INVESTIGATION OF *TRICHODERMA* SPECIES COLONIZATION OF NURSERY GRAPEVINES FOR IMPROVED MANAGEMENT OF BLACK FOOT DISEASE

by

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SUMMARY

Black foot disease (BFD) is one of the main fungal diseases associated with young grapevine decline. In recent years its incidence and severity has increased significantly, affecting both nurseries and young vineyards. The pathogens purported to contribute to this disease includes species from the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria* and *Thelonectria*. Currently there are no chemical control measures available to manage BFD in nurseries or vineyards. *Trichoderma* species are well-known biocontrol agents and offers the potential to be implemented as biological control agent (BCA) against this disease. Currently, no *Trichoderma* product is registered for root application on grapevines in South Africa. Previous studies, applying an imported *Trichoderma* product, showed that the *Trichoderma* colonization of nursery vines was poor and the control of black foot pathogens marginal. Therefore, the aim of this study was to investigate different *Trichoderma* products and application methods for the improved control of BFD in grapevine nurseries.

Ten *Trichoderma* spp. isolates were tested *in vitro* for their ability to inhibit the mycelial growth of four major BFD pathogens, namely *Ca. fasciculare*, *Ca. pseudofasciculare*, *D. macrodidyma* and *I. liriodendri* by means of volatile organic compounds, diffusible antibiotic compounds and direct antagonism. In most cases *Trichoderma* were able to inhibit the growth of BFD pathogens, though with variation in efficacy. Generally, the diffusible antibiotic compounds resulted in greater inhibition than the volatile organic compounds. For both classes of compounds tested, *D. macrodidyma* was found to be the most sensitive pathogen, while a number of *T. atroviride* isolates resulted in higher overall growth inhibition. The competitive growth study revealed all *Trichoderma* isolates to exert some form of antagonism towards BFD pathogens.

The efficacy of *T. atroviride* to endophytically colonize different grapevine rootstock cultivars were evaluated on dormant rootstock shoots of five cultivars including Ramsey, Richter 99, Richter 110, US8-7 and Paulsen. The lower 5 cm of the rootstock material was soaked in a *T. atroviride* conidial suspension for different time periods. The rootstock material was then incubated in sterile moist chambers followed by fungal isolations. *Trichoderma atroviride* was able to successfully colonize all five rootstocks to a depth of 10 cm. In general did a longer soaking period not significantly increase *T. atroviride* colonization.

In order to assess the effect of different application methods on *Trichoderma* colonization and control BFD, nine treatments were evaluated on nursery vines post callusing. One hundred graftlings were used per treatment, replicated five times and repeated over two seasons. In order to assess the efficacy of different *Trichoderma*-based

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products another trial was carried out on nursery vines post callusing using eight commercially produced products. One hundred graftlings were used per treatment, replicated four times and repeated over two seasons. For both trials the graftlings were uprooted after 7 months and the number of certifiable vines and total wet root mass determined. Fungal isolations were made from the xylem and pith in the basal end as well as at three sections of the roots. Subsequent *Trichoderma* isolates and BFD pathogens were identified based on colony morphology. In order to confirm the identify the BFD pathogens a subset of 703 isolates were selected for identification by means of genus-specific PCRs using a newly designed primer pair for the *Campylocarpon* genus in combination with two previously described primer pairs for *Dactylonectria* spp. and *Ilyonectria liriodendri*.

The different application methods clearly showed that a newly described method of application, that consists of dipping the basal ends in a dry formulation followed by monthly soil drenches, consistently gave the highest colonization of *Trichoderma*. Field drenching alone was significantly less effective than the dry dip application or a combination of these treatments. Soaking of the basal ends of vines in a conidial suspension for one hour was ineffective and did not differ from the untreated control. None of the application methods resulted in significant differences between percentage certifiable vines, total wet root mass or BFD pathogen incidence.

The trial evaluating different *Trichoderma*-based products showed products that contain isolates originating from grapevine to be the most effective in colonizing nursery vine rootstocks. In the 2016/17 season all of the products resulted in significantly lower black foot pathogen incidence in the basal ends of the vines. However, in the 2016/17 season three of the products resulted in significantly lower root mass than the untreated control, while one product resulted in significantly less certifiable vines in the 2017/18 season.

When comparing tissue parts, the base of the vine and top part of roots had significantly higher *Trichoderma* colonization than the middle and bottom parts of the roots, while significantly less black foot pathogens were isolated from the base in comparison to the roots. Even though *Trichoderma* spp. were not sufficient to prevent infections by BFD pathogens, a certain degree of protection was obtained in the basal ends. The effect of the *Trichoderma* spp. in the nursery vines post transplanting in relation to black foot development remains to be determined. Combining existing knowledge of *Trichoderma* spp. as BCA with the knowledge obtained from this research will assist in optimizing the application procedure in nurseries post callusing.

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OPSOMMING

Swartvoet is een van die belangerikste swamsiektes wat geassosieer word met jong wingerd afname. Die voorkoms daarvan het onlangs toegeneem en affekteer beide kwekerye en jong wingerde. Die patogene wat verantwoordelik is vir die siekte sluit spesies van die *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria* en *Thelonectria* genera in. Daar is tans geen chemiese beheermaatreëls beskikbaar om die siekte in kwekerye of wingerde te beheer nie. *Trichoderma* spesies is bekende biologiese beheeragente wat die potensiaal het om vir siektebeheer gebruik te word. Daar is egter geen *Trichoderma* produkte geregistreer vir worteltoediening op wingerde in Suid-Afrika nie. Vorige studies wat ’n ingevoerde produk ondersoek het, het bevind dat die toediening van die swam tot swak kolonisasie gelei het en net matige beheer van die siekte tot gevolg gehad het. Die doel van hierdie studie was dus om verskillende *Trichoderma* produkte, asook maniere van toediening te evalueer om die beheer van swartvoet te verbeter.

Tien *Trichoderma* isolate is ondersoek vir hul vermoë om die groei van miselia van vier belangrike swartvoet patogene, naamlik *Ca. fasciculare*, *Ca. pseudofasciculare*, *D. macrodidyma* en *I. liriodendri* te inhibeer deur middel van vlugtige organsiese verbindings, oplosbare antibiotiese verbindings, asook direkte antagonisme. *Trichoderma* was meestal in staat om die groei van swartvoet patogene te inhibeer, maar met variasie in die doeltreffendheid daarvan. Die oplosbare antibiotiese verbindings het dikwels meer inhibisie veroorsaak as die vlugtige organiese verbindings. *Dactylonectria macrodidyma* was die mees sensitiewe patogeen vir beide groepe verbindings. Die kompeterende groeistudie het daarop gedui dat alle *Trichoderma* isolate ’n mate van antagonisme toon.

Die potensiaal van *T. atroviride* om verskillende wingerdonderstok-kultivars endofities te koloniseer, was geëvalueer op dormante lote van vyf wingerdonderstok-kultivars insluitend Ramsey, Richter 99, Richter 110, US8-7 en Paulsen. Die basis van die onderstokmateriaal was in ’n *T. atroviride* spoorsuspensie geweek vir verskillende periodes. Die onderstokmateriaal was daarna in steriele vogkamers geïnkubeer, waarna swamisolasies gedoen was. *Trichoderma atroviride* was in staat om al vyf onderstok-kultivars suksesvol te koloniseer tot ’n hoogte van 10 cm. Oor die algemeen het ’n langer weekperiode nie tot hoër kolonisasie van *T. atroviride* gelei nie.

Ten einde die effek van verskillende toedieningsmetodes op *Trichoderma* kolonisasie en die beheer van swartvoet te evalueer, was nege behandelinge uitgevoer op kwekeryplante na kallus. Eenhonderd geënte plante was gebruik per behandeling en vier keer herhaal oor twee seisoene. Nog ’n veldproef was uitgevoer ten einde die doeltreffendheid van agt verskillende *Trichoderma*-gebaseerde produkte te evalueer op kwekeryplante na kallus. Eenhonderd geënte plante was gebruik per behandeling en vyf

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keer herhaal oor twee seisoene. Beide proewe was uitgehaal ná sewe maande waarna die aantal sertifiseerbare plante bepaal is asook die totale nat wortelmassa. Swam isolasies was gedoen vanuit die xileem en pit in die basis van die onderstokke asook van drie gedeeltes van die wortels. Die *Trichoderma* isolate en swartvoet patogene wat gevolglik geïsoleer was, is geïdentifiseer op grond van hul kolonie morfologie. Ten einde die identiteit van die swartvoet patogene te bevestig was ’n subgroep van 703 isolate gekies vir identifikasie deur middel van genus-spesifieke polimerasie kettingreaksies. Dit het gebruik gemaak van een inleier stel vir die *Campylocarpon* genus wat nuut ontwikkel is, asook twee inleier stelle vir *Dactylonectria* spesies en *Ilyonectria liriodendri* wat voorheen beskryf is.

Die proef wat verkillende toedieningsmetodes ondersoek het, het duidelik gewys dat die nuwe metode van toediening wat die doop van die basis van die wingerd onderstokke in ’n droë produk formulasie behels en dan opgevolg word deur maandelikse grond toedienings, konsekwent die hoogste kolonisasie van *Trichoderma* gelewer het. Veldtoediening alleen was noemenswaardig minder doeltreffend as die droë produk toediening of ’n kombinasie van hierdie behandelinge. Weking van die wingerdonderstokke in ’n spoorsuspensie vir een uur was ondoetreffend en het nie van die onbehandelde kontrole verskil nie. Geen toedieningsmetodes het gelei tot noemenswaardige verkille tussen die persentasie sertifiseerbare wingerde, totale nat wortelmassa of voorkoms van swartvoet nie.

Die proef wat verskillende *Trichoderma*-gebaseerde produkte geëvalueer het, het gewys dat die produkte wat isolate bevat wat oorspronklik van wingerde afkomstig is, lei tot die hoogste kolonisasie van kwekery wingerdonderstokke. In die 2016/17-seisoen het al die produkte tot noemenswaardig laer swartvoet voorkoms in die basis van die onderstokke van die wingerde gelei. Drie van die produkte het egter tot noemenswaardig laer wortelmassa gelei as die onbehandelde kontrole in die 2016/17-seisoen, terwyl een produk tot noemenswaardig minder sertifiseerbare wingerde in die 2017/18-seisoen gelei het.

Die verskillende weefseltipes is met mekaar vergelyk en het daarop gedui dat die basis van die onderstok van die wingerdplante en die boonste dele van die wortels noemenswaardig hoër vlakke van *Trichoderma* kolonisasie gehad het as die middelste en onderste dele van die wortels, terwyl noemenswaardig minder swartvoet patogene daaruit geïsoleer was. Selfs al was *Trichoderma* spesies nie voldoende om infeksie van swartvoet patogene te voorkom nie, het dit ’n mate van beskerming in die basis van die onderstokke gebied. Die effek van *Trichoderma* spesies op swartvoet patogene in kwekerywingerde nadat dit uitgeplant is, moet steeds ondersoek word. Die inligting wat versamel is in hierdie studie sal bydra tot die kennis van *Trichoderma* as biologiese beheeragent en sal help met die optimalisering van toedieningsprosedures van *Trichoderma* in die wingerdkwekeryproses.

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CHAPTER 1

A review of black foot disease of grapevine and the *Trichoderma* genus as possible biological control agent thereof

INTRODUCTION

In South Africa, the first wine was pressed in the Cape in February 1659 from cuttings imported from France. Since then, the South African wine industry has grown to become one of the major producers, now being the eighth largest worldwide (South African wine industry directory 2016/17). Today there are more than 119 181 ha of land under grapevine cultivation, consisting of approximately 292.1 million vines (SAWIS, 2018). Already at the start of the 19th century, South Africa had suffered epidemics of various fungal diseases. More recently a decrease in the survival rate of grafted vines in nurseries, and subsequent failure of young vines, has been noted. Among the factors contributing to this phenomenon are pathogenic microorganisms such as deleterious fungi, oomycetes and bacteria, insect and nematode pests, abiotic factors, as well as nutritional deficiencies and toxicities (Halleen *et al*., 2003).

One of the main fungal diseases associated with young grapevine decline is black foot disease (Halleen *et al*., 2006b; Gramaje *et al*., 2010; Dos Santos *et al*., 2016). The disease was first described in France in 1961, with its name referring to the characteristic black necrosis on the base of diseased rootstocks (Sheck *et al*., 1998b). In recent years its incidence and severity has increased significantly, affecting both nurseries and young vineyards (Halleen *et al*., 2004; Oliveira *et al*., 2004; Halleen *et al*., 2006a, 2006b, 2007; Abreo *et al*., 2010; Alaniz *et al*., 2010; Cabral *et al*., 2012; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Úrbez-Torres *et al*., 2014; Dos Santos *et al*., 2016). It is now considered as being one of the major destructive grapevine trunk diseases, causing substantial economic losses in industries worldwide (Petit *et al*., 2005; Rego *et al*., 2009; Compant *et al*., 2013; Úrbez-Torres *et al*., 2014). The pathogens purported to contribute to this disease includes species from the genera *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew., *Dactylonectri*a L. Lombard and Crous, *Ilyonectria* P. Chaverri and C. Salgado and *Thelonectria* P. Chaverri and C. Salgado (Agustí-Brisach *et al*., 2012; Agustí-Brisach and Armengol, 2013; Dos Santos *et al*., 2016; Carlucci *et al*., 2017).

At present, no curative control measures are available to eradicate black foot pathogens in nurseries or vineyards (Oliveira *et al*., 2004; Halleen *et al*., 2006a; Alaniz *et al*., 2010; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013), even though numerous agrochemicals have been investigated for this purpose. Agrochemicals can, however, have a detrimental effect on both the soil microbiota and environment (Samuels and Hebbar, 2015). Due to the aforementioned reason, there is an increasing trend to implement sustainable agricultural practices, using more environmentally friendly approaches that is

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less dependent on agricultural chemicals (Antal *et al*., 2000; Du Plessis, 2015; Halleen and Fourie, 2016). One of the fundamental components of such sustainable agricultural practices is the use of biological control agents for plant protection (Antal *et al*., 2000; Butt *et al*., 2001; Monte, 2001; Gal-Hemed *et al*., 2011; Chen *et al*., 2015).

A biological control agent can be defined as a microbe with the ability to directly parasitize a pathogen, produce a wide array of chemicals that constrain pathogen growth, stimulate host resistance, compete for nutrients and limiting resources or increase overall plant health (Samuels and Hebbar, 2015). Beneficial microbes, such as *Trichoderma* Persoon, have been greatly studied for this use as it can reproduce, persist and flourish in soils, without apparent negative effects (Harman, 2006; Harman and Shoresh, 2007; Samuels and Hebbar, 2015). *Trichoderma* spp. represents one of the most widely used biocontrol agents against several economically important plant pathogens (Mukherjee *et al*., 2013; Zaidi and Singh, 2013; Chen *et al*., 2015; López-Bucio *et al*., 2015). It is cosmopolitan and characterized by its rapid growth, capability of utilizing different substrates and resistance to noxious chemicals (Anees *et al*., 2010; Chen *et al*., 2015; Samuels and Hebbar, 2015). Species within this genus can therefore be considered as potential candidates to be used to control black foot disease in grapevine nurseries, ensuring the health and longevity of newly planted vineyards.

This chapter will provide an overview of black foot disease (BFD) and its causal agents, with emphasis on the taxonomy, etiology, epidemiology, symptoms, distribution and disease management. It will also provide an overview of the genus *Trichoderma* as a potential biological control agent. The recent history, taxonomy, mechanisms of action and formulation of commercial products will be discussed. Reviewing these aspects could contribute to a better understanding of BFD in South African grapevine nurseries and aid in the development of sustainable control methods for this disease.

THE CAUSAL AGENTS OF BLACK FOOT DISEASE IN GRAPEVINE

Taxonomy and etiology

The genus *Cylindrocarpon* was erected by Wollenweber in 1913 and broadly contained all species having *Cylindrocarpon*-like conidia (Rossman *et al*., 2013). In 1966, Booth divided the genus into four groups based on the presence or absence of microconidia and chlamydospores, with most of the teleomorphs (groups 1, 2 and 4) being congregated in the genus *Neonectria* (Reis *et al*., 2013). As taxonomic precision has increased in the past two decades, driven mostly by molecular phylogenetic analysis, notable taxonomic changes have since been made to the genera contributing to black foot disease. Several new genera that have asexual morphs belonging to *Cylindrocarpon* have recently been segregated from *Neonectria* (Rossman *et al*., 2013).

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Ilyonectria, being one of these novel genera, was erected to accommodate species associated with grapevine decline (Halleen *et al*., 2004, 2006; Chaverri *et al*., 2011; Cabral *et al*., 2012; Cardoso *et al*., 2013; Reis *et al*., 2013). *Ilyonectria radicola,* previously known as *Cylindrocarpon destructans* (Cabral *et al*., 2012; Rossman *et al*., 2013), was included in this genus, but later found to be a species complex and delineated into *I. europeaea*, *I. lusitanica*, *I. pseudodestructans*, *I. robusta* and *I. vitis* (Cabral *et al*., 2012a, b; Reis *et al*., 2013). Another species residing within this genus includes *I. liriodendri* (Úrbez-Torres *et al*., 2014).

Furthermore, species belonging to the *I. macrodidyma* species complex were delineated based on multi-gene phylogenies and consequently comprised of *I. alcacerensis*, *I. estremocensis*, *I. macrodidyma* sensu stricto, *I. novozelandica* and *I. torresensis* (Cabral *et al*., 2012b; Agustí-Brisach *et al*., 2013). Recently, however, multi-gene studies revealed the genus *Ilyonectria* to be paraphyletic and resulted in the introduction of the genus *Dactylonectria* (Cabral *et al*., 2012a; Lombard *et al*., 2014). Consequently, all species formally belonging to the *I. macrodidyma* complex, together with *I. vitis* and *C. pauciseptatum*, have been grouped within this genus (Dos Santos *et al*., 2016). The genus encompasses *D. alcacerensis*, *D. estremocensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, and *D. vitis* (Lombard *et al*., 2015). Species within this genus occur more often than other genera and is thought to be the primary cause of BFD (Agustí-Brisach *et al*., 2013; Dos Santos *et al*., 2016), with *D. macrodidyma* being reported as one of the major role players (Halleen *et al*., 2006a; Cardoso *et al*., 2013).

The genus *Campylocarpon* was proposed by Halleen *et al*. (2004) for species resembling *Cylindrocarpon* with 3–5-septate, curved macroconidia and lacking microconidia**.** Currently there are two species residing within this genus, *Ca. fasciculare* and *Ca. pseudofasciculare*, both of which have been associated with black foot disease (Halleen *et al*., 2006a; Cardoso *et al*., 2013). Despite its limited geographical distribution (Álvarez *et al*., 2012; Cardoso *et al*., 2013; Silva *et al*., 2017), Halleen *et al*. (2006b) still considered these species to be the primary causal agents of black foot disease in South Africa.

More recently the genera *Cylindrocladiella* and *Thelonectria* has also been associated with black foot disease. Up to date two species within the genus *Cylindrocladiella*, namely *Cy. parva* and *Cy. peruviana*, and one species within the genus *Thelonectria*, namely *T. blackeriella*, have been reported on grapevine (Agustí-Brisach *et al*., 2012; Agustí-Brisach and Armengol, 2013; Úrbez-Torres *et al*., 2014; Carlucci *et al*., 2017).

