al.*, 2015
	Ilyonectria lusitanica	CBS 129080 = Cy 197*	JF735570	Lombard *et al.*, 2014
	Ilyonectria mors-panacis	CBS 306.35*	JF735557	Lombard *et al.*, 2014
	Ilyonectria palmarum	DiGeSA-BRA1	HF922618	-
	Ilyonectria panacis	CDC-N-9a*	JF735572	Lombard *et al.*, 2014
	Ilyonectria pseudodestructans	CBS 117824 = IFFF 98	JF735562	Lombard *et al.*, 2014
	Ilyonectria robusta	CBS 308.35*	JF735518	Lombard *et al.*, 2014
	Ilyonectria rufa	CBS 153.37*	JF735540	Lombard *et al.*, 2014
	Ilyonectria venezuelensis	CBS 102032 = ATCC 208837 = AR 2553*	JF735571	Lombard *et al.*, 2014

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Neofusicoccum	*Neofusicoccum algeriense*	CBS 135704*	KX505893	Lopes *et al*., 2016
	Neofusicoccum andinum	CBS 117453 = PD 252*	GU251287	Lopes *et al*., 2016
	Neofusicoccum arbuti	CBS 116131*	KF531792	Lopes *et al*., 2016
	Neofusicoccum australe	CMW 6837*	AY339270	Lopes *et al*., 2016
	Neofusicoccum batangarum	CBS 124924 = CMW 28363*	FJ900653	Lopes *et al*., 2016
	Neofusicoccum brasiliense	CMM 1338*	JX513610	Lopes *et al*., 2016
	Neofusicoccum cordaticola	CBS 123634 = CMW 13992*	EU821868	Lopes *et al*., 2016
	Neofusicoccum cryptoaustrale	CMW 20738	FJ752710	Lopes *et al*., 2016
		CMW 23785	FJ752713	Lopes *et al*., 2016
	Neofusicoccum eucalypticola	CBS 115679 = CMW 6539*	AY615133	Lopes *et al*., 2016
	Neofusicoccum eucalyptorum	CBS 115791 = CMW 10125 = BOT 24*	AY236891	Lopes *et al*., 2016
	Neofusicoccum hellenicum	CERC 1947*	KP217061	Lopes *et al*., 2016
	Neofusicoccum kwambonambiense	CBS 123639 = CMW 14023*	EU821870	Lopes *et al*., 2016
	Neofusicoccum luteum	CBS 110299*	AY573217	Lopes *et al*., 2016
	Neofusicoccum macroclavatum	CBS 118223 = WAC 12444*	DQ093217	Lopes *et al*., 2016
	Neofusicoccum mangiferae	CBS 118531 = CMW 7024	DQ093221	Lopes *et al*., 2016

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Neofusicoccum	*Neofusicoccum mangiferae*	CBS 118532 = CMW 7797	DQ093220	Lopes *et al*., 2016
(cont.)	*Neofusicoccum mediterraneum*	CBS 121718 = PD 312*	GU251308	Lopes *et al*., 2016
	Neofusicoccum nonquaesitum	CBS 126655 = PD 484*	GU251295	Lopes *et al*., 2016
	Neofusicoccum parvum	CMW 9081 = ICMP 8003*	AY236888	Lopes *et al*., 2016
	Neofusicoccum protearum	MUCC 497	EF591965	Lopes *et al*., 2016
	Neofusicoccum ribis	CBS 115475 = CMW 7772*	AY236877	Lopes *et al*., 2016
	Neofusicoccum sp. 8	ID 847	n/a	Du plessis, unpublished
	Neofusicoccum stellenboschiana	CBS 282	KX464758	-
	Neofusicoccum umdonicola	CBS 123645 = CMW 14058*	EU821874	Lopes *et al*., 2016
	Neofusicoccum vitifusiforme	5H022	KF779059	Lopes *et al*., 2016
Didymosphaeriaceae	*Didymosphaeria rubi-ulmifolii* (*Paraconiothyrium brasiliense*)	CBS 100299*	AY642531	Verkley *et al*., 2014
	Didymosphaeria sp. *(Paraconiothyrium brasiliense)*	CBS 122321	JX496034	Verkley *et al*., 2014
		CBS 115.92	JX496022	Verkley *et al*., 2014
	Didymosphaeria variabile	CBS 121164	JX496028	Verkley *et al*., 2014
		CBS 120014	JX496026	Verkley *et al*., 2014
	Microsphaeropsis pseudaspera	CBS 113682	JX496021	Verkley *et al*., 2014

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Didymosphaeriaceae	*Microsphaeropsis* sp.	CBS 978.95	JX496120	Verkley *et al*., 2014
(cont.)	*Paracamarosporium fungicola*	CBS 113269*	JX496020	Verkley *et al*., 2014
	Paracamarosporium hawaiiense	CBS 120025*	JX496027	Verkley *et al*., 2014
	Paraconiothyrium archidendri	CBS 168.77*	JX496049	Verkley *et al*., 2014
	Paraconiothyrium cyclothyrioides	CBS 972.95*	AY642529	Verkley *et al*., 2014
	Paraconiothyrium estuarinum	CBS 109850*	AY642530	Verkley *et al*., 2014
	Paraconiothyrium fuckelii	MFLUCC 13-0073	KJ939278	Ariyawansa *et al*., unpublished
	Paraconiothyrium magnoliae	MFLUCC 10-0278 = HA-2014*	KJ939280	Ariyawansa *et al*., unpublished
	Paraphaeosphaeria angularis	CBS 167.70*	JX496047	Verkley *et al*., 2014
	Paraphaeosphaeria arecacearum	CBS 158.75*	JX496043	Verkley *et al*., 2014
	Paraphaeosphaeria michotii	MFLUCC 13-0349	KJ939279	Ariyawansa *et al*., unpublished
	Paraphaeosphaeria minitans	CBS 111750	JX496017	Verkley *et al*., 2014
	Paraphaeosphaeria neglecta	CBS 124078	JX496039	Verkley *et al*., 2014
	Paraphaeosphaeria pilleata	CBS 102207	JX496013	Verkley *et al*., 2014
	Paraphaeosphaeria sardoa	CBS 501.71*	JX496094	Verkley *et al*., 2014
	Paraphaeosphaeria sp.	CBS 101464	JX496012	Verkley *et al*., 2014

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Didymosphaeriaceae	*Paraphaeosphaeria sporulosa*	CBS 218.68*	JX496054	Verkley *et al*., 2014
(cont.)		CBS 690.70	JX496110	Verkley *et al*., 2014
	Paraphaeosphaeria verruculosa	CBS 263.85	JX496059	Verkley *et al*., 2014
	Paraphaeosphaeria viridescens	CBS 854.73*	JX496085	Verkley *et al*., 2014
	Pseudocamarosporium africanum	CBS 121166*	JX496029	Verkley *et al*., 2014
Phaeomoniellales	*Aequabiliella effusa*	CBS 120883 = STE-U 6121*	NR132005	Reviewed
	Celerioriella dura	CBS 120882 = STE-U 6122*	NR132004	Reviewed
	Celerioriella prunicola	CBS 120876 = STE-U 6118*	NR132003	Reviewed
	Paraphaeomoniella capensis	CBS 123535 = CPC 15416*	NR137711	Reviewed
	Paraphaeomoniella chlamydospora	CBS 229.95*	NR155612	Reviewed
	Paraphaeomoniella pinifoliorum	CBS 114903 = CW 202*	NR160218	Reviewed
	Phaeomoniella sp.	Z 133	JN628178	-
	Phaeomoniella sp.	CBS 121944	n/a	-
	Phaeomoniella sp. CFJ-2015f	CSN 1191	n/a	Spies *et al*., unpublished
	Phaeomoniella sp. PMM-2014b	PMM 1193	n/a	Spies *et al*., unpublished