Epidemiology

Environmental factors and host stress

Numerous stresses imposed on young vines in nurseries and vineyards favor the

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development of black foot disease (Oliveira *et al*., 2004; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013). The most significant of these being environmental factors and vineyard management practices such as poor water drainage, soil compaction, planting of vines in poorly prepared soil and improper planting holes, which cause poor root development, as well as malnutrition and heavy crop loads on young vines (Larignon, 1999; Fourie *et al.*, 2000; Gubler *et al*., 2004; Halleen *et al*., 2006a; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013). High summer temperatures also play a major role in symptom expression as it accentuates water stress on the vines (Larignon, 1999; Gramaje and Armengol, 2011). This phenomenon can be ascribed to gum inclusions and tyloses that block the xylem vessels and prevent the movement of water within the vascular system, ultimately preventing the plant to compensate for the high transpiration rate (Halleen *et al*., 2006a; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013).

Furthermore, young vines are placed under abiotic stress conditions throughout the nursery propagation process. The wounds produced during cutting and bench grafting, development of roots and shoots in the nursery field, uprooting and trimming as well as cold storage are just some of the stressors on these vines (Oliveira *et al*., 2004; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013). Once planted in the vineyards, environmental factors that are often not ideal for vine establishment (Probst *et al*., 2012; Agustí-Brisach *et al*., 2013), together with cultivation practices such as defoliation, can further stress the vines that results in increased susceptibility to the disease (Agustí-Brisach *et al*., 2013).

"*Cylindrocarpon*" species are often part of disease complexes with other fungi (Brayford, 1993; Rego *et al*., 2001; Halleen *et al*., 2006a), which may also increase the severity of subsequent disease development (Probst *et al*., 2012; Cardoso *et al*., 2013). Some of the pathogens isolated from the same diseased vines include *Botryosphaeria* spp., *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, *Phomopsis* spp., *Phytophthora* spp. and *Pythium* spp. (Halleen *et al*., 2003; Oliveira *et al*., 2004; Halleen *et al*., 2006a, 2007; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013). Indeed, a study by Úrbez-Torres *et al*. (2014) confirmed that BFD pathogens primarily coexist with fungal taxa associated with Petri disease.

Inoculum sources and disease development

Fungi belonging to these genera are common soil inhabitants occurring as saprophytes on dead plant material, root colonizers or weak plant pathogens (Brayford, 1993). Most of the species are able to produce chlamydospores, enabling it to survive in soils for extended periods of time (Halleen *et al*., 2004, 2006a; Agustí-Brisach *et al*., 2011; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Lombard *et al*., 2013), even after an infected crop has been removed (Probst *et al*., 2012; Úrbez-Torres *et al*.,

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2014). Its association with subsequent infection is, however, still unknown (Halleen *et al*., 2006a; Agustí-Brisach *et al*., 2011, 2013).

The nursery process consists of a number of steps in which contamination of propagation material can lead to infection (Rego *et al*., 2000, 2001; Rumbos and Rumbou, 2001; Halleen *et al*., 2003). At the end of the growing season cuttings from both the scion and rootstocks are collected from mother blocks, which are then used as grafting material (Fourie *et al*., 2001). Already at this step Agustí-Brisach *et al*. (2013) were able to isolate *Ilyonectria* spp. from symptomatic and asymptomatic rootstock mother-plants. These cuttings can be systemically infected by the pathogens without showing any visual symptoms (Halleen *et al*., 2003; Gramaje and Armengol, 2011; Cardoso *et al*., 2013; Reis *et al*., 2013). However, only a few reports of "*Cylindrocarpon*" spp. from rootstock mother vines and cuttings have been made before (Halleen *et al*. 2003; Fourie and Halleen 2004; Oliveira *et al*., 2004; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013) and is therefore regarded as a minor source of inoculum due to its low occurrence.

In South Africa, cuttings are grafted late winter to early spring, either by hand or by omega grafting machines (Fourie *et al*., 2001). After the grafting process, the vines are laid in pine sawdust drenched with a broad-spectrum fungicide to allow for formation of callus roots and callus tissue around the graft union (Fourie and Halleen, 2006). Cardoso *et al*. (2013), who investigated this process as possible source of inoculum, were unable to detect BFD pathogens at the callusing stage, confirming that the infection occurs later during the rooting phase.

From literature it is clear that propagation material get infected in the nursery fields. Various studies reported an increase of BFD infection after the rooting phase in the nurseries (Fourie *et al*., 2001; Halleen and Crous, 2001; Halleen *et al*., 2003, 2006a; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Reis *et al*., 2013). A study by Halleen and Crous (2001) have shown that the number of grafted vines infected with black foot pathogens could increase from 1% after callusing to a staggering 50% after the 7-month rooting phase in the nursery fields.

This phenomenon can be explained by various practices during the propagation process that result in wounding of the graftlings, making them extremely vulnerable to infection by these pathogens. Callus roots, for example, often break during the planting process that result in small wounds (Halleen *et al*., 2003, 2006a; Alaniz *et al*., 2011; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013). Moreover, the susceptible basal ends (especially the pith area) of most of the grafted cuttings are exposed to infection, as callus tissue does not typically cover the entire area (Halleen *et al*., 2003, 2006a; Agustí-Brisach *et al*., 2013). A cultural practice that is commonly used in South Africa that might further contribute to the high disease incidence involves covering the graft union with soil for

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a period of approximately five weeks to prevent drying of the callused tissue. During this period the graft union is fully exposed and can readily be infected by "*Cylindrocarpon*" spp. in the soil (Halleen *et al*., 2006a; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013).

Even though infection of vines mostly occurs during the rooting process in nursery soils, some studies have shown that infection can also occur in vineyards. Infected soils in vineyards may also serve as source of inoculum (Dubrovsky and Fabritius, 2007; Agustí-Brisach *et al*., 2013). In most cases, however, latent pathogens established within the xylem tissue of seemingly healthy vines and only become evident in the vineyard once the plants are placed under stress conditions (Gramaje and Armengol, 2011).

Host range

These genera are often associated with the roots of herbaceous woody plants (Brayford, 1993), emphasizing the potential for cross-infection of isolates from other hosts to grapevines (Cabral *et al*., 2012). In addition to grapevine (*Vitis* spp.), black foot pathogens have been reported on *Abies nordmanniana*, *Actinidia chinensis*, *Festuca duriuscula*, *Liriodendron tulipifera*, *Olea europaea*, *Panax quinquefolius*, *Persea americana*, *Picea glauca*, *Pinus radiate*, *P. sylvestris*, *Proteaceae* spp., *Prunus persica*, to name but a few hosts of economic importance (Rahman and Punja, 2005; Agustí-Brisach *et al*., 2011a; Erper *et al*., 2011; Cabral *et al*., 2012a; Vitale *et al*., 2012; Agustí-Brisach *et al*., 2013; Lombard *et al*., 2013; Úrbez-Torres *et al*., 2014b).

Moreover, Agustí-Brisach *et al*. (2011) demonstrated a wide range of vineyard weeds in Spain to harbor black foot pathogens and therefore serve as a source of inoculum. Of the 52 weed species sampled, *D. macrodidyma* could be isolated from at least 26 of those. More recently Langenhoven (2017) found five vineyard weed and grass species in South Africa to harbor these pathogens as well.

Symptoms

The disease typically expresses a range of vascular and foliar symptoms. The diagnoses of black foot disease can, however, be challenging considering that its symptomology closely resembles that of Petri disease (Scheck *et al*., 1998a; Rego *et al*., 2000).

Symptoms in the nurseries

In nurseries, characteristic symptoms of this disease are often expressed shortly after transplantation in infected soils. External symptoms that may be expressed by affected graftlings include delayed bud break, reduced vigor, shortened internodes, sparse foliage, and small leaves with interveinal chlorosis and necrosis (Halleen *et al*., 2006a; Abreo *et al*.,

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2010; Gramaje and Armengol, 2011; Cabral *et al*., 2012; Cardoso *et al*., 2013; Dos Santos *et al*., 2016). The most apparent internal symptoms of the graftlings include black discoloration (Grasso and Magnano di San Lio, 1975; Larignon, 1999), that will be revealed in cross-sections exhibiting internal necrosis and longitudinal sections that appear as dark brown to black vascular streaking (Halleen *et al*., 2006a; Cardoso *et al*., 2013; Dos Santos *et al*., 2016). Below ground symptoms comprise of a reduction in root biomass and root hairs with sunken, necrotic root lesions (Petite *et al*., 2005; Halleen *et al*., 2006a; Mohammadi *et al*., 2009; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Dos Santos *et al*., 2016).

Symptoms in the vineyards

Young vineyards between the ages of 1 and 5 years old are most susceptible to this disease (Gubler *et al*., 2004; Petite *et al*., 2005; Halleen *et al*., 2006a; Dubrovsky and Fabritius, 2007; Úrbez-Torres *et al*., 2014). Disease symptoms usually manifest early in the growing season as affected vines display retarded sprouting after winter dormancy and die by mid-summer (Oliveira *et al*., 2004; Halleen *et al*., 2006a; Rego *et al*., 2006; Abreo *et al*., 2010; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013). However, older vines demonstrate a gradual decline and death might only occur during the subsequent winter period (Halleen *et al*., 2004; Halleen *et al*., 2006a; Agustí-Brisach *et al*., 2013; Dos Santos *et al*., 2016). This decline becomes apparent as stunted growth, shortened internodes, sparse foliage and reduced vigor as well as small leaves with interveinal chlorosis and necrosis (Halleen *et al*., 2004; Petite *et al*., 2005; Halleen *et al*., 2006a; Alaniz *et al*., 2007; Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013). Nevertheless, not all infected vines expresses external symptoms.

Removal of the rootstock bark reveals black discoloration developing from the base that extends upwards affecting most of the rootstock wood (Fig. 1) (Sweetingham, 1983; Larignon, 1999; Rego *et al*., 2001; Halleen *et al*., 2006a; Rego *et al*., 2006; Gramaje and Armengol, 2011). A cross section through the lesions will reveal development of necrosis and congested xylem vessels spreading from the bark to the compacted pith (Gubler *et al*., 2004; Halleen *et al*., 2004; Oliveira *et al*., 2004; Halleen *et al*., 2006a; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013; Úrbez-Torres *et al*., 2014), while a longitudinal section would reveal brown to black vascular streaks (Fig. 1) (Oliveira *et al*., 2004; Alaniz *et al*., 2007; Dubrovsky and Fabritius, 2007). The necrosis eventually extends across the whole rootstock and results in death of the vine (Halleen *et al*., 2006a; Probst *et al*., 2012).

Below ground symptoms includes sunken necrotic root lesions and reduction in root biomass with characteristic black discoloration (Scheck *et al.,* 1998b; Larignon, 1999; Halleen *et al*., 2006a; Alaniz *et al*., 2007; Dubrovsky and Fabritius, 2007; Armengol *et al*., 2011; Reis *et al*., 2013; Úrbez-Torres *et al*., 2014). Consequently, a second crown of

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horizontally growing roots occasionally develop on an upper level of the rootstock to compensate for the loss of functional roots, while the rootstock diameter below the second tier thins (Scheck *et al.,* 1998b; Larignon, 1999; Fourie and Halleen, 2001; Halleen *et al*., 2004, 2006; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013).

Distribution

After the first report in France (Maluta and Larignon, 1991), the disease has been reported in all major grapevine-growing regions worldwide (Table 1). Each country, however, has its own BFD pathogen profile (Agustí-Brisach and Armengol, 2013). In South Africa the main purported species belongs to the genera *Campylocarpon*, *Dactylonectria* and *Ilyonectria*, namely *Ca. fasciculare* and *Ca. pseudofasciculare*, *D. alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis* and *I. liriodendri* (Langenhoven, 2017). *Cylindrocladiella parva* and *Cy. peruviana* has also been isolated from grapevine in South Africa (Van Coller *et al*., 2005), though their role in the disease has not been established.

Management and control

Suitable control measures are necessary to prevent infections by BFD pathogens (Gramaje *et al*., 2010), considering that it infects grafted grapevine plants from nursery soils (Halleen *et al*., 2003). Currently there are no curative control measures available to eradicate black foot pathogens in nurseries or vineyards (Oliveira *et al*., 2004; Halleen *et al*., 2006a; Alaniz *et al*., 2010; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013), rendering the management strategies merely preventive (Halleen *et al*., 2006a; Fontaine *et al*., 2016). This is a consequence of the soil-borne nature of this disease that makes it difficult to control (Dos Santos *et al*., 2016). Integrated disease management programs that include physical, chemical and biological treatments have the potential to reduce infection by fungal trunk pathogens (Gramaje *et al*., 2010; Úrbez-Torres *et al*., 2014) and should therefore be implemented in grapevine nurseries.

Cultural practices

The majority of grapevine nursery soils in South Africa are contaminated with BFD pathogens (pers com., Francois Halleen), especially in areas where the same soils have been used for grapevine propagation for decades (Halleen *et al*., 2006a). Mostly a two-year rotation system is used, whereby cuttings are planted every second year and alternated with a rotation crop (Halleen *et al*., 2006a). The rotation system can, however, not be prolonged due to limitation in space and economic viability of grapevine nurseries. To accommodate for this shortcoming, soil biofumigation can be applied to decrease the incidence of BFD pathogens in nursery soils (Bleach *et al*., 2010; Berlanas *et al*., 2018).

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In vineyards, management strategies mainly focus on limiting predisposing stress factors that makes the vines more susceptible to disease (Fourie *et al*., 2001; Halleen *et al*., 2006a, 2007; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013). Soil preparation, for example, forms an integral part of disease management. Compacted layers of soil should be broken up to make the subsoil accessible to roots (Larignon, 1999), while plant holes should be deep and big enough to facilitate proper root development (Fourie *et al*., 2000). When dealing with poorly drained soils vines should be planted on berms; drip irrigation emitters should be moved away from the vines (Gubler *et al*., 2004) and flood irrigation should be avoided, though it is not universal practice (Gramaje and Armengol, 2011). Good hygiene and wound protection is also of utmost importance (Gramaje and Armengol, 2011), while maintaining a proper sanitation program to ensure disease-free grapevine planting material (Agustí-Brisach *et al*., 2013).

Physical control

Hot water treatment (HWT) is widely used for the proactive management of black foot disease (Rego *et al*., 2006; Halleen *et al*., 2007) as it has been proven to reduce the occurrence of black foot pathogens in dormant nursery propagation material (Fourie *et al*., 2004, Halleen *et al*., 2006a, 2007; Rego *et al*., 2009; Alaniz *et al*., 2011; Gramaje and Armengol, 2011). The high temperatures are thought to reduce fungal inoculum by damaging the fungal cells and facilitate a stress-response in the vines (Halleen *et al*., 2006a, 2007).

These treatments are generally performed at 50°C for 30 min, which is considered effective to control "*Cylindrocarpon*" spp. (Alaniz *et al*., 2011; Agustí-Brisach *et al*., 2013). Other studies have, however, suggested that HWT needs to be conducted at 53°C for 30 min (Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013) or the period needs to be extended to 45 min at 50°C to sufficiently control black foot pathogens (Gramaje and Armengol, 2011; Agustí-Brisach and Armengol, 2013). In accordance, Gramaje *et al*. (2010) showed that conidial germination and mycelial growth of "*Cylindrocarpon*" spp. are inhibited after 45 min above 45°C and 48°C, respectively, while acknowledging that higher temperatures are required when pathogens are introduced into the wood of 1-year old cuttings. Recently HWT regimes in South African nurseries were extended from 30 min to 45 min at 50°C in order to combat aster yellows, but the effect of this HWT regime on BFD pathogens adapted to South African conditions is still unknown.

Alas, the treatment only has a short-term effect (Rego *et al*., 2009) as vines can easily become re-infected once planted in the vineyard. Moreover, different cultivars vary in sensitivity to HWT and can also be affected by the temperature during the previous growing season (Fontaine *et al*., 2016), resulting in vine failure due to damage caused to the more

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sensitive cultivars (Rego *et al*., 2009). While it is recommended to incorporate HWT in an integrated disease management strategy (Halleen *et al*., 2006a, 2007; Rego *et al*., 2009), the risks of vine failure should be carefully considered (Fontaine *et al*., 2016).

Chemical control

Chemical control strategies for fungal trunk pathogens can be challenging in the nursery process (Gramaje and Armengol, 2011), as conventional chemical sprays and dips are not able to penetrate dormant grapevine cuttings sufficiently and therefore have limited efficacy towards fungal pathogens within the phloem and xylem tissue (Gramaje and Armengol, 2011). It can, however, act as protection against pathogen attack (Rego *et al*., 2009) and should therefore be implemented to decrease the incidence and severity of infection by "*Cylindrocarpon*" spp. during the nursery propagation process (Alaniz *et al*., 2011). A fungicide soak prior to grafting can significantly reduce these pathogens in grapevine cuttings (Rego *et al*., 2009), as it protects the wounds from infection during the rooting stage in nursery soils. Numerous studies have looked at the efficacy of fungicides towards BFD pathogens, often with promising results. Yet there are no fungicides currently registered in South Africa for the control of black foot disease in vineyards (Halleen *et al*., 2006a).

For example, benomyl was found to have exceptional efficacy against various "*Cylindrocarpon*" spp. (Oliveira *et al*., 2004; Halleen *et al*., 2006a; Rego *et al*., 2006; Halleen *et al*., 2007; Gramaje and Armengol, 2011). Moreover, its efficacy has also been shown in semi-commercial field trials by Halleen *et al*. (2007), while Fourie and Halleen (2004) proposed this fungicide to be used in hydration tanks. This fungicide is, however, not available for use anymore (Oliveira *et al*., 2004). It is recommended that benomyl be replaced with carbendazim when benomyl is no longer available (Halleen and Fourie, 2016).

The mycelial growth of both "*C*". *liriodendri* and "*C*". *macrodidymum* was inhibited by flusilazole in a study by Halleen *et al*. (2006, 2007), while Alaniz *et al*. (2011) found similar results for carbendazim. The latter mentioned fungicide also decreased the root disease severity and significantly reduced "*C*". *liriodendri* when compared to the control (Gramaje and Armengol, 2011). Furthermore, a combination of these fungicides was particularly effective in decreasing disease incidence (Oliveira *et al*., 2004; Rego *et al*., 2006; Gramaje and Armengol, 2011) and improving plant growth (Gramaje and Armengol, 2011). A combination of cyprodinil with fludioxonil yielded similar results (Oliveira *et al*., 2004; Rego *et al*., 2006; Rego *et al*., 2009; Gramaje and Armengol, 2011), even under high inoculum pressure (Rego *et al*., 2009). Another mixture which efficacy has been shown is that of pyraclostrobin and metiram, also being able to reduce the incidence and severity of "*Cylindrocarpon*" spp. (Rego *et al*., 2009).