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Phaeomoniellales (cont.)	*Phaeomoniella* sp. WVJ-2015a	CSN 801	n/a	Spies *et al*., unpublished
		CSN 1091	n/a	Spies *et al*., unpublished
	Pseudophaeomoniella oleae	CBS 139191 = FV 84*	NR137966	Reviewed
	Pseudophaeomoniella oleicola	CBS 139192 = M 24*	NR137965	Reviewed
	Pseudophaeomoniella sp.	CSN 18 = STE-U 7946	n/a	Spies *et al*., unpublished
		CSN 185 = STE-U 7951*	n/a	Spies et al., unpublished
		CSN 808 = STE-U 7963	n/a	Spies *et al*., unpublished
	Xenocylindrosporium sp. CFJS-2015b	CSN 1179	n/a	Spies *et al*., unpublished
		CSN 1216	n/a	Spies *et al*., unpublished
	Xenocylindrosporium sp. CFJS-2015c	CSN 1203	n/a	Spies *et al*., unpublished
		CSN 1180	n/a	Spies *et al*., unpublished
	Xenocylindrosporium sp. CFJS-2015e	CSN 1222	n/a	Spies *et al*., unpublished
Phaeoacremonium	*Phaeoacremonium africanum*	CSN 946	KY906773	Spies *et al*., 2018
		CSN 871	KY906755	Spies *et al*., 2018
	Phaeoacremonium album	CBS 142688 = STE-U 8379 = PMM 1938*	KY906885	Spies *et al*., 2018
	Phaeoacremonium alvesii	PMM 744	KY906823	Spies *et al*., 2018

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Phaeoacremonium	*Phaeoacremonium alvesii*	CSN 1239	KY906785	Spies *et al*., 2018
(cont.)	*Phaeoacremonium aureum*	CBS 142691 = STE-U 8372 = CSN 23*	KY906657	Spies *et al*., 2018
	Phaeoacremonium australiense	PMM 1826	KY906849	Spies *et al*., 2018
	Phaeoacremonium australiense	CSN 490	KY906729	Spies *et al*., 2018
	Phaeoacremonium bibendum	CBS 142694 = STE-U 8365 = CSN 894*	KY906759	Spies *et al*., 2018
	Phaeoacremonium fraxinopennsylvanicum	CSN 66	KY906681	Spies *et al*., 2018
	Phaeoacremonium gamsii	CBS 142712 = STE-U 8366 = CSN 670*	KY906741	Spies *et al*., 2018
	Phaeoacremonium geminum	CBS 142713 = STE-U 8402 = C 741 = CSN 1944*	KY906649	Spies *et al*., 2018
	Phaeoacremonium globosum	CSN 1258	KY906797	Spies *et al*., 2018
		CSN 471	KY906725	Spies *et al*., 2018
	Phaeoacremonium griseo-olivaceum	PMM 1829	KY906853	Spies *et al*., 2018
	Phaeoacremonium griseorubrum	PMM 1895	KY906875	Spies *et al*., 2018
		PMM 1828	KY906851	Spies *et al*., 2018

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Phaeoacremonium	*Phaeoacremonium inflatipes*	PMM 739	KY906821	Spies *et al*., 2018
(cont.)		CSN 47	KY906665	Spies *et al*., 2018
	Phaeoacremonium inflatipes			
	Phaeoacremonium iranianum	PMM 2248	KY906913	Spies *et al*., 2018
		CSN 170	KY906695	Spies *et al*., 2018
	Phaeoacremonium italicum	PMM 731	KY906819	Spies *et al*., 2018
	Phaeoacremonium italicum	CSN 59	KY906677	Spies *et al*., 2018
	Phaeoacremonium junior	CBS 142697 = STE-U 8397 = CSN 273*	KY906709	Spies *et al*., 2018
	Phaeoacremonium longicollarum	CBS 142699 = STE-U 8393 = CSN 84*	KY906689	Spies *et al*., 2018
	Phaeoacremonium meliae	CBS 142710 = STE-U 8392 = PMM 975*	KY906825	Spies *et al*., 2018
	Phaeoacremonium minimum	PMM 1305	KY906837	Spies *et al*., 2018
		CSN 668	KY906739	Spies *et al*., 2018
	Phaeoacremonium oleae	CBS 142704 = STE-U 8385 = PMM 2440*	KY906937	Spies *et al*., 2018
	Phaeoacremonium parasiticum	PMM 1978	KY906889	Spies *et al*., 2018
		CSN 24	KY906659	Spies *et al*., 2018

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
***Phaeoacremonium* (cont.)**	*Phaeoacremonium paululum*	CBS 142705 = STE-U 8389 = PMM 1914*	KY906881	Spies *et al.*, 2018
	Phaeoacremonium proliferatum	CBS 142706 = STE-U 8368 = PMM 2231*	KY906903	Spies *et al.*, 2018
	Phaeoacremonium prunicola	PMM 1318	KY906841	Spies *et al.*, 2018
		ID 230	KY906817	Spies *et al.*, 2018
	P. prunicola	CSN 398	KY906717	Spies *et al.*, 2018
	Phaeoacremonium rosicola	CBS 142708 = STE-U 8390 = PMM 1002*	KY906831	Spies *et al.*, 2018
	Phaeoacremonium scolyti	PMM 1853	KY906861	Spies *et al.*, 2018
		CSN 27	KY906661	Spies *et al.*, 2018
	Phaeoacremonium spadicum	CBS 142711 = STE-U 8386 = PMM 1315*	KY906839	Spies *et al.*, 2018
	Phaeoacremonium subulatum	PMM 1839	KY906855	Spies *et al.*, 2018
		CSN 42	KY906663	Spies *et al.*, 2018
	Phaeoacremonium venezuelense	PMM 1138	KY906835	Spies *et al.*, 2018
	Phaeoacremonium viticola	PMM 1863	KY906863	Spies *et al.*, 2018
		CSN 678	KY906745	Spies *et al.*, 2018

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Pleurostoma	*Pleurostoma ochraceum*	CBS 131321*	NR136033	Reviewed
	Pleurostoma ootheca	CBS 115329*	NR136009	Reviewed
	Pleurostoma repens	CBS 294.39*	NR135925	Reviewed
	Pleurostoma richardsiae	CBS 270.33 = IFM 50539*	AB364696	Reviewed
Outgroups	*Alloconiothyrium aptrootii*	CBS 981.95	JX496122	Verkley *et al*., 2014
	Calosphaeria africana	STE-U 6182	EU367464	Spies *et al*., 2018
	Collophora paarla	CBS 120877 = STE-U 6114*	NR119749	Reviewed
	Collophora rubra	CBS 120873 = STE-U 6109*	NR119747	Reviewed
	Cosmospora arxii	CBS 748.69*	NR145062	Reviewed
	Cosmospora butyri	CBS 301.38*	NR145028	Reviewed
	Diaporthella corylina	CBS 121124 = AR 4131*	KC343972	Guarnaccia and Crous, 2017
	Dothiorella iberica	CBS 115041*	AY573222	Lopes *et al*., 2016
	Dothiorella sarmentorum	IMI 63581b*	AY573235	Lopes *et al*., 2016
	Gliocladiopsis irregularis	CBS 755.97	JQ666023	Lombard *et al*., 2015
	Gliocladiopsis pseudotenuis	CBS 116074	JQ666030	Lombard *et al*., 2015
	Hymenopleella endophytica	EML-AS5-1*	KX216520	Liu *et al*., 2019