Studies conducted by Alaniz *et al*. (2011) and Halleen *et al*. (2006, 2007) showed

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that mycelial growth of "*C*". *liriodendri* and "*C*". *macrodidymum* could be inhibited by prochloraz and prochloraz manganese chloride, respectively. Prochloraz was also able to reduce the root disease severity in another study by Gramaje and Armengol (2011), though they found the percentage of re-isolation values only to be significantly different from the control treatment in the case of "*C*". *macrodidymum*. Likewise, thiram and hydroxyquinoline sulphate also reduced mycelial growth of these two pathogens (Alaniz *et al*., 2011) with the latter being able to decrease the root disease severity (Gramaje and Armengol, 2011). Imazalil was able to reduce mycelial growth of these pathogens and showed some efficacy in semi-commercial field trials (Halleen *et al*., 2007). Contrasting results were obtained by Alaniz *et al*. (2011) for imazalil, who found that a significant decrease in root disease severity could only be obtained for "*C*". *liriodendri*, but not for "*C*". *macrodidymum*.

Copper oxichloride, captan and didecyldimethylammonium chloride were the most effective to inhibit conidial germination of "*C*". *liriodendri* and "*C*". *macrodidymum* (Alaniz *et al*., 2011). Similarly, Gramaje and Armengol (2011) found the two latter mentioned fungicides to yield a percentage re-isolation significantly less from that of the control treatment in the case of "*C*". *liriodendri*. The fungicides were also able to decrease the root disease severity of the vines (Gramaje and Armengol, 2011).

This control strategy is favorable in comparison to HWT, because of its feasibility (Rego *et al*., 2006). Nonetheless, significant variation occurs in fungicide sensitivity both between and within black foot species (Alaniz *et al*., 2011), illustrating the necessity for integrated disease management.

Biological control

Fourie *et al*. (2001) conducted experiments in South African nurseries in which they investigated the effects of *Trichoderma* drenching before and after grafting, as well as soil amendments against "*Cylindrocarpon*" spp. Although inconsistent results were obtained in this study, it did show the potential of *Trichoderma* spp. to control the disease in grapevine nurseries. A reduction of 42.9% in "*Cylindrocarpon*" spp. was observed, though not statistically different from the control. Dos Santos *et al*. (2016) hypothesized that this inconsistency may have been a result of the short periods of immersion in the *Trichoderma* suspensions. These authors also investigated the effect of *Trichoderma* spp. against *D. macrodidyma* and observed mycelial growth inhibition of up to 40%, consolidating the case. Furthermore, numerous studies have shown the efficacy of *Trichoderma* as soil amendments, not only contributing to the control of fungal pathogens, but also improving overall plant health. The improved tolerance of grapevines to black foot disease pathogens when subjected to stress (Fourie *et al*., 2001; Halleen *et al*., 2006a; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013; Agustí-Brisach and Armengol, 2013) can be ascribed to

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the growth stimulating effects, enhanced root development, induced resistance instigated by *Trichoderma* (Fourie *et al*. 2001; Fourie and Halleen, 2004; Oliveira *et al*., 2004; Rego *et al*., 2006) and direct antagonism towards the pathogens (Di Marco and Osti, 2007; Dos Santos *et al*., 2016).

Chemical and physical control should therefore be combined with biological control in all stages of the grapevine propagation process to form an integrated disease management strategy (Fourie and Halleen, 2006; Rego *et al*., 2006; Halleen *et al*., 2007; Agustí-Brisach *et al*., 2013) that can effectively decrease the incidence and severity of BFD pathogens during the nursery propagation process (Agustí-Brisach *et al*., 2013). A recent study by Halleen and Fourie (2016) investigated different integrated strategies for the proactive management of GTDs, applying chemical, physical and biological treatments at all stages of the nursery process including before cold storage, before and after grafting, before planting and after uprooting. The authors reported that the only treatment able to eradicate BFD completely was HWT (50°C for 30 min) of dormant nursery vines, though they concluded the best integrated strategy for grapevine nurseries to be a combination of Benomyl before cold storage, HWT and Sporekill before grafting and the application of *Trichoderma* after grafting and before planting. However, *Trichoderma* needs to be further investigated to develop application methods that may ensure more consistent efficacy (Fourie *et al*., 2006; Halleen *et al*., 2007; Agustí-Brisach *et al*., 2013).

THE GENUS *TRICHODERMA* AND ITS POTENTIAL AS BIOLOGICAL CONTROL AGENT

Taxonomy and ecology

This is a well-studied genus of filamentous deuteromycetes that was erected by Persoon in 1794. It consists of more than 200 molecularly defined species (Atanasova *et al*., 2013) that is well known for its biochemical abilities (Samuels and Hebbar, 2015), rapid growth, ability to utilize diverse substrates (Anees *et al*., 2010; Chen *et al*., 2015) and resistance to both biotic and abiotic stresses (Chen *et al*., 2015). *Trichoderma* are predominantly known as soil fungi (Vinale *et al*., 2008b; Anees *et al*., 2010; Gal-Hemed *et al*., 2011; Atanasova *et al*., 2013; Zaidi and Singh, 2013; Chen *et al*., 2015; John *et al*., 2015; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015), being among the most prevalent culturable fungi therein (Harman and Shoresh, 2007), though it is cosmopolitan and adapted to various other ecological niches. For example, it has been isolated from marine habitats (Gal-Hemed *et al*., 2011; Atanasova *et al*., 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015), decaying bark and fruiting bodies of basidiomycete fungi (Atanasova *et al*., 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015).

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Trichoderma species can be found in diverse climatic zones globally (Vinale *et al*, 2008b; López-Bucio *et al*., 2015). The optimum temperature for *Trichoderma* differs between species and may even be isolate dependent (Antal *et al*., 2000). Most *Trichoderma* species are mesophilic and are therefore unable to provide protection in colder periods (Antal *et al*., 2000; Hjeljord *et al*., 2000), while the fewer cold tolerant strains will have greater efficacy in both cool field situations and after cold storage (Hjeljord *et al*., 2000) as it possesses the ability to continue normal biological activity at temperatures as low as 5°C (Antal *et al*., 2000).

It engages in a range of opportunistic lifestyles and interactions with other plants and fungi (Karagiosis and Baker, 2013), either as biotrophs or saprotrophs (Atanasova *et al*., 2013). Consequently it is commonly associated with plant root systems (Contreras-Cornejo *et al*., 2013; Karagiosis and Baker, 2013) where it can colonize healthy plant roots and internal tissue as endophytes (Harman *et al*., 2004; Bailey and Melnick, 2013; Karagiosis and Baker, 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015). The establishment of a symbiotic relationship begins with root colonization and infection of the outer cortical layers of the roots where a zone of chemical interactions are established (Harman and Shoresh, 2007; Samuels and Hebbar, 2015) that significantly contributes to overall plant health (Harman *et al*., 2004; Harman and Shoresh, 2007; Samuels and Hebbar, 2015).

Trichoderma *species as biological control agents*

Fungi in this genus have long been investigated as biocontrol agents (Monte, 2001; Harman, 2006; Harman and Shoresh, 2007). Already in the 1930's Weindling demonstrated these fungi as mycoparasites, producers of antibiotics and biocontrol agents (Mukherjee *et al*., 2013; Samuels and Hebbar, 2015). In the 1970's Dennis and Webster indicated that volatile organic compounds produced by certain species could inhibit the growth of wood decay fungi (Samuels and Hebbar, 2015). Following these findings, the agricultural application of *Trichoderma* to increase growth of crops was further instigated in the 1980's and 1990's (Samuels and Hebbar, 2015). Ever since, these filamentous fungi have had a major impact on agriculture, now being the most widely used biofungicides and plant growth promoters contributing to approximately 60% of all registered biofungicides worldwide (Mukherjee *et al*., 2013; Zaidi and Singh, 2013; Chen *et al*., 2015; López-Bucio *et al*., 2015). However, despite this prodigious amount of commercial *Trichoderma* based products available, none has as of yet been registered against black foot disease of grapevine.

This genus includes a number of species that are known to be beneficial in agricultural production systems with select strains being used for biological control of plant diseases. Prominent species that exhibit high biostimulant action and have been successfully commercialized for this purpose include *T. asperelloides* (Harman *et al*., 2004),

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T. asperellum (Karagiosis and Baker, 2013; Mukherjee *et al*., 2013; Qualhato *et al*., 2013; López-Bucio *et al*., 2015), *T. atroviride*, *T. harzianum* (Karagiosis and Baker, 2013; Mukherjee *et al*., 2013; Qualhato *et al*., 2013; López-Bucio *et al*., 2015), *T. virens* (Karagiosis and Baker, 2013; Mukherjee *et al*., 2013; López-Bucio *et al*., 2015) and *T. viride* (Harman *et al*., 2004; Mukherjee *et al*., 2013; López-Bucio *et al*., 2015). However, Samuels and Hebbar (2015) proposed that biological ability cannot be based solely on phylogenetic relationships considering that strains within a species are genetically different, though acknowledging that species in some groups might have greater potential than that of other groups. Similarly, a number of other studies concluded that antagonistic mechanisms, mycoparasitic capability and secreted secondary metabolites (volatile and diffusible compounds) are strain-reliant (Vinale *et al*., 2009; Anees *et al*., 2010; López-Mondéjar *et al*., 2011; John *et al*., 2015) and is therefore characteristic of a population, and not a species (Anees *et al*., 2010).

Numerous *Trichoderma*-based products are available in South Africa (Table 2), with a select few registered for use on grapevine in South Africa. In a study by Mutawila *et al*. (2015), a local isolate of *Trichoderma atroviride*, USPP-T1, were identified and evaluated against GTD's on grapevine. The authors continued to generate a benzimidazole resistant mutant, which proved to be more effective when applied in combination with carbendazim. However, none of these are registered against BFD, nor for root application of grapevine in South Africa.

A wide array of phytopathogens belonging to taxonomically distinct groups can be controlled by species in the *Trichoderma* genus. These comprise a number of soilborne pathogens, including species of *Armillaria*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotinia* and *Verticillium*, among others. The efficacy, however, greatly depends on the specific *Trichoderma* isolates, the pathogens as well as the host involved. This suggests that *Trichoderma* employs various mechanisms of action against phytopathogens, perhaps even specific to the phytopathogens present.

Mechanisms of *Trichoderma* biological control

Biocontrol is multifaceted and mediated by various antimicrobial activities and factors that elicits plant growth promotion. Beneficial actions that have been studied extensively includes the production of secondary metabolites and its role in antibiosis, direct mycoparasitism of plant pathogens (hyphae and resting structures or fruiting bodies) and competition for iron and other limiting resources (Harman, 2006; Vinale *et al*., 2009a; Anees *et al*., 2010; Karagiosis and Baker, 2013; Mukherjee *et al*., 2013; Qualhato *et al*., 2013; Du Plessis, 2015; John *et al*., 2015; Samuels and Hebbar, 2015; Saravanakumar *et al*., 2016). However, other factors contributing to overall plant health and productivity only recently became a subject of

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interest. Plant growth promotion, for instance, can mainly be attributed to the induction of plant host resistance (Harman *et al*., 2004; Harman, 2006; Vinale *et al*., 2008a; Vinale *et al*., 2009; Anees *et al*., 2010; Zaidi and Singh, 2013; Du Plessis, 2015; John *et al*., 2015; Samuels and Hebbar, 2015; Saravanakumar *et al*., 2016) and growth stimulation (Küçük and Kivanç, 2003; Vinale *et al*., 2008a; Vinale *et al*., 2009; Karagiosis and Baker, 2013; Mukherjee *et al*., 2013; Du Plessis, 2015; John *et al*., 2015; Samuels and Hebbar, 2015). A single biocontrol agent can exude several of the latter mentioned modes of action that act synergistically to improve overall plant health (Harman *et al*., 2004; Vinale *et al*., 2008a; Samuels and Hebbar, 2015).

Antibiosis

Antibiosis is a process mediated by the secretion of a wide arsenal secondary metabolites, which suppress the growth of other microorganisms (Vinale *et al*., 2008a; Vinale *et al*., 2008b; Anees *et al*., 2010; Kotze *et al*., 2011; Karagiosis and Baker, 2013; Zaidi and Singh, 2013). The composition of these compounds is subject to the specific strain (Vinale *et al*., 2008a; Vinale *et al*., 2008b; Anees *et al*., 2010; Atanasova *et al*., 2013a; López-Bucio *et al*., 2015) and includes a variety of classes of chemical compounds that can be grouped into volatile organic compounds and diffusible compounds.

The volatile organic compounds (VOCs) are low molecular weight, non-polar compounds (Vinale *et al*., 2008a; Vinale *et al*., 2008b) that can permeate soil pores and travel long distances (Arjona-Girona *et al*., 2014; Chen *et al*., 2015). These compounds can diffuse across cell membranes (Mukherjee *et al*., 2013) and significantly contribute towards antagonism (Chet *et al*., 1981; Gal-Hemed *et al*., 2011; Zeilinger and Schuhmacher, 2013; Samuels and Hebbar, 2015). Some of the VOCs produced by fungi in this genus include alcohols, aldehydes, alkanes, furanes, ketones, pyrones (alkyl pyrones) and terpenes (Contreras-Cornejo *et al*., 2013; Zaidi and Singh, 2013; Chen *et al*., 2015; López-Bucio *et al*., 2015), all of which have fluctuating levels of antagonistic activity against phytopathogens. These profiles also vary based on the environmental, biological and growth conditions (López-Bucio *et al*., 2015). Of these, unsaturated lactone 6-Pentyl-alpha-pyrone (6-PP) is among the most studied and constitutes the principle pyrone of certain species (Vinale *et al*., 2008a; Vinale *et al*., 2008b; Contreras-Cornejo *et a*l., 2013; Mutawila *et al*., 2016). 6-PP has been shown to inhibit mycelial growth of various pathogenic fungi, though some can metabolize it into less toxic products (Zeilinger and Schuhmacher, 2013). Nonetheless, *Trichoderma* are very effective antagonists in the soil environment (Mukherjee *et al*., 2013).

The diffusible compounds include a number of water-soluble antibiotic compounds, peptaibols and cell wall degrading enzymes (CWDE) that act in close proximity of

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Trichoderma due to its polar nature (Vinale *et al*., 2008b). The antibiotics can be divided into three distinct groups (Kotze, 2008) namely gliovirin, gliotoxin and viridin (Zaidi and Singh, 2013), which is central to antibiosis (Vinale *et al*., 2008a; Mukherjee *et al*., 2013; Samuels and Hebbar, 2015). A wide arsenal of CWDE is produced and significantly contributes to the evident success of this genus as competitor. The most notable of these includes chitinolytic- and glucanolytic enzymes, pectinases, phospholipases, polygalacturonases, proteases and xylanases (Baek *et al*., 1999; Antal *et al*., 2000; Monte, 2001; López-Mondéjar *et al*., 2011; Qualhato *et al*., 2013; John *et al*., 2015; Da Mota *et al*., 2016; Saravanakumar *et al*., 2016). These enzymes also exhibit antibiotics activity (Saravanakumar *et al*., 2016) that can inhibit mycelial growth and spore germination of various phytopathogens (Monte, 2001). This is achieved by hydrolyzing the immature walls of hyphal apices (Monte, 2001), mature cell walls (López-Mondéjar *et al*., 2011) and survival structures such as sclerotia and chlamydospores (Monte, 2001).

It is well documented that a synergistic effect exists between CWDE and different classes of antibiotics (Monte, 2001; Vinale *et al*., 2008a), though it is strictly related to the mechanism of action (Vinale *et al*., 2008b). For instance, Baek *et al*. (1999) found that the production of extracellular endochitinase by *T. virens* inhibited spore germination of *B. cinerea* conidia, caused cell wall damage, and lead to the eventual burst of hyphal tips, which acted synergistically in combination with gliotoxin. The synergistic affect between CWDE and peptaibols can be ascribed to the inhibition of β-glucan synthase activity that impedes the growth of the phytopathogens (Baek *et al*., 1999; Harman, 2006; Mutawila, 2010; Atanasova *et al*., 2013; Zaidi and Singh, 2013) and prevents the reconstruction of its cell walls (Vinale *et al*., 2008b; Mutawila, 2010).

Mycoparasitism

Mycoparasitism is an innate property of the genus (Atanasova *et al*., 2013) and can be described as the ability of *Trichoderma* species to directly parasitize other filamentous fungi and oomycetes (Harman and Shoresh, 2007). It comprises of sequential actions including recognition, attack and subsequent penetration (Monte, 2001; Harman, 2006; Vinale *et al*., 2008b; Kotze *et al*., 2011; López-Mondéjar *et al*., 2011; Zaidi and Singh, 2013; Saravanakumar *et al*., 2016), collectively referred to as “coiling”. For the latter mentioned event to occur, *Trichoderma* actively grows towards the target fungi using remote sensing (Harman, 2006; Harman and Shoresh, 2007; Vinale *et al*., 2008b; Zaidi and Singh, 2013), whereby constitutive levels of CWDE (mainly β-1,3-glucanase) are secreted (Harman, 2006; Vinale *et al*., 2008b; López-Mondéjar *et al*., 2011; Qualhato *et al*., 2013; Zaidi and Singh, 2013; John *et al*., 2015; Da Mota *et al*., 2016; Saravanakumar *et al*., 2016). These CWDE hydrolyzes fungal cell walls (consisting of chitin and β-glucan fibrils) of target fungi that are in

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close proximity to the *Trichoderma* (Monte, 2001; López-Mondéjar *et al*., 2011; Qualhato *et al*., 2013; John *et al*., 2015; Da Mota *et al*., 2016; Saravanakumar *et al*., 2016), causing the release of oligomers from the pathogen cell walls (Vinale *et al*., 2008b; López-Mondéjar *et al*., 2011; Zaidi and Singh, 2013). The oligomers then activate exochitinases that subsequently induce attack by the *Trichoderma* (Zaidi and Singh, 2013). The growth of the host fungus is impeded by the CWDE even before contact is made (Harman and Shoresh, 2007). Once the *Trichoderma* is in contact with the host fungi, its hyphae coils around the host and forms appresoria that is mediated by cell wall carbohydrates that binds to lectins of the target fungus (Harman and Shoresh, 2007; Anees *et al*., 2010; Zaidi and Singh, 2013). During this process many hydrolytic enzymes (CWDE)[including cellulases, (Harman, 2006; Zaidi and Singh, 2013; Samuels and Hebbar, 2015), chitinases, β-(1,4)-, β-(1,3)- and β-(1,6)-glucanases, proteases (Atanasova *et al*., 2013; Karagiosis and Baker, 2013; Zaidi and Singh, 2013; Samuels and Hebbar, 2015) and xylanases (Zaidi and Singh, 2013)] are produced that facilitates *Trichoderma* hyphae to penetration the lumen of the host fungus and absorb its nutrients (Harman and Shoresh, 2007; Zaidi and Singh, 2013; Saravanakumar *et al*., 2016).

Competition

Another mechanism of action is competition between *Trichoderma* and the phytopathogens for space or specific infection sites, iron and other limiting nutrients (Harman and Shoresh, 2007; Vinale *et al*., 2008; Kotze *et al*., 2011; Zaidi and Singh, 2013; Du Plessis, 2015; Samuels and Hebbar, 2015). The common occurrence of *Trichoderma* species in agricultural and natural soil systems worldwide suggests it to be a good competitor (Zaidi and Singh, 2013). Fourie *et al*. (2001) hypothesized that *Trichoderma* colonization in the rhizosphere prevented root pathogens from attacking developing roots of grapevine by means of this mode of action. It can possibly be ascribed to the consumption of germination stimulants (Mukherjee *et al*., 2013; Vargas *et al*., 2013) and the fast uptake and metabolism of sucrose from the rhizosphere, rendering less thereof available for utilization by phytopathogens (Vargas *et al*., 2013). Another limiting resource, for which *Trichoderma* can actively compete and result in this scenario, is iron. *Trichoderma* has the ability to rapidly mobilize and metabolize this element, due to its excellent capability to produce siderophores (Zaidi and Singh, 2013) that assist in the uptake thereof.

Systemic resistance

Trichoderma can colonize root surfaces and penetrate the root epidermis as avirulent plant symbionts, where it secretes a range of bioactive metabolites (Harman *et al*., 2004; Harman, 2006; Vinale *et al*., 2008a; Vinale *et al*., 2008b; Bailey and Melnick, 2013; Karagiosis and

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Baker, 2013). Some of these bioactive metabolites are known as microbe-associated molecular patterns (MAMPs), which includes VOCs (Qualhato *et al*., 2013; Chen *et al*., 2015)[mainly 6-PP (Harman *et al*., 2004; Vinale *et al*., 2008a; Vinale *et al*., 2008b; Mukherjee *et al*., 2013; Samuels and Hebbar, 2015)] and diffusible compounds [incl. peptaibiols (Vinale *et al*., 2008a Bailey and Melnick, 2013; Samuels and Hebbar, 2015), CWDE such as chitinases (Bailey and Melnick, 2013; Samuels and Hebbar, 2015), xylanases (Harman *et al*., 2004; Vinale *et al*., 2008b)] and avirulence-like gene products that can induce defense mechanisms (Harman *et al*., 2004; Vinale *et al*., 2008b). These MAMPs are recognized by plant receptors as being potentially dangerous (Harman *et al*., 2004; Harman, 2006; Perazzolli *et al*., 2008; Mukherjee *et al*., 2013; Zaidi and Singh, 2013; Du Plessis, 2015; Samuels and Hebbar, 2015), inducing either systemically acquired resistance (SAR)(Anees *et al*., 2010; Perazzolli *et al*., 2011; Contreras-Cornejo *et al*., 2013; Banani *et al*., 2014), localized acquired resistance (LAR)(Vinale *et al*., 2008b; Anees *et al*., 2010) or induced systemic resistance (ISR) (Vinale *et al*., 2008a; Vinale *et al*., 2008b; Anees *et al*., 2010; Gal-Hemed *et al*., 2011; Perazzolli *et al*., 2011; Contreras-Cornejo *et al*., 2013; Banani *et al*., 2014; Samuels and Hebbar, 2015). Colonization by *Trichoderma* mostly results in the two latter mentioned defense mechanisms (LAR and ISR)(Harman *et al*., 2004; Anees *et al*., 2010).

Various regulatory genes that are involved in plant defense get activated (Harman, 2006; Perazzolli *et al*., 2008; Vinale *et al*., 2008a; Samuels and Hebbar, 2015), resulting in more than 300 proteins with altered expression (Harman and Shoresh, 2007). Some of these include the enhanced production of anthocyanin (Contreras-Cornejo *et al*., 2013), proteins associated with pathogen resistance and stress (Harman and Shoresh, 2007; Vinale *et al*., 2008a) and structural barriers such as lignification (Baek *et al*., 1999). The transport of compounds such as phospholipids and ions are also stimulated during this process (Bailey and Melnick, 2013; Contreras-Cornejo *et al*., 2013). Furthermore, it also results in the activation of the jasmonic acid (JA) and salicylic acid (SA) pathways (Bailey and Melnick, 2013; Contreras-Cornejo *et al*., 2013). These cycles in turn regulate the ISR and SAR in the plant, respectively (Banani *et al*., 2014; Perazzolli *et al*., 2011). Induced systemic resistance, that is more commonly associated with *Trichoderma* symbiosis, offers broad-spectrum protection (Perazzolli *et al*., 2008, 2011; Banani *et al*., 2014) and involves the activation of a priming state (Perazzolli *et al*., 2008; Banani *et al*., 2014) without significant energy costs to plant metabolism and growth (Perazzolli *et al*., 2011). Interestingly, the JA cycle functions in conjunction with the ethylene cycle (ET) and inhibits the SA cycle (Perazzolli *et al*., 2011), resulting in less expenditure to the plant.

This mode of action has been detected in grapevine following inoculation with *T. viride* and *T. harzianum* (Perazzolli *et al*., 2008; Banani *et al*., 2014) and has previously been

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used to argue the reduction of decline pathogens (including "*Cylindrocarpon*" spp.) in nursery vines in a study by Fourie *et al*. (2001). Another study by Mutawila *et al*. (2017) showed increased expression of genes involved in both SAR and ISR following inoculation with *T. atroviride*. Interestingly, similar results were obtained when cells of *V. vinifera* were treated with autoclaved filtrates of *T. atroviride*, indicating that elicitation was mostly not dependent on enzymatic activity. Indeed, Harman *et al*. (2004) proposed that this is the main effect exerted by *Trichoderma* that contributes to its biocontrol activity. However, Banani *et al*. (2014) suggested that it is essential to use responsive grapevine cultivars to maximize the efficacy of induced resistance as it is affected by genetic factors of different cultivars.

Growth stimulation

Growth stimulation attributed to root colonization by *Trichoderma* and the molecules exerted by it also comprises an integral part of crop management, though it received little attention in the past. Studies mainly focused on the biocontrol abilities of these fungi, though it extends beyond plant disease management to abiotic stress management and improved overall plant health (Harman *et al*., 2004; Harman and Shoresh, 2007; Vinale *et al*., 2008b; Zaidi and Singh, 2013; López-Bucio *et al*., 2015).

Growth stimulation results in increased root development, secondary root formation and shoot size (Harman, 2006; Di Marco and Osti, 2007; Harman and Shoresh, 2007; Zaidi and Singh, 2013; Du Plessis, 2015; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015) that not only improves nutrient and water uptake (López-Bucio *et al*., 2015), but simultaneously provides more niches for the growth of the fungus (Harman and Shoresh, 2007). *Trichoderma* species decomposes organic matter in the soil that increases organic acids [incl. citric, fumaric, gluconic and humic acids acid (Vinale *et al*., 2008b ; Zaidi and Singh, 2013)] lowering the soil pH (Vinale *et al*., 2008b). This results in the solubilization of various minerals and nutrients (e.g. phosphate and iron)(Harman *et al*., 2004; Harman, 2006; Harman and Shoresh, 2007; Vinale *et al*., 2008b; Mukherjee *et al*., 2013; Zaidi and Singh, 2013; López-Bucio *et al*., 2015) that increases the plants ability to take it up (Vinale *et al*., 2008b; Qualhato *et al*., 2013; Zaidi and Singh, 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015; Gal-Hemed *et al*., 2011). This would otherwise not have been available to crops in agricultural systems (Samuels and Hebbar, 2015), and in effect making the plants more tolerant under nutrient stress conditions (Contreras-Cornejo *et al*., 2013; Zaidi and Singh, 2013; López-Bucio *et al*., 2015). Moreover, tolerance to abiotic stresses such as drought, salinity and temperature are also enhanced (Contreras-Cornejo *et al*., 2013; Zaidi and Singh, 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015). This phenomenon has been observed in the absence of disease, implying that it is not a side effect of disease suppression (Zaidi and Singh, 2013).

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The symbiotic relationship seems to influence an array of metabolic pathways that exerts growth promotion. Energy metabolism of both the glycolysis and tricarboxylic acid cycle is also increased, resulting in increased shoot starch content (Harman and Shoresh, 2007; Zaidi and Singh, 2013). The alteration of these pathways also enhances photosynthetic activity (Mukherjee *et al*., 2013; Samuels and Hebbar, 2015) by reducing oxidized glutathione and ascorbate (reactive species involved with photosynthesis) to less toxic forms (Zaidi and Singh, 2013).

Trichoderma spp. produces phytohormones and certain other secondary metabolites that influence plant growth regulation and stimulates growth (Contreras-Cornejo *et al*., 2013; López-Bucio *et al*., 2015). A mycorrhizal-like characteristic between *Trichoderma* spp. and its host plant involves the exchange of auxin-related signals, which is responsible for various aspects of plant development (Contreras-Cornejo *et al*., 2013; López-Bucio *et al*., 2015). *Trichoderma* spp. has the ability to produce a range of auxins, including indole-3-acetic acid (IAA)(Contreras-Cornejo *et al*., 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015) and its precursors indole-3-ethanol, indole-2-acetaldehyde, indole-3-acetaldehyde and indole-3-carboxaldehyde (Contreras-Cornejo *et al*., 2013). The VOCs exerted by these fungi, with emphasis on 6-PP, also promotes plant growth (Zeilinger and Schuhmacher, 2013; Chen *et al*., 2015) by acting in a similar fashion to that of auxin or as auxin inducer (Vinale *et al*., 2008a). These molecules influences colonization of the roots by the fungus (Contreras-Cornejo *et al*., 2013), further improving plant nutrition (N, P, K) and micronutrient uptake, enhancing photosynthesis and carbon metabolism and stimulates plant growth and root formation (Contreras-Cornejo *et al*., 2013; López-Bucio *et al*., 2015) as well as various other changes at proteome level (López-Bucio *et al*., 2015).

This effect has been shown in *Arabidopsis*, where inoculation with a strain of *T. harzianum* that produces IAA-related indoles, resulted in increased lateral root formation, plant biomass and foliar area (Contreras-Cornejo *et al*., 2013). Collectively, these alterations in plant metabolism increase its tolerance towards biotic and abiotic stresses (Zaidi and Singh, 2013). It is, however, both species and host cultivar specific (Zaidi and Singh, 2013).

Formulation of biological control agents

Extensive research has been conducted to develop *Trichoderma*-based agricultural remedies, resulting in the commercialization of countless products that is currently available on the market. This can mainly be attributed to the antagonistic abilities of this fungus (Zaidi and Singh, 2013; Samuels and Hebbar, 2015), ease of manipulation and ubiquity thereof (Samuels and Hebbar, 2015). These products may contain a variety of fungal propagules such as conidia, chlamydospores and mycelium (Samuels and Hebbar, 2015). However, for the biocontrol remedies to be effective it is essential that these propagules stay viable during

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the process of formulation, storage and application (López-Bucio *et al*., 2015; Samuels and Hebbar, 2015). Various techniques and substrates are used for the production of inoculum, each of which impacts the final type of propagule being produced.

The majority of *Trichoderma*-based products contain conidia as inoculum that is produced in mass fermentation (Mukherjee *et al*., 2013; Samuels and Hebbar, 2015) through asexual reproduction known as conidiation (Mukherjee *et al*., 2013). The reason for this being that it germinates much faster than chlamydospores and are easier to harvest, formulate, and apply (Samuels and Hebbar, 2015). However, the germination is affected by various abiotic factors such as nutrients, temperature and light (Kotze, 2008). Moreover, products containing conidia as propagules need to be refrigerated to extend the shelf life of the product (Samuels and Hebbar, 2015). Alternatively, chlamydospores can be used as propagules in biocontrol remedies. It has a longer shelf life at ambient temperatures and is more stable in the environment than conidia (Samuels and Hebbar, 2015), but requires a longer cultivation period making it more costly to produce (Mutawila, 2010).

Unfortunately some biocontrol remedies fail to work because of poor quality control and short shelf life (Butt *et al*., 2001; Zaidi and Singh, 2013), loss of viability, contamination during the propagation process, inappropriate storage conditions as well as improper methods of application (Samuels and Hebbar, 2015). In a study conducted by Hjeljord *et al*. (2000) it was found that commercially formulated conidia lost its capacity to germinate and effectively colonize nutrient-poor natural substrates, eventually resulting in poor disease control. These failures can, however, be minimized with the improvement of biocontrol remedies in terms of higher initial colony forming units, extended shelf life and improved efficacy under both biotic- [antagonists factors (Butt *et al*., 2001)] and abiotic stress conditions [high soil pH, salinity, low moisture (Mukherjee *et al*., 2013), suboptimal temperatures and nutrient deficiencies (Hjeljord *et al*., 2000)]. It is therefore of utmost importance that the initial screening for *Trichoderma* isolates as biocontrol agent is done comprehensively, both *in vitro* and under field conditions.

CONCLUSION

From the literature it is clear that black foot disease is an ever-increasing problem in grapevine nurseries and newly established vineyards worldwide. This results in infected vines being planted in vineyards, ultimately contributing to young vine failure. With the disease having such great economical importance, and no effective management strategies implemented in South Africa, it is necessary to investigate possible control methods. The *Trichoderma* genus, being one of the most widely used biofungicides, offers the potential to be used as a biological control agent against this disease. Further investigation is, however, necessary to determine its efficacy against BFD pathogens. With such research, it is

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possible to develop a sustainable and eco-friendly method to systematically lower the disease incidence both in grapevine nurseries and vineyards.

AIM OF THIS STUDY

The aim of this study was to investigate the efficacy of *Trichoderma* spp. colonization of grapevine nursery plants for the improved control of black foot disease. The specific objectives of this study were i) to determine the efficacy of *Trichoderma* spp. against black foot pathogens *in vitro*, ii) to evaluate different *Trichoderma* products and methods of application to nursery vines post callusing and iii) to evaluate the extent of *Trichoderma* colonization of rootstocks and roots.

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TABLES AND FIGURES

Table 1. Distribution of black foot pathogens isolated from *Vitis* spp.

Species	Associated name(s)	Distribution	Reference
Campylocarpon fasciculare Schroers, Halleen & Crous		Brazil, New Zealand, South Africa, Spain, Uruguay, Turkey	Halleen *et al*., 2004; Abreo *et al*., 2010; Alaniz *et al*., 2011b; Correia *et al*., 2012; Silva *et al*., 2017
Campylocarpon pseudofasciculare Halleen, Schroers & Crous		Brazil, New Zealand, Perú, South Africa; Spain, Uruguay, Turkey	Halleen *et al*., 2004; Abreo *et al*. 2010; Álvarez *et al*., 2012; Correia *et al*., 2012; Silva *et al*., 2017
Cylindrocladiella parva (P.J. Anderson) Boesewinkel	= *Cylindrocladium parvum*	New Zealand, South Africa, Spain	Van Coller *et al*., 2005; Agustí-Brisach *et al*., 2012; Jones *et al*., 2012; Úrbez-Torres *et al*., 2014; Varela *et al*., 2017
Cylindrocladiella peruviana (Bat., J.L. Bezerra & M.P. Herrera) Boesewinkel		Perú, South Africa, Spain	Van Coller *et al*., 2005; Agustí-Brisach *et al*., 2012; Álvarez *et al*., 2012; Úrbez-Torres *et al*., 2014
Dactylonectria alcacerensis (A. Cabral, H. Oliveira & Crous) L. Lombard & Crous	=*C. macrodidymum* complex =*I. macrodidyma* complex =*I. alcacerensis*	Iberian Peninsula, Portugal, South Africa, Spain	Cabral *et al*., 2012a, c; Agustí-Brisach *et al*., 2013a, b; Berlanas *et al*., 2017; Varela *et al*., 2017
Dactylonectria estremocensis (A. Cabral, T. Nascim. & Crous) L. Lombard & Crous	=*C. macrodidymum* complex =*I. macrodidyma* complex =*I. estremocensis*	Iberian Peninsula, Portugal	Cabral *et al*., 2012a, c

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Table 1. Continued.

Species	Associated name(s)	Distribution	Reference
Dactylonectria macrodidyma (Halleen, Schroers & Crous) L. Lombard & Crous	= *Neonectria macrodidyma* = *Ilyonectria macrodidyma* = *Cylindrocarpon macrodidymum complex*	Australia, British Columbia, California, Canada, Chile, France, New Zealand, Portugal, South Africa, Spain, Turkey, Uruguay	Halleen *et al*., 2004, 2006b; Petit and Gubler, 2005; Auger *et al*., 2007; Abreo *et al*., 2010; Petit *et al*., 2011; Cabral *et al*., 2012c; Özben *et al*., 2012; Agustí-Brisach *et al*., 2013a, b; Úrbez-Torres *et al*., 2014; Varela *et al*., 2017
Dactylonectria novozelandica (A. Cabral & Crous) L. Lombard & Crous	=*C. macrodidymum* complex =*I. macrodidyma* complex =*I. novozelandica*	New Zealand, South Africa, Spain, USA	Halleen *et al*., 2004; Cabral *et al*., 2012a, c; Agustí-Brisach *et al*., 2013a, b
Dactylonectria pauciseptata (Schroers & Crous) L. Lombard & Crous	=*C. pauciseptatum*	British Columbia, Slovenia, New Zealand, Portugal, Slovenia, South Africa, Spain, Uruguay, USA	Schroers *et al*., 2008, Abreo *et al*., 2010; Martín *et al*., 2011; Cabral *et al*., 2012a; Reis *et al*., 2013, Úrbez-Torres *et al*., 2014, Langenhoven, 2017; Varela *et al*., 2017
Dactylonectria torresensis (A. Cabral, Rego & Crous) L. Lombard & Crous	=*C. macrodidymum* complex =*I. macrodidyma* complex =*I. torresensis*	Australia, British Columbia, Czech Republic, Italy, New Zealand, Portugal, South Africa, Spain, USA	Cabral *et al*., 2012a, c; Agustí-Brisach *et al*., 2013a, b; Reis *et al*., 2013; Úrbez-Torres *et al*., 2014; Berlanas *et al*., 2017; Carlucci *et al.,* 2017; Varela *et al*., 2017; Pečenka *et al*., 2018
Dactylonectria vitis (A. Cabral, Rego & Crous) L. Lombard & Crous	=*C. destructans* complex =*I. radicola* =*I. vitis*	Portugal	Cabral *et al*., 2012a

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Table 1. Continued.

Species	Associated name(s)	Distribution	Reference
Ilyonectria europaea A. Cabral, Rego & Crous	=*C. destructans* complex =*I. radicola*	France, Iberian Peninsula Portugal	Cabral *et al*., 2012a
Ilyonectria liriodendri (Halleen, Rego & Crous) P. Chaverri & Salgado	=*C. destructans* complex =*C. liriodendri*	Australia, Brazil, British Columbia; California, France, Iran, Italy, New Zealand, Portugal, South Africa, Spain, Switzerland, Uruguay, USA	Sheck *et al*., 1998; Halleen *et al*. 2006b; Alaniz *et al*., 2007; Petit *et al*., 2007; Whitelaw-Weckert *et al*., 2007; Abreo *et al*., 2010; Casieri *et al*., 2009; Mohammadi *et al*., 2009; Russi *et al*., 2010; Abreo *et al*., 2010; Petit *et al*., 2011; Cabral *et al*., 2012a; Úrbez-Torres *et al*., 2014; Berlanas *et al*., 2017; Carlucci *et al.,* 2017; Varela *et al*., 2017
Ilyonectria lusitanica A. Cabral, Rego & Crous	=*C. destructans* complex =*I. radicola*	Australia, Portugal	Cabral *et al*., 2012a
Ilyonectria pseudodestructans A. Cabral, Rego & Crous	=*C. destructans* complex =*I. radicola*	Iberian Peninsula, Portugal	Cabral *et al*., 2012a
Ilyonectria robusta (A.A. Hildebr.) A. Cabral & Crous	=*C. destructans* complex =*I. radicola*	British Columbia, Portugal, Spain	Cabral *et al*., 2012a; Úrbez-Torres *et al*., 2014; Varela *et al*., 2017
Thelonectria blackeriella M.L. Raimondo and A. Carlucci		Italy	Carlucci *et al.,* 2017

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Table 2. List of commercially available *Trichoderma*-based products in South Africa.