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Taxon	Species	Strains[a]	Accession number[b]	Publication[c]
Outgroups (cont.)	*Hymenopleella hippophaeicola*	CBS 140410*	NR154078	Reviewed
	Jattaea algeriensis	STE-U 6201	EU367466	Spies *et al*., 2018
	Kalmusia ebuli	CBS 123120	KF796674	Zhang *et al*., unpublished
	Melanconis alni	CBS 109773 = AR 3500	EU219102	-
	Phialocephala dimorphospora	CBS 300.62 = P 59	AY249075	Harrington and Mcnew, 2003
	Phialocephala fortinii	CBS 443.86 = P 58*	AY249076	Harrington and Mcnew, 2003
	Phialophora tarda	CBS 111589*	NR146251	Reviewed
	Phialophora verrucosa	CBS 140325 = BMU 07506*	NR146242	Reviewed

[a] A, C and P = Culture collection of T. C. Harrington, Iowa State University, U.S.A.; AR = Collection of A.Y. Rossman; ATCC: American Type Culture Collection, U.S.A.; BBA = Biologische Bundesanstalt für Land-und Forstwirtschaft, Berlin-Dahlem, Germany; CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CERC = China Eucalypt Research Center, Beijing, China; CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC = Culture collection of Pedro Crous housed at CBS; CSN = collection of Chris Spies at ARC-Nietvoorbij, Stellenbosch, South Africa; Cy = *Cylindrocarpon* collection housed at Laboratório de Patologia Vegetal "Veríssimo de Almeida" - ISA, Lisbon, Portugal; HJS = Collection of H.-J. Schroers; HKUCC = University of Hong Kong Culture Collection, Department of Ecology and Biodiversity, Hong Kong, China; ICMP = International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; IFFF = Institute of Forest Entomology, Forest Pathology and Forest Protection, Austria; IFO = Institute for Fermentation, Osaka, Japan; IMI = International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; LGMF = Culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; LYN = Lynchburg College, Biology Department, U.S.A.; MFLU(CC) = Mae Fah Luang University Culture Collection; MUCC = Murdoch University Culture Collection, Perth, Australia; MUCL = Mycothèque de l'Université Catholique de Louvain, Belgium; NBRC = NITE Biological Resource Center, Japan; NRRL = Agricultural Research Service Culture Collection, U.S.A.; NZ = Collection of L. Castlebury; PD: Collection of the Dutch National Plant Protection Organization (NPPO-NL), Wageningen, The Netherlands; PMM = collection of Providence Moyo at Stellenbosch University, Department of Plant Pathology, Stellenbosch; STE-U = fungal

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collection of Stellenbosch University, Department of Plant Pathology, Stellenbosch; WAC = Department of Agriculture, Western Australia Plant Pathogen Collection, Perth. Type and ex-type strains are notated with an asterisk (*).

[b] Genbank accession number.

[c] Reference strain selected from the respective articles. Reviewed = accession curated by NCBI staff.

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APPENDIX D: Supplementary statistical analyses output of Chapter 5

Table 1. Analysis of variance of the incidence of *Pseudophaeomoniella* sp. isolated from the experimental treatments during the winter pruning wound susceptibility field trials.

Source	Degrees of Freedom	Sum of Squares (Type I)	Mean Square	F-value	Pr > F
Trial	1	89.20	89.20	0.58	0.45
Trial (Replicates)	4	2153.09	538.27	3.52	0.03
Treatments	5	27890.43	5578.09	36.44	0.00
Trial x Treatments	5	668.21	133.64	0.87	0.52

Table 2. Analysis of variance of the incidence of *Pseudophaeomoniella* sp. isolated from the experimental treatments during the spring pruning wound susceptibility field trials.

Source	Degrees of Freedom	Sum of Squares (Type I)	Mean Square	F-value	Pr > F
Trial	1	25.00	25.00	0.12	0.73
Trial (Replicates)	4	735.80	183.95	0.88	0.49
Treatments	5	22668.21	4533.64	21.79	0.00
Trial x Treatments	5	976.85	195.37	0.94	0.48

Table 3. Analysis of variance of the percentage *Pseudophaeomoniella* sp. isolated from the experimental treatments during the pruning wound susceptibility field trials over winter and spring combined.