Product name	Company	Species (commercial isolate)	Crop	Target disease or pathogen
Awegenic Tri-cure™	MBFi	*T. harzianum* (MIT04)	Dry beans, Groundnuts, Maize, Potatoes, Soybeans, unspecified vegetables, Wheat	*Fusarium*, *Pythium*, *Rhizoctonia*
Bio-Tricho	AgroOrganics	*T. atroviride* (Vitic 2) *T. harzianum* (Sp)	Various crops	Various diseases
Eco 77®	Plant Health Products	*T. atroviride B77*	Cucumber, grapevine, tomatoes	*Botrytis*, *Eutypa*
Eco T®	Plant Health Products	*T. asperellum* (kd)		*Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*
Excalibur Gold™	Advanced Biological Marketing	*Trichoderma harzianum* (K2) *Trichoderma atroviride* (K4) *Trichoderma virens* (K1)	Unspecified vegetables	Unspecified
Graph-Ex SA™	Advanced Biological Marketing	Unspecified *Trichoderma* isolate	Corn, Soybeans, Wheat	Unspecified
Hygromix-T	Hygrotech	*T. harzianum* (BD103)	Unspecified	Unspecified
Romulus	Dagutat Biolab	*T. harzianum* (DB 104)	Sugarcane	Unspecified
Rootshield	Bioworks inc	*T. harzianum* (Rifai T-22) *T. harzianum* (Rifai KRL-AG2)	Unspecified	Soilborne diseases
SabrEx®	Advanced Biological Marketing	*Trichoderma* spp.	Corn, wheat	Unspecified
Trichodex	Adama	*T. harzianum* (T39)	Grapevine	*Botrytis*

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Table 2. Continued.

Product name	Company	Species (commercial isolate)	Crop	Target disease or pathogen
TrichoPlus™	BASF	*T. fertile*	Beans, Cucumber, Maize, Potatoes, Strawberries, Tomatoes	Soil borne diseases
Trykocide	Axiom bio-product	*T. harzianum*	Various	Soil borne diseases

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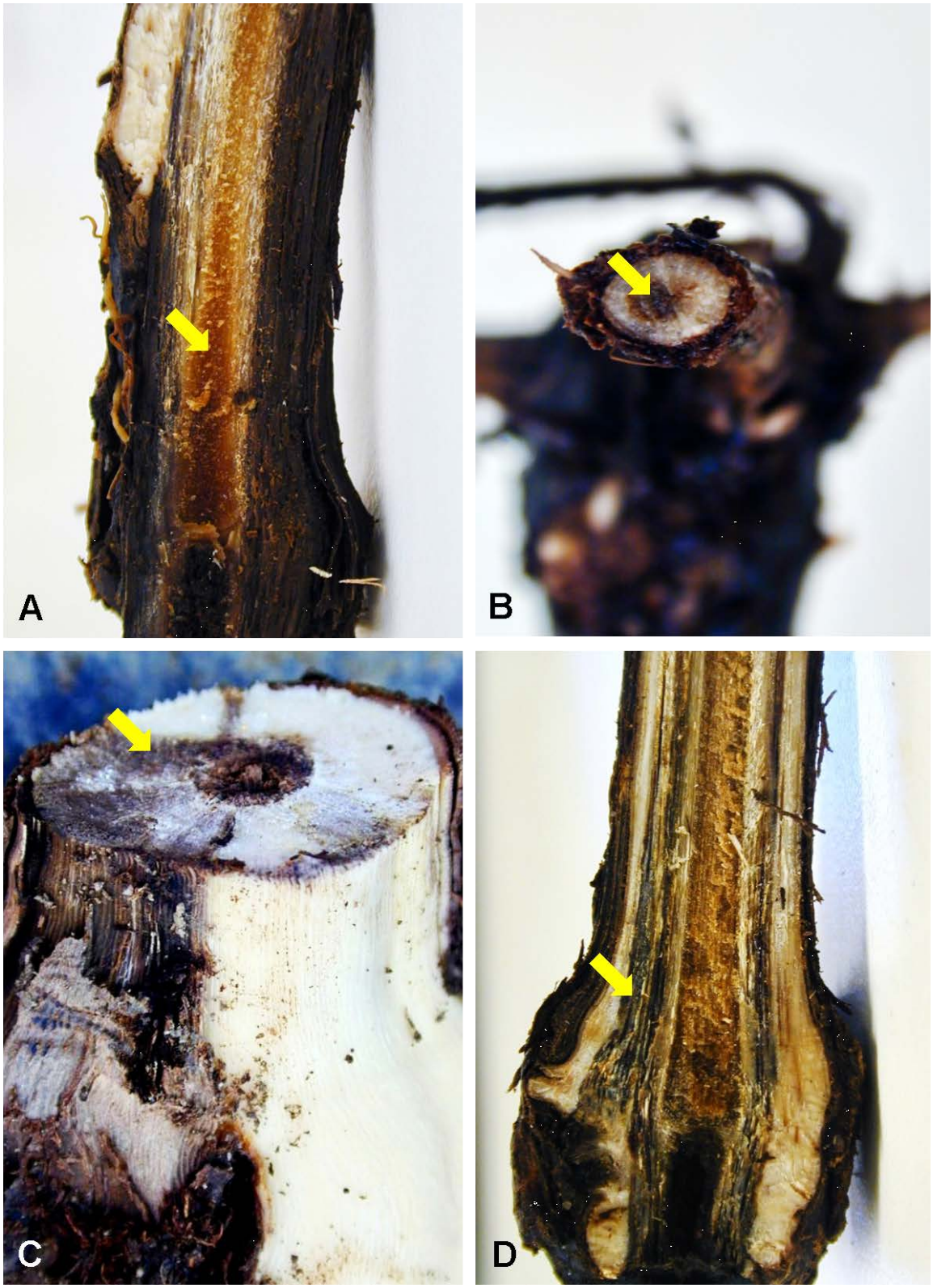

Figure 1. Internal symptoms of *Vitis* spp. associated with black foot disease. A) Cross section through a black foot disease infected rootstock revealing severe necrosis from the bark to the pith; B) brown discoloration under the bark that extends upward from base of the rootstock; C) dark vascular streaking as seen in longitudinal section of young grapevine and D) a cross section of an infected root that reveals necrosis.

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CHAPTER 2

Investigation of *Trichoderma* species colonization of nursery grapevines for improved management of black foot disease

ABSTRACT

Black foot disease (BFD) is one of the main fungal diseases associated with young grapevine decline and is caused by species from the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria* and *Thelonectria*. Apart from hot water treatment of dormant nursery vines, no treatment is available to prevent nursery plants from becoming infected with BFD pathogens after planting. *Trichoderma* species are well-known biocontrol agents (BCAs) and have shown good efficacy to prevent grapevine trunk disease infections of pruning wounds. However, no *Trichoderma* product is registered for root application on grapevine in South Africa. Therefore the aim of this study was to investigate different *Trichoderma* products and application methods for the improved control of BFD in grapevine nurseries. Ten *Trichoderma* spp. isolates were tested *in vitro* for their ability to inhibit the mycelial growth of four major BFD pathogens, namely *Ca. fasciculare*, *Ca. pseudofasciculare*, *D. macrodidyma* and *I. liriodendri* by means of volatile organic compounds (VOCs), diffusible antibiotic compounds (DACs) and direct antagonism. In most cases *Trichoderma* inhibited the growth of BFD pathogens, though higher growth inhibition was obtained with the DACs than the VOCs. For both classes of compounds *D. macrodidyma* were found to be more sensitive (maximum of 100% inhibition by DACs and 66.94% by VOCs). The efficacy of *Trichoderma* species to endophytically colonize grapevine rootstock xylem vessels, phloem elements and pith were evaluated on rootstock shoots of five grapevine cultivars including Ramsey, Richter 99, Richter 110, US8-7 and Paulsen. The lower 5 cm of the rootstock material was soaked in a *Trichoderma atroviride* conidial suspension (1×10^6 CFU.mL^{-1}) for 1 min, 10 min, 1 hr and 2 hrs and then incubated in sterile moisture chambers followed by fungal isolations from the xylem and pith. *Trichoderma atroviride* was able to successfully colonize all five rootstock cultivars to a depth of 10 cm. In general did a longer soaking period not significantly increase *T. atroviride* colonization. In order to assess the effect of different application methods on *Trichoderma* colonization and BFD control, nine treatments were evaluated on nursery vines post callusing. One hundred graftlings were used per treatment, replicated five times and repeated over two seasons. To assess the efficacy of different commercially available *Trichoderma*-based products another trial was carried out on grapevine nursery vines post callusing using eight commercially produced products. One hundred graftlings were used per treatment, replicated four times and repeated over two seasons. For both trials the graftlings were uprooted after 7 months and the number of certifiable vines and total wet root mass determined. Fungal isolations were made from the xylem and pith in the basal end as well as at three sections of the roots.

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Subsequent *Trichoderma* isolates and BFD pathogens were identified based on colony morphology and BFD pathogens verified by means of genus-specific primers using a newly designed primers pair for *Campylocarpon* in combination with two previously described primer pairs for *Dactylonectria* spp. and *Ilyonectria liriodendri*. The different application methods clearly showed that dipping the basal ends in a dry formulation followed by monthly soil drenches, consistently gave higher colonization of *Trichoderma* (39.20% in the base and 26.40% in the roots in 2016/17; and 28.00% in the base and 15.73% in the roots in 2017/18). Field drenching alone was significantly less effective than the dry dip application or a combination of these treatments (19.20% in the base and 8.80% in the roots in 2016/17; and 9.60% in the base and 7.47% in the roots in 2017/18). Soaking of the basal ends of vines in a conidial suspension for one hour was ineffective (8.80% in the base and 5.87% in the roots in 2016/17; and 4.00% in the base and 2.40% in the roots in 2017/18) and did not differ from the untreated control (6.40% in the base and 5.87% in the roots in 2016/17; and 2.00% in the base and 1.33% in the roots in 2017/18). None of the application methods resulted in significant differences between percentage certifiable vines, total wet root mass or BFD pathogen incidence. The trial evaluating different *Trichoderma*-based products showed products that contain *T. atroviride* originating from grapevine, namely Eco 77®, USPP-T1 and USPP-MT1 to be the more effective in colonizing nursery vine rootstocks. In the 2016/17 season all of the products resulted in significantly lower BFD pathogen incidence in the basal ends of the vines (ranging from 1.00% to 2.00% vs. 6.50% in the untreated control). When comparing tissue parts, the base of the vine and top part of roots had significantly higher *Trichoderma* colonization (maximum of 29.25% in 2016/17; and 24.11% in 2017/18) than the middle and bottom parts of the roots (maximum of 13.88% in 2016/17; and 11.11% in 2017/18). Significantly less BFD pathogens were isolated from the base in comparison to the roots (maximum of 5.49% vs. 21.14 in 2016/17; and 11.47% vs. 28.22% in 2017/18). Even though *Trichoderma* spp. were not sufficient to prevent infections by BFD pathogens, a certain degree of protection was obtained in the basal ends. The effect of the *Trichoderma* spp. in the nursery vines post transplanting in relation to black foot development remains to be determined. Combining existing knowledge of *Trichoderma* spp. as BCA with the knowledge obtained from this research will assist in optimizing the application procedure in nurseries post callusing.

INTRODUCTION

One of the main fungal diseases associated with young grapevine decline is black foot disease (BFD) (Halleen *et al*., 2006; Gramaje *et al*., 2010; Dos Santos *et al*., 2016). In recent years its incidence and severity has increased significantly, affecting both nurseries and young vineyards (Scheck *et al*., 1998b; Halleen *et al*., 2004; Halleen *et al*., 2006a). It is now

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considered as being one of the major destructive grapevine trunk diseases, causing substantial economic losses in grapevine industries worldwide (Scheck *et al*., 1998b; Petit *et al*., 2005; Rego *et al*., 2009). The genera purported to contribute to this disease includes *Campylocarpon* Halleen, Schroers and Crous, *Cylindrocladiella* Boesew., *Dactylonectri*a L. Lombard and Crous, *Ilyonectria* P. Chaverri and C. Salgado and *Thelonectria* P. Chaverri and C. Salgado. Species associated with BFD in South Africa includes *Campylocarpon fasciculare* Schroers, Halleen and Crous and *Campylocarpon pseudofasciculare* Halleen, Schroers and Crous (Halleen *et al*., 2004; 2006; Cabral *et al*., 2012c; Carlucci *et al*., 2017); *Dactylonectria alcacerensis* (A. Cabral, Oliveira and Crous) L. Lombard and Crous, *Dactylonectria macrodidyma* (Halleen, Schroers and Crous) L. Lombard and Crous, *Dactylonectria novozelandica* (A. Cabral and Crous) L. Lombard and Crous, *Dactylonectria pauciseptata* (Schroers & Crous) L. Lombard and Crous and *Dactylonectria torresensis* (A. Cabral, Rego and Crous) L. Lombard and Crous as well as *Ilyonectria liriodendri* (Halleen, Rego and Crous) Chaverri and C. Salgado. *Cylindrocladiella parva* (P.J. Anderson) Boesew. and *Cylindrocladiella peruviana* (Bat., J.L. Bezerra and M.P. Herrera) Boesew., have been isolated from grapevine in South Africa (Van Coller *et al*., 2005) though its role in BFD in South Africa remains unknown.

Fungi belonging to these genera are common soil inhabitants occurring as saprophytes, root colonizers or weak plant pathogens that can survive in soil for extended periods of time due to the formation of chlamydospores (Brayford, 1993; Halleen *et al*., 2004). In South Africa the majority of grapevine nursery soils are contaminated with BFD pathogens (pers com., Francois Halleen), especially in areas where the same soils have been used for grapevine propagation for decades (Halleen *et al*., 2006a). Numerous studies reported an increase of BFD infection after the rooting phase in the nurseries (Halleen *et al*., 2003; Dubrovsky and Fabritius, 2007; Rego *et al*., 2009; Agustí-Brisach *et al*., 2013; Cardoso *et al*., 2013; Reis *et al*., 2013), confirming it as the main source of infection in vines (Fourie *et al*., 2001, Halleen *et al*., 2003; Rego *et al*., 2006; Gramaje and Armengol, 2011).

Due to the soilborne nature of the disease, infection of the roots occurs first, followed by infection of the basal ends of the rootstocks (Halleen *et al*., 2003; 2006). Below ground symptoms includes sunken necrotic root lesions and reduction in root biomass with characteristic black discoloration (Scheck *et al*., 1998a; Larignon, 1999) that often result in the formation of a second crown of horizontally growing roots on the upper level of the rootstock (Scheck *et al*., 1998b), while the rootstock diameter below the second tier thins (Fourie and Halleen, 2001; Halleen *et al*., 2004; 2006; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013). Internal symptoms include black discoloration developing from the base and extending upwards in the rootstock (Sweetingham, 1983; Larignon, 1999), necrotic lesions and congested xylem vessels that spread from the bark to the compacted pith

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(Halleen *et al*., 2004). Vine decline becomes apparent as stunted growth, shortened internodes, sparse foliage and reduced vigor as well as small leaves with interveinal chlorosis and necrosis (Halleen *et al*., 2004; Rego *et al*., 2006; Alaniz *et al*., 2007; Dubrovsky and Fabritius, 2007; Alaniz *et al*., 2011; Cabral *et al*., 2012).

In most cases, however, latent pathogens established within the xylem tissue of seemingly healthy vines only become evident once the plants are placed under stress conditions (Gramaje and Armengol, 2011). Such conditions, that favor the development of BFD during the nursery process, includes wounds created during cutting and grafting, uprooting, trimming and cold storage, to name but a few (Oliveira *et al*., 2004; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013). Once planted in the vineyards, bad vineyard management practices (including poor water drainage, soil compaction, planting of vines in poorly prepared soil and improper planting holes, malnutrition, heavy crop loads on young vines) (Gubler *et al*., 2004; Halleen *et al*., 2006; Gramaje and Armengol, 2011; Probst *et al*., 2012; Agustí-Brisach *et al*., 2013) and unfavorable environmental conditions (including high summer temperatures) can further stress the vines that ultimately results in death (Gramaje and Armengol, 2011).

Currently there are no curative control measures available to eradicate BFD in nurseries or vineyards (Halleen *et al*., 2006; Alaniz *et al*., 2010; Gramaje and Armengol, 2011; Agustí-Brisach *et al*., 2013) and, therefore, preventative management is of utmost importance (Di Marco *et al*., 2004). Various studies have investigated the use of chemical fungicides with little success, due to its inability to penetrate phloem and xylem tissue (Waite and May, 2005; Gramaje and Armengol, 2011), fluctuating levels of sensitivity of BFD pathogens (Alaniz *et al*., 2011) and the shortage of registered products. Hot water treatment (HWT) of dormant nursery vines, a process in which rooted vines are heated to 50°C for 30 min, have been considered effective to control BFD (Halleen *et al*., 2006, 2007; Alaniz *et al*., 2011; Agustí-Brisach *et al*., 2013), though its effect is short-term and may result in subsequent vine failure if not executed properly and if low quality propagation material is used (Rego *et al*., 2009). As an alternative, the use of *Trichoderma* spp. as biological control agents (BCA) offers more sustainable and lasting protection against numerous grapevine trunk diseases (Fourie and Halleen, 2001; Mondello *et al*., 2018). Currently there are no *Trichoderma*-based products registered for root application on grapevine in South Africa.

Fourie *et al*. 2001 investigated the application of *Trichoderma* by soaking rootstocks prior to grafting, applying pellets in the planting row and applying soil drenches on a monthly basis. It was clear from this study that the *Trichoderma* treated plants had less *Cylindrocarpon* spp. (although not statistically significant) and larger root mass than the controls. In a further study, comparing different chemical and biological treatments (which included *Trichoderma*), hot water treatment of dormant nursery plants after uprooting

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showed to be more effective in the management of BFD (Halleen *et al*. 2007). The effect of HWT of dormant material on *Trichoderma* colonization was questioned and therefore, also included in this study. Two HWT regimes were included because of the more recent application of 45 min at 50°C for the elimination of aster yellows.

The application method of *Trichoderma* spp. is essential to ensure good colonization. In a study by Halleen *et al*. (2007) low re-isolation percentages of *Trichoderma* spp. was ascribed to insufficient systemic colonization of the basal ends of rootstocks, which according to the authors, could be alleviated by prolonging the 1 min dip treatment prior to planting in the nursery. Di Marco and Osti (2008) compared application during callusing, before planting in nurseries and drenches, and found that before planting gave the best results in terms of root development. The efficiency of soil drenches after planting in nurseries needs to be determined. It also needs to be determined if *Trichoderma* applied before planting in nurseries is present in new roots formed in the soil.

The aim of this study was to investigate the efficacy of *Trichoderma* spp. colonization of grapevine nursery plants for the improved control of black foot disease. The specific objectives of this study were i) to determine the efficacy of *Trichoderma* spp. isolates against black foot pathogens *in vitro*, ii) to evaluate different *Trichoderma* products and methods of application to nursery vines post callusing and iii) to evaluate the extent of *Trichoderma* colonization of rootstocks and roots.

MATERIALS AND METHODS

In vitro evaluation

Fungal isolates

Ten *Trichoderma* spp. isolates, of which eight are from commercial products (Table 1), were tested *in vitro* for their ability to inhibit the mycelial growth of BFD pathogens according to *in vitro* assays by Dennis and Webster (1971a; b) with some amendments. The BFD pathogens included three isolates of *Ca. fasciculare*, *Ca. pseudofasciculare*, *D. macrodidyma* and *I. liriodendri*, respectively (Table 2).

Screening Trichoderma *isolates for the production of volatile organic compounds*

Trichoderma isolates and BFD pathogens were grown on potato dextrose agar (PDA) (Biolab, Midrand, South Africa) in the dark at 25°C for three and seven days, respectively. Mycelial plugs (5 mm) were cut from the growing margins of the colonies and placed face down in the center of 90 mm PDA Petri dishes. The Petri dishes containing the pathogens were then inverted over the ones containing the *Trichoderma* isolates in all combinations and sealed with Parafilm® M (Bemis, Neenah, Wisconsin). The Petri dishes were incubated in the dark at 25°C for seven days. Control plates were set up in the same manner, with the

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exception that BFD pathogen inoculated dishes were combined with sterile PDA Petri dishes. Following the incubation period, the colony diameters of BFD pathogens from each Petri dish were measured twice, perpendicular across the colony.

Screening Trichoderma *isolates for the production of diffusible antifungal compounds*

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for three and eight days, respectively. Mycelial plugs (5 mm) cut from the growing margins of the *Trichoderma* colonies were placed face down on autoclaved 50 µm thick cellophane membranes (Sigma, Germany) (85 mm in diameter) covering PDA Petri dishes. The Petri dishes were then incubated in the dark at 25°C until a colony diameter of 50 mm was reached (ranging from 42 to 48 hr). Following the incubation period the cellophane membranes were removed ensuring that the Petri dishes were completely free of *Trichoderma* spores and mycelia. The Petri dishes were then re-inoculated with mycelial plugs (5 mm) cut from the growing margins of the BFD pathogen colonies and incubated in the dark at 25°C for a duration of six days. Control plates were set up in the same manner, with the exception that *Trichoderma* were not inoculated on the cellophane membranes. Following the incubation period, the colony diameters of BFD pathogens from each Petri dish were measured twice, perpendicular across the colony.

Competitive growth

Trichoderma isolates and BFD pathogens were grown on PDA in the dark at 25°C for three and seven days, respectively. Mycelial plugs (5 mm) of the pathogen were cut from the growing margins of the colonies and placed face down on one side of 90 mm Petri dishes. The Petri dishes were then incubated in dark conditions at 25°C for four days. Following this incubation period mycelial plugs (5 mm) were cut from the growing margins of the *Trichoderma* colonies and placed face down opposing the BFD pathogen colonies and incubated for another six days. The duel-inoculated plates were then used to observe the interaction zones macroscopically. Hyphal interactions were observed by mounting an agar block cut from the interaction of zone between the *Trichoderma* isolates and one isolate of each BFD pathogen on a slide and viewing with a Nikon Eclipse E600 compound microscope at various levels of magnification (200×, 400× and 1000× in oil immersion).

Dormant rootstock shoot assay

Grapevine cultivars and fungal isolates

The endophytic growth of *T. atroviride* in grapevine rootstock xylem vessels, phloem elements and pith were evaluated using one-year-old dormant rootstock shoots of five grapevine rootstock cultivars including Ramsey (*Vitis champinii*), Richter 99 (*V. berlandieri* ×

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V. rupestris), Richter 110 (*V. berlandieri* × *V. rupestris*), US 8-7 [Jacquez (*V. aestivalis* × *V. cinerea* × *V. vinifera*) × Richter 99] and Paulsen 1103 (*V. berlandieri* × *V. rupestris*). *Trichoderma atroviride* (T2) was formulated as a wet product formulation (ABM Africa Division).

Treatment

All of the plant material was disinfected with a quaternary ammonium product Sporekill® (ICA International Chemicals, South Africa) and underwent hot water treatment (HWT) for a period of 45 min at 50°C. Rootstock shoots were then surface sterilized (30 s in 70% ethanol, 1 min in 1% sodium hypochlorite and 30 s in 70% ethanol) and air dried in a laminar flow cabinet. The basal ends (50 mm) of the rootstock material were soaked in a *Trichoderma atroviride* conidial suspension (1×10^6 CFU.mL^{-1}) for 1 min, 10 min, 1 hr and 2 hrs, respectively. Five shoots of each cultivar were used per treatment and the trial was repeated. The rootstock material was then incubated in sterile moisture chambers at 25°C for 7 days.

Fungal isolations

Following the incubation period wood tissue isolations were made. The plant material was surface sterilized as described above and air-dried in a laminar flow cabinet. The shoots were sectioned longitudinally to reveal the xylem and pith regions. Four small sections (2 × 1 mm) were cut from both the xylem and pith tissue at five intervals (20 mm apart) and transferred to PDA amended with streptomycin sulphate (PDA-S) (40 mg.L^{-1}, Calbiochem, Merck). The Petri dishes were then incubated at 25°C and inspected daily for subsequent fungal growth to determine the depth of endophytic colonization by *Trichoderma*. The fungal growth in each rootstock was determined as a percentage of the isolated segments colonized.

Nursery field trials

The evaluation of different methods of application of Trichoderma *to nursery vines post callusing*

Sauvignon blanc scion (*V. vinifera*, clone SB316G) grafted onto rootstock cultivar Ramsey (*V. champinii*, clone SC18AB) callused graftlings were used. All hand grafting, standard cold callusing and HWT prior to grafting took place according to standard nursery practices at a commercial grapevine nursery located in the Wellington region (Western Cape, South Africa). *Trichoderma atroviride* (T2) was formulated as wet- and dry product formulations (ABM Africa Division).

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Graftlings were randomly selected and individually labeled before treatments commenced. Treatments consisted of i) coating the basal ends with dry product, ii) coating the basal ends with dry product followed by one soil drench per month for 6 months, iii) HWT (of dormant rootstock and scion shoots) at 50°C for 30 min and coating the basal ends with dry product, iv) soil drenching directly after planting followed by one soil drench per month for 6 months, v) soaking for 1 hr in conidial suspension, vi) HWT at 50°C for 30 min and soaking for 1 hr in conidial suspension, vii) HWT at 50°C for 45 min and soaking for 1 hr in conidial suspension, viii) HWT at 50°C for 30 min, ix) HWT at 50°C for 45 min and x) an untreated control. For the respective treatments that received dry product applications (i, ii, iii) the basal ends (approximately 30 mm) of the rootstock material were dipped in water and then in 1 kg dry product formulation (2×10^8 CFU.g^{-1}), while for soaking (v, vi, vii) the basal ends (approximately 150 mm) of the rootstock material were soaked in a conidial suspension (1×10^6 CFU.mL^{-1}) for 1 hr. However, a number of treatments were adapted or added in the second growing season and, therefore, treatments vi, vii, viii and ix were not repeated over two growing seasons.

Following on site treatment, the graftlings were planted in a nursery field for rooting according to a randomized block design with 100 graftlings per treatment, replicated five times and repeated over two seasons, 2016/17 (October 2016 to May 2017) and 2017/18 (October 2017 to May 2018). Due to the nursery practice of rotation of fields, the two seasons' trials were planted on different sites of the same nursery. Graftlings were planted at 5 cm spacing within rows, and 60 cm between rows. For treatments receiving soil drenches (ii, iv) the root zones of the respective plots were drenched with 10 L of conidial suspension (1×10^6 CFU.mL^{-1}) at monthly intervals for six months after planting. Normal nursery practices (irrigation, nutrition, cultivation practices and disease- and pest management) were followed for the duration of each growing season.

The evaluation of different Trichoderma*-based products to nursery vines post callusing*

The efficacy of different commercially produced *Trichoderma* products to colonize nursery vines and control BFD was assessed. ABM Africa Division formulated isolate T1 and T2, while the majority of products were sourced locally and one internationally. Sauvignon blanc scion (*V. vinifera*, clone SB316G) grafted onto rootstock cultivar Ramsey (*V. champinii*, clone SC18AB) callused graftlings were used. All hand grafting and standard cold callusing took place according to standard nursery practices at a commercial grapevine nursery located in the Wellington region (Western Cape, South Africa).

Graftlings were randomly selected and individually labeled before treatments commenced. Treatments included eight commercially produced *Trichoderma* products namely i) Awegenic Tri-cure™, ii) Bio-Tricho, iii) Eco 77®, iv) Excalibur Gold™, v)

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Trichoflo™, vi) TrichoPlus™, vii) USPP-MT1, viii) USPP-T1 and ix) an untreated control (Table 1). With the exception of the USPP-MT1 treatment, the basal ends (approximately 30 mm) of the rootstock material for an entire treatment were dipped in water and then in 1 kg of the respective dry products. For the USPP-MT1 treatment, the bottom ends of the graftlings were dampened with Bendazid® 500 SC before it was dipped in 1 kg of the dry formulation thereof.

Following on site treatment, the graftlings were planted in a nursery field for rooting according to a randomized block design with 100 graftlings per treatment, replicated four times and repeated over two seasons, 2016/17 (October 2016 to May 2017) and 2017/18 (October 2017 to May 2018). Graftlings were planted at 5 cm spacing within rows, and 60 cm between rows. The root zones of the treated plots were drenched with 10 L of conidial suspension (1×10^6 CFU.mL^{-1}) of the respective *Trichoderma* products at monthly intervals for the first three months after planting. Normal nursery practices (irrigation, nutrition, cultivation practices and disease- and pest management) were followed for the duration of each growing season.

Determination of growth parameters

For both trials the graftlings were uprooted after 7 months according to standard nursery practice. The number of certifiable vines, according to the standards of the Vine Improvement Association (PO Box 166, Paarl 7622, South Africa), was determined as a percentage of the total number of grafted cuttings planted. The total wet root mass (g) of all vines was determined.

Determination of fungal incidence

For both trials 25 vines per replicate (in total 1675 vines in 2016/17 and 2025 vines in 2017/18) were randomly selected and used to determine the incidence of BFD pathogens and *Trichoderma* spp. by means of destructive sampling. The basal section (within 50 mm of the rootstock base) and four roots were removed from each rootstock, surface sterilized (30 s in 70% ethanol, 1 min in 3% sodium hypochlorite and 30 s in 70% ethanol) and air-dried in a laminar flow cabinet. The basal section was sectioned longitudinally to reveal the xylem and pith regions. Four small sections (2 × 1 mm) were cut from both the xylem and pith (10 – 50 mm from the basal end). A set of four pieces was placed per PDA dish amended with chloramphenicol (PDA-C; 250 mg.L^{-1}). One section from each root at three intervals (four pieces from 20 mm from the rootstock attachment, the central part and root tips, respectively) were cut and transferred to PDA-C. The Petri dishes were then incubated at 25°C under 12 hr fluorescent white light/dark regime and inspected for new fungal growth daily.

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Identification of fungal isolates according to colony morphology

The fungal isolates were arranged into different taxonomic groups based on its cultural growth characteristics. The cultural characteristics included colony size, color, texture and shape. The occurrence of *Trichoderma* spp. was notated and isolates resembling BFD pathogens were sub-cultured. After a colony diameter of approximately 60 mm was reached, mycelia were scraped and stored at −80°C for molecular identification at a later stage. The occurrence of both *Trichoderma* spp. and BFD pathogens were recorded as a percentage of the total number of tissue sections colonized (xylem, pith and three root intervals).

Molecular identification of BFD isolates

DNA extraction

In order to verify the cultural identification of BFD pathogens a subgroup consisting of 27% of the total isolates were selected for molecular identification up to genus level. Genomic DNA was extracted from fungal mycelium as described by Damm *et al*. (2008) with some amendments. Mycelia was placed in 2 mL Eppendorf tubes with 0.5 g glass beads and 600 µL CTAB (2% CTAB; 1M Tris, pH 7.5; 5M NaCl; 0.5M EDTA, pH 8.0). Samples were homogenized in a Retsch® mill (Verder Scientific, Haan, Germany) for 5 min at 30 Hz. Following an incubation step at 65°C for 30 min, 400 µL chloroform:isoamylalcohol (24:1) was added and centrifuged at 13100 rpm for 15 min. The supernatant was transferred to new Eppendorf tubes containing 250 µL ammonium acetate (7.5 M) and 600 µL isopropanol, followed by an incubation period of 30 min at –20°C. The Eppendorf tubes were again centrifuged at 13100 rpm for 15 min and the supernatant discarded. One milliliter of 70% ethanol was added and centrifuged for a further 5 min. The supernatant was discarded and the pellets dissolved in 100 µL double distilled water (ddH_2O).

Primer design

Genus-specific primers for *Campylocarpon* spp. were developed from the internal transcribed spacers 1 and 2 as well as the 5.8S rRNA gene (ITS). Reference sequences were obtained from the nucleotide database GenBank of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/nucleotide/) and aligned using MAFFT v. 7 (Katoh and Standley, 2013). Primers were designed from sites within the ITS that i) showed homology between *Campylocarpon* spp. sequences, and simultaneously ii) heterogeneity between *Campylocarpon* and other closely related species sequences. In this manner the primer pair, CamF and CamR were designed (Table 3).

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Polymerase chain reaction (PCR) and electrophoresis

PCR-parameters for the newly designed *Campylocarpon* spp. primer set were evaluated and optimized. All isolates were screened with the primer set. In a total reaction volume of 20 µL the PCR reaction contained 2 µL of DNA, 10 µL GoTaq® green master mix (Promega Corporation, Madison, Wisconsin, USA), 0.8 µL CamF (10 µM), 0.8 µL CamR (10 µM), and 6.4 µL ddH_2O. Reaction conditions consisted of an initial denaturation step at 94°C for 4 min followed by 35 cycles of 45 s at 94°C, 30 s at 67°C and 1 min at 72°C with a final extension step at 72°C for 6 min.

All isolates that did not amplify with the latter primer set (including *Dactylonectria* spp. and *Ilyonectria* spp.) were identified by amplifying the partial β-tubulin gene region using the primers YT2F (Tewoldemedhin *et al*., 2011) and CylR (Dubrovsky and Fabritius, 2007) (Table 3). In a total reaction volume of 20 µL the PCR reaction contained 2 µL of DNA, 10 µL GoTaq® green master mix (Promega Corporation, Madison, Wisconsin, USA), 0.6 µL YT2F (10 µM), 0.6 µL CylR (10 µM), and 6.8 µL ddH_2O. Reaction conditions consisted of an initial denaturation step at 94°C for 4 min followed by 35 cycles of 45 s at 94°C, 30 s at 60°C and 1 min at 72°C with a final extension step at 72°C for 6 min.

To distinguish *Ilyonectria liriodendri* the partial β-tubulin gene region were amplified using the primers CyliF1 and CyliR1 (Mostert *et al*., 2010) (Table 3). For the primer set a total reaction volume of 20 µL the PCR reaction contained 2 µL of DNA, 10 µL GoTaq® green master mix (Promega Corporation, Madison, Wisconsin, USA), 0.6 µL CyliF1 (10µM), 0.6 µL CyliR1 (10 µM), and 6.8 µL ddH_2O. Reaction conditions consisted of a touchdown cycling program with an initial denaturation step at 94°C for 5 min and then 94°C for 45 s with 6 cycles at 66°C for 30 s, 5 cycles at 62°C for 30 s and 20 cycles at 60°C for 30 s, with an extension step at 72°C for 1 min and a final extension step at 72°C for 6 min.

In order to validate specificity of the primer sets PCR assays were performed using genomic DNA of eight BFD pathogens including *Ca. fasciculare*, *Ca. pseudofasciculare*, *D. alcacerensis*, *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, *D. torresensis*, *I. liriodendri* and five negative controls including *Diplodia seriata*, *Fusarium solani*, *Phaeoacremonium minimum*, *Phaeomoniella chlamydospora*, *Phytophthora niederhauserii* and *Pythium irregulare* (Table 4). All PCR reactions were performed in an Applied Biosystems 2700 PCR machine (Carlsbad, California, USA).

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

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Sequencing of PCR products and identification

In order to verify the specific primer identifications, eight PCR products were sequenced for the primer sets CamF/CamR and YT2F/CylR and two PCR products for the primer set CyliF1/CyliR1. A subset of 32 isolates that did not amplify was sequenced to determine its identity. The PCR products were purified using the MSB® Spin PCRapase kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The PCR products were prepared for forward and reverse sequencing using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California, United States) with the primers used in the initial PCR reactions. Reaction conditions consisted of 1 minute at 95˚C, 30 cycles of 10 s at 95˚C, 5 s at 50˚C and 4 min at 60˚C, with a final extension of 30 s at 60˚C. The nucleotide order of samples was read in an ABI 3130xl DNA sequencer (Perkin-Elmer, Norwalk, California, United States) at the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University. Consensus sequences were made from the forward and reverse sequences in Geneious R10.1.3. (Biomatters Ltd., Auckland, New Zealand). The identity of the sequences was determined through the Basic Local Alignment Search Tool (BLAST) of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

In vitro *evaluation*

Six replicates were done for the volatile organic compound and diffusible antibiotic compound experiments. The percentage inhibition for each treatment was calculated by using the formula: Percentage inhibition of pathogen colony = [(Colony radius of control – Colony radius of treatment)/ Colony radius of control] × 100. Normality of standardized residuals was confirmed by Shapiro-Wilk test (Shapiro and Wilk, 1965). Levene's test was used to verify the homogeneity of factor (treatment) variances (Levene, 1960). The data were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS Version 9.2 (SAS Institute Inc., Cary, USA). Fisher's least significant difference (LSD) was calculated at the 5% level to compare factor means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

Dormant rootstock shoot assay

The data from the five cultivars were combined in split-split plot design. The data were logit transformed prior to analysis. The data were subjected to ANOVA using General Linear Models Procedure (PROC GLM) of SAS. Normality of standardized residuals was confirmed by the Shapiro-Wilk test (Shapiro and Wilk, 1965). Levene's test was used to verify the homogeneity of factor (cultivars) variances (Levene, 1960). Fisher's least significant

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difference (LSD) was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

Nursery field trials

The data of certifiable plant yield were analyzed as is. For the root mass the averages were determined across experimental units (Treatment × Block). In order to determine the percentage incidence of *Trichoderma*- and BFD isolates total counts were calculated across plants per experimental unit (Treatment × Block) and determined by applying the following rules: (If *Trichoderma* per tissue type > 1, then *Trichoderma* Incidence per tissue type = 1; If Black foot per tissue type > 1, then Black foot Incidence per tissue type = 1). It was then calculated as the percentage of plants in which the fungi were present by using the following formula: Percentage *Trichoderma* incidence = (*Trichoderma* Incidence / Graftlings) × 100; Percentage BFD incidence = (Black foot Incidence / Graftlings) × 100. Normality of standardized residuals was confirmed by Shapiro-Wilk test (Shapiro and Wilk, 1965). Levene's test was used to verify the homogeneity of factor (treatment) variances (Levene, 1960). If experiments were not of comparable precision a weighted ANOVA was performed (John and Quenouille, 1977). ANOVA were performed per season and tissue group; per season with tissue groups as subplotfactor and with seasons combined using General Linear Models Procedure (PROC GLM) of SAS. Fisher's least significant difference was calculated at the 5% level to compare treatment means for significant effects (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

RESULTS

In vitro evaluation

Screening Trichoderma *isolates for the production of volatile organic compounds (VOC)*

Analysis of variance revealed a significant pathogen × *Trichoderma* interaction ($P < 0.0001$; Appendix A, Table 1), therefore the data is presented per isolate (Tables 5 – 6). Higher inhibition of *D. macrodidyma* isolates (maximum inhibition of 66.94%) was found in comparison to the other species (maximum inhibition of 39.61% for *Ca. fasciculare*, 29.39% for *Ca. pseudofasciculare* and 23.85% for *I. liriodendri*). *Trichoderma* isolate T1 gave the highest inhibition of *D. macrodidyma* STE-U 8702 (66.94%), though not statistically different from T2 (57.98%), T5 (61.40%) and T8 (57.17%). *Dactylonectria macrodidyma* STE-U 8264 was significantly more inhibited by T3 (60.17%), T4 (60.68%) and T5 (64.21%), while *D. macrodidyma* STE-U 8265 were more inhibited by isolates T1 to T5 (ranging from 49.72% to 58.31%).