Source	Degrees of Freedom	Sum of Squares (Type I)	Mean Square	F-value	Pr > F
Season	1	672.22	672.22	3.72	0.06
Season (Trial)	2	114.20	57.10	0.32	0.73
Season x Trial (Rep)	8	2888.89	361.11	2.00	0.07
Treatments	5	47453.09	9490.62	52.56	0.00
Season x Treatments	5	3105.56	621.11	3.44	0.01
Season x Treatments (Trial)	10	1645.06	164.51	0.91	0.53

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Table 4. Analysis of variance of the incidence of *Pseudophaeomoniella* sp. isolated from the experimental treatments during the pruning wound protectant field trials performed during 2017/2018.

Source	Degrees of Freedom	Sum of Squares (Type 1)	Mean Square	F-value	Pr > F
Trial	1	3680.88	3680.88	24.69	0.00
Trial (Replicate)	4	1090.48	272.62	1.83	0.13
Protectant	11	3432.55	312.05	2.09	0.02
Challenge	2	5027.57	2513.79	16.86	0.00
Protectant x Challenge	22	4874.28	221.56	1.49	0.09
Protectant x Trial	11	667.43	60.68	0.41	0.95
Challenge x Trial	2	242.39	121.19	0.81	0.45
Protectant x Challenge x Trial	22	6186.63	281.21	1.89	0.01

Table 5. Analysis of variance of the incidence of *Trichoderma* spp. isolated from the *Trichoderma*-based experimental treatments during the pruning wound protectant field trials performed during 2017/2018.

Source	Degrees of Freedom	Sum of Squares (Type 1)	Mean Square	F-value	Pr > F
Trial	1	2721.19	2721.19	9.75	0.00
Trial (Replicate)	4	4403.29	1100.82	3.94	0.01
Protectant	2	1676.95	838.48	3.00	0.06
Challenge	2	534.98	267.49	0.96	0.39
Protectant x Challenge	4	2057.61	514.40	1.84	0.14
Protectant x Trial	2	442.39	221.19	0.79	0.46
Challenge x Trial	2	3004.12	1502.06	5.38	0.00
Protectant x Challenge x Trial	4	823.05	205.76	0.74	0.57

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Table 6. Analysis of variance of the incidence of *Pseudophaeomoniella* sp. isolated from the experimental treatments during the pruning wound protectant field trials performed during 2018/2019.

Source	Degrees of Freedom	Sum of Squares (Type 1)	Mean Square	F-value	Pr > F
Trial	1	398.35	398.35	1.22	0.27
Trial (Replicate)	4	1964.40	491.10	1.50	0.20
Protectant	11	14959.52	1359.96	4.16	0.00
Challenge	2	51095.78	25547.89	78.21	0.00
Protectant x Challenge	22	7542.49	342.84	1.05	0.41
Protectant x Trial	11	1645.17	149.56	0.46	0.93
Challenge x Trial	2	230.04	115.02	0.35	0.70
Protectant x Challenge x Trial	22	4644.03	211.09	0.65	0.88

Table 7. Analysis of variance of the incidence of *Trichoderma* spp. isolated from the *Trichoderma*-based experimental treatments during the pruning wound protectant field trials performed during 2018/2019.

Source	Degrees of Freedom	Sum of Squares (Type 1)	Mean Square	F-value	Pr > F
Trial	1	2489.71	2489.71	8.86	0.00
Trial (Replicate)	4	3786.01	946.50	3.37	0.02
Protectant	2	72.02	36.01	0.13	0.88
Challenge	2	504.12	252.06	0.90	0.42
Protectant x Challenge	4	1131.69	282.92	1.01	0.42
Protectant x Trial	2	3899.18	1949.59	6.94	0.00
Challenge x Trial	2	1059.67	529.84	1.89	0.17
Protectant x Challenge x Trial	4	144.03	36.01	0.13	0.97