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Campylocarpon fasciculare STE-U 8693 was significantly more inhibited by *Trichoderma* isolate T8 (39.61%), in comparison to the other *Trichoderma* isolates. *Trichoderma* isolate T8 caused higher inhibition of *Ca. fasciculare* STE-U 8692 (36.08%), though not significantly different from T1 to T5 (ranging from 24.13% to 33.87%). Likewise, *Ca. fasciculare* STE-U 8691 was most inhibited by T8 (28.59%) and T3 (28.51%), though not differing significantly from T1 (27.82%), T2 (23.87%) and T5 (24.71%).

In a few cases growth stimulation occurred for *Ca. pseudofasciculare* isolates. For *Ca. pseudofasciculare* STE-U 8694 the growth was significantly more inhibited by T1 (28.64%), T2 (29.39%) and T3 (27.05%), though not differing from T4 (21.31%) and T5 (21.27%). In contrast T9 induced growth stimulation (−2.56%). Lower levels of inhibition were observed for *Ca. pseudofasciculare* STE-U 8279, which were significantly more inhibited by T1, T2, T3 and T7 (ranging from 9.72% to 15.66%). *Campylocarpon pseudofasciculare* STE-U 8280 was similarly inhibited by T1, T2, T3, T7 and T8 (ranging from 7.51% to 15.75%). *Trichoderma* isolate T6 resulted in growth stimulation of both these isolates (−1.47% and −1.58%, respectively), while T8 stimulated the growth of STE-U 8279 (−2.49%) and T9 for STE-U 8280 (−1.15%).

For *I. liriodendri* STE-U 8699 was inhibited the most (ranging from 17.29% to 23.8% for isolates T1, T3, T7 and T8), though not statistically different from T2 (14.46%). Also for this pathogen isolate, *Trichoderma* isolates T6 and T9 resulted in growth stimulation (−0.64% and −4.09%, respectively). For STE-U 8266 higher inhibition was obtained with T1, T3 and T8 (ranging from 15.07% to 21.79%), while T9 resulted in growth stimulation (−1.62%). For *I. liriodendri* STE-U 8267 growth inhibition was only obtained with T1, T3 and T8 (ranging from 5.68% to 14.55%), while growth stimulation occurred with T2, T4, T5, T6, T7, T9 and T10 (ranging from −4.17% to −18.58%).

Screening Trichoderma *isolates for the production of diffusible antifungal compounds*

Analysis of variance revealed a significant pathogen × *Trichoderma* interaction ($P < 0.0001$; Appendix A, Table 2). In general higher inhibition was obtained when investigating the effect of diffusible antifungal compounds on the different pathogen isolates in comparison to that obtained with the VOCs (Table 6). *Dactylonectria macrodidyma* isolates showed higher sensitivity to the compounds produced by the *Trichoderma* isolates. For all three isolates of this pathogen *Trichoderma* isolate T1 resulted in 100.00% growth inhibition. Growth inhibition by T8 was not significantly different from T1 over all isolates (96.36% for 8264, 100.00% for STE-U 8265 and 94.62% for STE-U 8702) and T9 for STE-U 8264 (99.07%) only.

In the case of *Ca. fasciculare*, T1 showed higher inhibition against all three isolates, with growth inhibition of 47.98% for STE-U 8691, 52,75% for 8693 and 53.82% for STE-U

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8692. For T8 growth inhibition was not significantly different from T1 in the case of STE-U 8693 (39.66%) and STE-U 8692 (48.44%) and T9 for STE-U 8693 (52.01%) and STE-U 8691 (39.58%). The diffusible compounds of T6 resulted in growth stimulation of isolate STE-U 8692 (−6.28%) and STE-U 8693 (−14.07%), while T7 resulted in a similar outcome for STE-U 8693 (−2.83%).

In contrast to the results obtained from the experiment investigating the effect VOCs on *Ca. pseudofasciculare* no growth stimulation occurred following the exposure of the pathogen to diffusible antifungal compounds. A 100% growth inhibition was obtained with T8 towards STE-U 8694, though not observed in the case of the other two isolates. For STE-U 8279, T1, T8 and T9 caused higher inhibition ranging from 25.17% to 36.38%. For STE-U 8280 an inhibition of 97.77% was caused by T9.

In the case of *I. liriodendri* isolate T8 performed better over all three isolates, with 37.99% inhibition for STE-U 8267, 57.46% for STE-U 8266 and 65.51% for 8699, though not differing significantly from T1 in the case of STE-U 8267 (35.92%) and STE-U 8266 (70.70%). Growth stimulation of this pathogen only occurred in one instance, in the case of T6 with STE-U 8266 (−1.13%).

Competitive growth

Several interactions were macroscopically observed between *Trichoderma* and the pathogen isolates, which included overgrowth or partial overgrowth, often associated with sporulation of the *Trichoderma* species, and to a lesser extent arrested growth or the formation of inhibition zones between *Trichoderma* and the pathogen isolates (Table 7; Fig. 1). Interestingly, these results coincide with those obtained in the previous *in vitro* assays that showed different pathogen isolates to react differently to *Trichoderma* spp. isolates. All of the *Trichoderma* isolates overgrew the isolates of *Dactylonectria macrodidyma* and *Campylocarpon fasciculare* and, with only a few exceptions, sporulated amply. In the case of *Campylocarpon pseudofasciculare*, however, this was only the case for STE-U 8694, while arrested growth or the formation of inhibition zones was mostly observed for STE-U 8280 and STE-U 8279. Likewise, for *Ilyonectria liriodendri* STE-U 8267 and STE-U 8699 arrested growth was mostly observed, while inhibition zones occurred between STE-U 8266 and most of the *Trichoderma* isolates.

Similar hyphal interactions were observed between isolates of the same species. Adhesion of the *Trichoderma*- to pathogen hyphae and disintegration of pathogen hyphae were often observed, while coiling of the *Trichoderma*- around pathogen hyphae and swelling and malformation of the pathogen hyphae were seen less often (Fig. 2). Often these interactions were observed in conjunction with one another, as was the case for nine of the ten *Trichoderma* isolates and were therefore shown per *Trichoderma* isolate (Table 8).

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Dormant rootstock shoot assay

Trichoderma atroviride (T2) was able to colonize all five rootstock cultivars to a depth of 10 cm following all periods of soaking (Table 9). Significant interaction was found between the depth of isolation and soaking time (P = 0.0008; Appendix A, Table 3). At 2 cm and 6 cm there was not a significant difference in colonization whether the shoots were soaked 1 min, 10 min, 60 min or 120 min (Table 9). At 4 cm the shoots that were soaked for 10 min had significantly more colonization than the other soaking times. At 8 cm, 60 min soaking resulted in significantly less colonization than the other soaking times. At 10 cm, 120 min soaking resulted in significantly more colonization. Comparing the different depths of isolation for 1 min soak, the 2 cm area were significantly more colonized than 4 to 10 cm depths. For 10 min both 2 cm and 4 cm were significantly more colonized than 6 to 10 cm. For 60 min, 2 cm and 6 cm were more colonized than the other depths and lastly at 120 min soaking time 2 cm, 6 cm, 8 cm and 10 cm were not significantly different from each other and were more colonized than 4 cm. In general did a longer soaking time not significantly increase the *T. atroviride* colonization, except at a depth of 10 cm. Furthermore, significant differences were found between the colonization of the xylem and pith (P = 0.0061; Appendix A, Table 3). The xylem was more readily colonized (8.37%) throughout the shoot than the pith elements (5.82%) (Table 10).

Nursery field trials

The data of the two seasons were not combined due to additional treatments in both trials in the second season. Low natural infection levels of black foot were recorded during both seasons in which the trials were executed. Due to great variation in incidence of *Trichoderma* spp. and BFD pathogens between tissue from the basal ends and root sections the data were analyzed per tissue group (Appendix A, Tables 4 – 11).

The evaluation of different methods of application of Trichoderma *to nursery vines post callusing*

Determination of growth parameters

The respective application methods of *T. atroviride* to nursery vines post callusing did not have a significant effect, neither positive or negative, on the total number of certifiable vines produced over the two seasons (P = 0.6855 in 2016/17; P = 0.6010 in 2017/18) (data not shown). Likewise, none of the application methods resulted in a significant effect on the total wet root mass in either of the seasons (P = 0.5044 in 2016/17; P = 0.3094 in 2017/18) (data not shown).

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Determination of fungal incidence

Analysis of variance revealed significant differences in the *Trichoderma* incidences in the basal ends and roots following different treatments over both seasons with a *P*-value of less than 0.0001 (Appendix A, Tables 12 – 15). For the respective seasons the mean incidence of *Trichoderma* within the basal ends and roots are summarized per treatment in Table 11. When comparing different treatments the application of dry product formulation resulted in the highest colonization of *Trichoderma atroviride* in the first season (50.40% in the basal end and 32.53% in the roots), though not differing significantly from the same treatment in combination with monthly soil drenches (39.20% in the basal end and 26.40% in the roots). This was followed by 30 min HWT prior to grafting in combination with the dry product application (30.80% in the basal end and 20.00% in the roots) that did not differ significantly from the latter. *Trichoderma* could be re-isolated from the vines in all treatments in the 2017/18 season, though generally less than the previous season. The highest re-isolation percentages were obtained with the dry product application in combination with monthly soil drenches (28.00% in the basal end and 15.73% in the roots), or in combination with 30 min HWT prior to grafting (20.40% in the basal end and 12.00% in the roots). Re-isolation percentages from the basal ends in the treatment only receiving a dry product application (16.40%) did not differ significantly from the latter. In both seasons re-isolation percentages following monthly soil drench applications were lower than that of the treatments combined with a dry product application, though not always significantly different from the lowest re-isolation percentages yielded by these treatments. The 1-hr soaking treatment and combinations thereof, however, did not result in *Trichoderma* colonization significantly different from the untreated control in either of the seasons.

Despite no significant differences in the incidence of BFD pathogens between different treatments in either of the seasons ($P = 0.3665$ and $P = 0.9976$ for the base; and $P = 0.3443$ and $P = 0.6423$ for the roots of the 2016/17 and 2017/18 seasons, respectively) (data not shown) an interesting trend were observed in the tissue types that harbored the pathogens. Throughout all treatments, and in both seasons, the incidence of pathogens were higher in the different sections of the root (ranging from 17.83% to 21.14% in 2016/17; and from 15.55% to 24.00% in 2017/18), while occurring significantly less in the basal ends of the vines (2.97% and 5.49%, and 6.58% and 11.47% in the pith and xylem of the respective seasons) (Table 12). The highest re-isolation percentages of *T. atroviride*, in the contrary, were obtained from the basal ends (21.37% and 25.60%, and 9.51% and 9.69% in the pith and xylem of the respective seasons), whereas it was less frequently re-isolated from the roots (ranging from 10.29% to 20.91%, and 3.20% to 6.84% in the respective seasons) (Table 12).

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The evaluation of different Trichoderma*-based products to nursery vines post callusing*

Determination of growth parameters

For the total number of certifiable vines produced there were significant differences between treatments (products) in both seasons (P = 0.0469 in 2016/17; P = 0.0229 in 2017/18) (Appendix A, Tables 16 – 17). In the first season, none of the products were significantly different from the control (73.25%). The percentage take of products 5 and 7 were, however, significantly lower than products 1 and 3 (Table 13). In the following season, the majority of the products did not differ significantly from the control (77.25%), except product 7 (62.75%) that resulted in a percentage take significantly lower than the untreated control (Table 13).

For the total wet root mass significant differences between treatments (products) were observed in the first season only (P = 0.0326; Appendix A, Table 18). The total wet root mass for products 1, 3 and 6 were significantly less than the untreated control (29.50 g) (Table 14). The total wet root mass of the other products was not significantly different from the untreated control.

Determination of fungal incidence

Analysis of variance revealed significant differences in the *Trichoderma* incidences in the basal ends and roots following different treatments over both seasons with a P-value of less than 0.0001 (Appendix A, Tables 19 – 22). For the respective seasons the mean incidence of *Trichoderma* spp. within the basal ends and roots are summarized per treatment in Table 15. Comparing the different products that were evaluated, in both seasons product 5 resulted in higher re-isolation percentages from the basal ends (58.50% and 43.50%, respectively), though not differing significantly from product 3 (47.50% and 40.50%, respectively). Product 5 resulted in higher re-isolation percentages from the roots in the first season (37.67%), but in the second season product 5 (20.33%) was not significantly different from product 1, 3 and 8. Product 1 and 2 resulted in intermediate re-isolation percentages in most cases not differing significantly from product 3. Product 7, however, did not result in re-isolation percentages significantly different from the untreated control in the first season, while the same was true for product 4 and 6 over both seasons.

Interestingly, analysis of variance revealed significant differences in the incidence of black foot infections within the basal ends following treatments over both seasons (Appendix A, Tables 23 – 24), while no significant differences could be observed in the roots at 5% confidence level (P = 0.9236 and P = 0.6676 for the respective seasons) (data not shown). The infection was found to be significantly lower in all product-treated vines (1.00 – 2.50%) than in the untreated control (6.50%) in the first season, while products 1, 2, 3, 5 and 7 resulted in lower incidence in the second season (ranging between 2.50% and 5.00%), though not significantly different from the untreated control (7.50%) (Table 16).

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When comparing different tissue types the same trend was observed than in the previous trial. Throughout all treatments, and in both seasons, the incidence of pathogens were higher in the different sections of the root (ranging from 14.00% to 19.00%; and from 15.56% to 28.22% in the respective seasons), while occurring significantly less in the basal ends of the vines (1.38% and 3.13%, and 3.44% and 9.22% in the pith and xylem of the respective seasons) (Table 17). In contrast, higher re-isolation percentages of *Trichoderma* spp., were obtained from the basal ends (28.13% and 29.25%, and 24.11% and 21.00% in the pith and xylem of the respective seasons), whereas it was less frequently re-isolated from the central part of the roots and root tips (ranging from 12.38% to 13.88%, and 7.22% to 11.11% in the respective seasons).

Molecular identification of BFD isolates

The newly designed primer pair (CamF/CamR) resulted in successful genus-specific amplification of *C. fasciculare* and *C. pseudofasciculare* (Fig. 3). The previously described primer sets were also specific for amplification of the BFD pathogens, except for the YT2F and CylR primer set that amplified *F. solani* (Fig. 3). This was not considered problematic as only the isolates with BFD pathogen cultural growth characteristics were recorded and accordingly used for molecular confirmation.

The subgroup of putative BFD pathogens following isolations in the first season was identified as *Campylocarpon* (104 isolates), *Dactylonectria* (185 isolates) and *I. liriodendri* (2 isolates). For the following season it were identified as *Campylocarpon* (407 isolates), *Dactylonectria* (180 isolates) and *I. liriodendri* (1 isolate). The BLAST results of the sequences of the positive PCR products confirmed the identities of *Campylocarpon*, *Dactylonectria* and *I. liriodendri* (Appendix A, Table 25). A subset of 32 isolates that did not amplify with the specific primers was identified as *C. fasciculare*, *D. macrodidyma* or *D. novozelandica* (Appendix A, Table 25).

DISCUSSION

This study investigated the efficacy of *Trichoderma* spp. to colonize grapevine rootstocks for the improved control of black foot disease in grapevine nurseries. *Trichoderma* spp. have varying levels of growth inhibition towards the four BFD pathogen species tested *in vitro* and in a few cases even caused growth promotion. Results from the detached shoot assay showed that *T. atroviride* are able to colonize five major grapevine rootstock cultivars used in South Africa. The evaluation of different application methods to nursery vines post callusing revealed that a dry *Trichoderma*-product application followed by monthly soil drenches resulted in the highest colonization, while soaking of graftlings in a conidial suspension were found to be ineffective. Furthermore, all *Trichoderma*-based products tested in the field were

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able to colonize the grapevine rootstock evaluated, but with variation in percentage colonization. Limited efficacy of *Trichoderma* spp. was observed in the field towards BFD pathogen infection and was only able to lower infection in the base of the vines in one of the two seasons.

Antibiosis, as a result of the production of volatile and diffusible compounds, is an important mode of action of some *Trichoderma* species and can be observed by confronting the pathogens and antagonists in Petri dishes (Samuels and Hebbar, 2015). After a series of *in vitro* experiments by Dennis and Webster (1971a, b) that investigated the efficacy of *Trichoderma* antibiosis (including the production of VOCs and DACs) against wood rot fungi, screening for biocontrol activity in such manner became standard practice. From the current study the *in vitro* assays provided an indication of the possible effectiveness of *Trichoderma* spp. isolates tested. Some *Trichoderma* spp. isolates were found to have greater efficacy against certain BFD isolates, and in some cases only showed efficacy with the volatile or diffusible compounds. In the case of an isolate of *T. virens* (T9), for instance, moderate growth inhibition were obtained against all BFD pathogens following exposure to is diffusible compounds, while its VOCs were found to be less effective and, in the case of *I. liriodendri*, resulted in growth stimulation of all isolates.

Results from the assay investigating the efficacy of VOCs against BFD pathogens, however, differed to a large extent from those obtained in the assay investigating the efficacy of DACs. In the case of two isolates of *T. atroviride* (T2 and T3) overall good inhibition was obtained by means of VOCs, while the efficacy of its diffusible compounds were less notable. The best overall mycelial inhibition, when taking both classes of antibiotic compounds into account, was obtained with *T. atroviride* (T1 and T8). Both of these isolates were good producers of diffusible compounds, while only resulting in moderate inhibition by their VOC-profiles. Generally, *D. macrodidyma* were found to be the more sensitive species, with complete inhibition obtained in more than one instance following exposure to the diffusible compounds of the latter isolates.

The results from the *in vitro* competitive growth study demonstrated that all of isolates were able to exert some form of antagonism towards the pathogens. The formation of inhibition zones and arrested growth, which can likely be ascribed to the production of secondary metabolites by the BFD pathogens, were only observed in a few instances. *Trichoderma* spp. mostly overgrew the pathogens followed by profuse sporulation, a phenomenon that was not observed without direct contact to the pathogens. Numerous factors, including the metabolic rate of hyphal cells and the production of antimicrobial metabolites, can result in the induction of conidiation as observed here (Steyaert *et al*., 2013). These interactions often coincided with mycoparasitic actions including adhesion and coiling of *Trichoderma* hyphae to that of the pathogens' on a microscopic level. Indeed,

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CWDE and secondary metabolites are produced during this process to facilitate the entry of *Trichoderma* hyphae into the lumen of the parasitized fungi (Zaidi and Singh, 2013). The occurrence of hyphal disintegration and swelling and malformation of the pathogens' hyphae can be ascribed to antibiosis mediated by *Trichoderma* spp. (Benhamou and Chet, 1997). A single biocontrol agent can pertain several modes of action that act synergistically to improve overall plant health (Harman *et al*., 2004; Vinale *et al*., 2008a; Samuels and Hebbar, 2015).

The current study found numerous isolates of *T. atroviride* (T1, T3 and T8) to exhibit good antagonistic efficacy against BFD pathogens, though emphasizing that it is a property of the isolate and not the species. This can be ascribed to the composition of bioactive metabolites involved in antagonistic mechanisms and mycoparasitic capability that are strain-reliant (Vinale *et al*., 2009; Anees *et al*., 2010). Combining these isolates that exhibited good efficacy towards different BFD pathogen species in the respective *in vitro* assays can, therefore, offer promising prospective in managing the disease. This is, however, a highly artificial system that is not necessarily indicative of the effect in nature (Samuels and Hebbar, 2015). While Petri dish- and detached shoot assays may yield some knowledge regarding mycoparasitism and antibiosis (Samuels and Hebbar, 2015), *in planta* assays are fundamental to comprehend plant-microbe interactions.

Previous studies by Halleen *et al*. (2003) reported black foot infections of more than 50% in nursery vines following the rooting stage in South African nursery soils, while the current study revealed lower infection incidences of 28.22% or less. The low disease incidence during the two seasons in which the field trials were executed can be ascribed to the extreme drought that the country experienced the years preceding the trials as well as to the rootstock cultivar used. In both field trials Ramsey (*V. champinii*, clone SC18AB) were used, as it is one of the preferred cultivars due to its strong vigor, drought tolerance and ability to grow well in poor quality soils (Loubser and Uys, 1997). Sieberhagen (2016) found this cultivar to be most resistant to a number of fungal trunk pathogens, including black foot, after comparing it to other major rootstock cultivars planted in South Africa. Samuels and Hebbar (2015) noted that clear differences cannot necessarily be observed between BCAs if the disease incidence is too low. However, even when *Trichoderma* spp. application in grapevine nurseries was done when black foot infection was higher, marginal control of black foot was obtained (Fourie *et al*., 2001; Halleen *et al*., 2007). The low levels of control is, therefore, likely not due to the level of the pathogen, but rather the complex nature of the large root system within the diverse soil environment to provide effective protection. Moreover, the majority of the isolates identified by means of genus-specific PCRs comprised of *Campylocarpon*, which were one of the genera found to be least sensitive to *Trichoderma* isolates *in vitro* and likely also contributed to the observable effects in the field trials.

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Furthermore, the cultivar selection likely also influenced the observable effects of *Trichoderma* on the plants. Even though the results from the detached shoot assay indicated that the percentage *Trichoderma* colonization did not vary significantly between cultivars, cultivar-specific *Trichoderma* interactions cannot be excluded. The detached shoots were dormant at the time of the experiment and in this state wound healing occur at a slower rate, which could be the reason for better colonization by *Trichoderma* spp. (Mutawila *et al*., 2016). The trial might also have been too small to show differences in colonization between cultivars. Mutawila *et al*. (2011) demonstrated that the efficacy and colonization of *Trichoderma* spp. on pruning wounds under field conditions are largely influenced by the grapevine cultivar and phenological stages in which the vines reside, more so than by the *Trichoderma* isolate itself. Fungal-plant interactions play a major role in the ability of *Trichoderma* spp. to colonize its host and exert effects on it (Zaidi and Singh, 2013; López-Bucio *et al*., 2015; Samuels and Hebbar, 2015; Mondello *et al*., 2018). In the current study, low frequencies of *Trichoderma* spp. were also recovered from untreated controls, indicating low levels of natural *Trichoderma* colonization.

Results from the second seasons trials showed lower *Trichoderma* incidences than the first, again likely due to the worsening drought. In an attempt to save water in the 2017/18 season the nursery changed from a sprinkler irrigation system to a drip irrigation system, likely resulting in a dryer soil environment in the second season. As relative high moisture content is necessary for the germination and growth of *Trichoderma* conidial inoculum, it likely had an effect on the incidence of *Trichoderma*. In most cases, however, consistent trends could be observed in both trials over both seasons following analysis of the data.

Following the field trials' analysis of the incidence of BFD pathogens in different tissue types from which were isolated revealed an interesting phenomenon. In both trials BFD infections of the roots were significantly higher than in the basal ends of the rootstocks, while the opposite was true for *Trichoderma*. This effect was, however, variable over the two years. It appears as if *Trichoderma* are able to prevent pathogen infection of the basal ends where its colonization was higher, most likely due to the dry dip treatment application. This can likely be ascribed to two different modes of action employed by *Trichoderma* including antibiosis as well as competition for limiting resources, likely starches in this case. Starch reserves are stored in the ray cells of the phloem and younger xylem, which can serve as a readily metabolizeable carbon source to the BFD pathogens due to its ability to produce extracellular amylases (Halleen *et al*., 2006). Interestingly, in both the detached shoot assay and in most cases in the field *Trichoderma* colonized the xylem more extensively, while the same was true for black foot in the field. It is, however, unclear whether endophytic growing *Trichoderma* can parasitize pathogens from within living plant tissue (Bailey and Melnick,

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2013). Considering that pathogen infection of the roots occur first, and then followed by infection of the rootstock (Gubler *et al*., 2004; Halleen *et al*., 2006; Cardoso *et al*., 2013), excluding it from the base might ensure the longetivity of vines even if the roots are infected.

Comparing different methods of application of *T. atroviride* to nursery vines post callusing clearly showed which methods are superior in obtaining better colonization. This is essential as Halleen *et al*. (2007) ascribed the inconsistent results obtained with *Trichoderma* to insufficient systemic colonization of the basal ends of rootstocks. The newly described application technique that consists of an application of dry product formulation to the basal ends, either singularly or in combination with other treatments, consistently resulted in the highest mean *Trichoderma* colonization. When taking the results of both seasons into account, a dry product application followed by monthly soil drenches seem to yield the best results. Different from the standard methods of application, this method places high inoculum loads at the basal end of the graftlings before planting while the additional carriers in the formulation provide plentiful nutrients to the fungus during germination and initial colonization (Hjeljord *et al*., 2000). This is advantageous as *Trichoderma* conidia are often subject to fungistasis in the absence of nutrients in the soil environment (Hjeljord *et al*., 2000). At the time of planting the basal ends are often not completely healed or covered by callus tissue, and callus roots brake off during the process, providing direct infection sites into the xylem and pith for *Trichoderma* hyphae. Those tissues are otherwise not commonly reached when applied to the roots at a later stage due to its composition and complex structures that needs to be penetrated (Bailey and Melnick, 2013). The exposed basal ends are also one of the major infection sites of BFD pathogens during the rooting stage in the nursery process and therefore the protection offered by *Trichoderma* by means of competition for infection sites and antibiosis could aid in controlling the disease.

Furthermore, the application of *Trichoderma* by means of soil drenches continuously add inoculum to the newly developing roots, enabling it to re-infect the epidermis and cortex and colonize the developing root systems, while actively competing against phytopathogens. Less *Trichoderma* spp. were found in the central and root tips than in the root parts attached to the base. The colonization closest to the basal end can be ascribed to the initial colonization that occurred after the vines were planted and the lower infection in the developing root due to the large root surface area of the newly established root system.

The results from the field trial revealed that soaking of graftlings in a *Trichoderma* conidial suspension prior to planting were mostly ineffective and resulted in an outcome not different from the untreated control. The contrasting results obtained in the detached shoot assay can again be ascribed to the phenological stage in which the shoots resided. Soaking of dormant rootstock material at different time intervals in the detached shoot assay also indicated that the time of exposure did not have a significant effect on the colonization of

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Trichoderma. In this case, the higher percentage of re-isolation closer to the basal ends can be ascribed to higher spore loads accumulating at these ends as only this part were exposed to the *Trichoderma* spore suspension. The colonization from there on upward can either be ascribed to fungal hyphae growing upward in the dormant shoots or spores that moved upward during the soaking process by means of capillary activity and then germinated to colonize the upper part of the shoots. Regarding application techniques of *Trichoderma* this is an important finding as the label instructions of numerous products recommend soak application to perennial crops in such manner.

The HWT of grafting material in combination with the dry product application was done in both seasons and showed that *Trichoderma* could successfully colonize the 'sterile' material, and only resulting in significantly less colonization in the first season from the dry product treatment. The HWT with soaking for 1 hr were not significantly different from the untreated control. HWT did not have an effect in the colonization of BFD pathogens. Though not investigated in this study it is well documented that BFD is often more severe when occurring together with other GTDs of which infections stems from infected propagation material (Halleen *et al*., 2016). It is, therefore, important to implement HWT of dormant nursery vines after uprooting as it has been proved to be effective in eradicating many superficial and endophytic pathogens (Gramaje *et al*., 2010; Alaniz *et al*., 2011; Halleen and Fourie, 2016).

Even though different methods of application of one *Trichoderma* product resulted in great variation in the percentage colonization of *Trichoderma atroviride*, the results obtained in this study were in accordance with those obtained by Pertot *et al*. (2016) that observed no significant differences in percentage certifiable vines or mean wet root mass following the application of one *Trichoderma* isolate. On the contrary, the application of different products, and therefore different *Trichoderma* isolates, resulted in a mean root mass and percentage certifiable vines significantly less than the untreated controls in different seasons. This finding also suggests that physiological responses of grapevine are, in fact, influenced by specific *Trichoderma* isolates and not by the incidence of the biological control agent present within the plants. Indeed, Bae *et al*. (2011) showed heavy colonization of roots not to be critically linked to induced resistance.

Previous studies have reported an increase in root biomass following treatment with *Trichoderma* spp. (Di Marco *et al*. 2004; Di Marco and Osti, 2007). In the 2016/17 season three of the treatments resulted in significantly lower root biomass in comparison to the untreated control. The effect on growth parameters are likely influenced by the plant growth stage in which *Trichoderma* are applied. Di Marco and Osti (2007) reported that the use of *Trichoderma* in all the first steps of production (grafting, callusing, rooting) resulted in more plant growth failures, though noting more vigorous growth and increased tolerance to stress

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related diseases, especially for vines treated at the rooting stage. Likewise, Mutawila *et al*. (2011) considered the phenological stages of grapevine as a major factor responsible for the variation in biocontrol efficacy. This phenomenon can likely be ascribed to *Trichoderma* that metabolizes available starches in the graftlings during colonization (Samuels and Hebbar, 2015), even while it is still dormant. Between budburst and flowering the young grafted vines are highly dependent on these stored reserves as it has very few leaves and a small developing root system with photosynthetic ability at a minimum (Sieberhagen, 2016). Furthermore, to induce a host reaction to the *Trichoderma* the vine needs to utilize resources and nutrients to successfully compartmentalize the fungi present in the vine (Sieberhagen, 2016). In the case of *Trichoderma* being applied once the plants are established starch reserves are more readily available and does not affect the initial development as much as it would when the BCA are applied to dormant plants. Growth stimulation in plants is, in fact, ascribed to *Trichoderma* that colonize and penetrate the root epidermis where it metabolizes the nutrients and exert MAMPs, finally stimulating resistance responses and increase photosynthetic rate (Samuels and Hebbar, 2015). During this process a wide range of bioactive metabolites are also secreted by *Trichoderma* that acts as phytohormones and influences plant growth regulation and stimulates growth (Contreras-Cornejo *et al*., 2013).

The one-year growth period in grapevine nurseries is most probably too short to observe the growth promotion attributes of *Trichoderma* spp. Increased growth by means of nutrient solubilization and improved photosynthetic ability, for instance, will likely only be observed in the vineyards. Di Marco and Osti (2007) reported that growth promotion of the roots only increased in the year after the treatment was applied in the nursery, suggesting that the *Trichoderma* treatment had a delayed effect on growth. Likewise, Mondello *et al*. (2018) found that isolates of *T. atroviride* and a combination of *T. asperellum* and *T. gamsii* were able to be reduce the incidence and mortality of esca affected vineyards only after the second or third year of continuous treatment of vineyards.

When comparing the different *Trichoderma*-based products 3 and 5 (Eco 77® and TrichoPlus™, respectively) resulted in the highest colonization of vines, followed by products 1 and 2 (USPP-T1 and USPP-MT1, respectively). Noteworthy, the isolates contained in Eco 77®, USPP-T1 and USPP-MT1 were originally isolated from *Vitis* spp. in South Africa and are likely adapted to the climatic conditions and to colonizing *Vitis* spp. In fact, Askew and Laing (1994) reported that local strains of *Trichoderma* often perform better than imported strains. Products 4 and 6 (Bio-Tricho and Excalibur Gold™, respectively) that contained mixtures of different *Trichoderma* spp. isolates least colonized the vines and did not show greater efficacy in controlling BFD. Often the efficacy of *Trichoderma* spp. under field conditions was found to be inconsistent with those observed *in vitro*. This is most likely

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influenced by the inability of selected isolates to colonize the roots of *Vitis* spp. or compete in the rhizosphere (López-Bucio *et al*., 2015).

CONCLUSION

The success of *Trichoderma* spp. as biopesticide under field conditions depends not only on its antagonistic activity alone, but a combination of several other factors including the method of application, ability of the isolate to colonize grapevine as well as environmental conditions such as soil moisture content and temperature. Results from this study showed that the newly developed application technique consisting of a dry product application to the basal ends of vines post callusing in combination with monthly soil drenches gave consistent better colonization of *Trichoderma* spp. over the two season, while simultaneously providing a certain degree of protection against infection of the basal ends of vines to BFD pathogens. This is a practical and fast method of applying *Trichoderma* to nursery vines post callusing that can be incorporated into the nursery process without great difficulty. Unfortunately the use of a dry product formulation would be a costly endeavor, as the amount of dry product per plant (ranging between 0.195g and 0.302g on average) greatly exceeds that used for soaking or drenching. However, with further research and development of such formulations an affordable and marketable product can likely be developed.

It also showed the value in selecting *Trichoderma* isolates that are adapted to the specific niches and are able to survive and proliferate endophytically and in the rhizosphere of *Vitis* spp., while stressing the importance of field evaluation of prospective BCAs. Further research is necessary to evaluate the efficacy of these *Trichoderma* isolates, and combinations thereof, on other rootstocks cultivars used in South Africa by applying the dry product dip technique. It is suggested that growth parameters and incidence of both *Trichoderma* spp. and black foot, as well as other major GTDs needs to be assessed several years after vines have been transplanted into vineyards, taking into account the time it takes for *Trichoderma* to show efficacy. How long *Trichoderma* would remain in nursery vines post vineyard transplant also remains a question to be answered. In the case of hot water treatment of dormant nursery vines, the resident *Trichoderma* spp. would be eliminated. Therefore the application of *Trichoderma* spp. pre-planting in vineyards and the effect thereof on growth and disease suppression needs to be investigated.

The two fields trials showed that *Trichoderma* spp., even after more effective colonization, were not sufficient to prevent infections by BFD pathogens. A certain degree of protection was obtained in the base of the vines for one season. This can likely be attributed to the strong growth characteristics of the rootstock that was used in the trials that suppresses the effects of *Trichoderma*. Furthermore, the majority of BFD pathogens were of

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the *Campylocarpon* genus, which was found to be less sensitive to *Trichoderma* spp. *in vitro*.

Successful crop management is a multifaceted process, and despite the bias towards the use of biological control agents alone, integrated disease and crop management should not be neglected. *Trichoderma* offers promising prospective as part of such a strategy where it should be used as a component together with HWT, chemical and cultural measures. Its synergistic action of different biocontrol mechanisms and ability to colonize saprotrophic and endophytic niches make it an ideal candidate as BCA. Combining existing knowledge of *Trichoderma* as BCA with the knowledge obtained from this research will assist in optimizing the application procedure in nurseries and the sustainability of the South African grapevine industry as well as worldwide.

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TABLES AND FIGURES

Table 1. List of *Trichoderma* products and isolates used in the current study.

Product name	Product number[1]	Company	Species (commercial isolate)	Isolate number[2]	CFU.g^{-1}
Awegenic Tri-cure™	P7	MBFi	*Trichoderma harzianum* (MIT04)	T10	2×10^{9}
Bio-Tricho	P4	Agro-Organics	*Trichoderma atroviride* (Vitic 2) *Trichoderma harzianum* (Sp)	T4 T5	1.2×10^{7}
Eco 77®	P3	Plant Health Products	*Trichoderma atroviride* (MIT04)	T3	2×10^{9}
Excalibur Gold™	P6	Advanced Biological Marketing®	*Trichoderma harzianum* (K2) *Trichoderma atroviride* (K4) *Trichoderma virens* (K1)	T7 T8 T9	2×10^{8}
TrichoPlus™	P5	BASF	*Trichoderma fertile*	T6	2×10^{9}
Trichoflow™[3]	P8	Agrimm Technologies®	*Trichoderma harzianum*	–	1×10^{8}
USPP-MT1	P2	Stellenbosch University	*Trichoderma atroviride* (M1)	T1	2×10^{8}
USPP-T1	P1	Stellenbosch University	*Trichoderma atroviride*	T2	2×10^{8}

[1] Product numbers used in the field trial.

[2] Isolates numbers used in the *in vitro* assay.

[3] Product from New Zealand.

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Table 2. List of black foot disease isolates from *Vitis* spp. used in the *in vitro* evaluation.

	Accession number				
Fungal species	**STE-U[1]**	**Other**	**Location**	**Isolation date**	**Collector**
Campylocarpon fasciculare	8691	SL26-15	Nursery 1, Wellington, South Africa	2015	S. Langenhoven
	8692	SL86-15	Nursery 2, Wellington, South Africa	2015	S. Langenhoven
	8693	SL24-15	Nursery 1, Wellington, South Africa	2015	S. Langenhoven
Campylocarpon pseudofasciculare	8694	SL88-15	Nursery 2, Wellington, South Africa	2015	S. Langenhoven
	8280	FH-C397	Nursery 3, Wellington, South Africa	2013	F. Halleen
	8279	FH-C558	Nursery 3, Wellington, South Africa	2013	F. Halleen
Dactylonectria macrodidyma	8264	FH-C106	Farm 1, Paarl, South Africa	2011	F. Halleen
	8265	FH-C241	Nursery 4, Wellington, South Africa	2012	F. Halleen
	8702	SL94-15	Nursery 2, Wellington, South Africa	2015	S. Langenhoven
Ilyonectria liriodendri	8267	FH-C226	Nursery 2, Wellington, South Africa	2012	F. Halleen
	8266	FH-C204	Farm 2, Wellington, South Africa	2012	F. Halleen
	8699	SL110	Nursery 4, Wellington, South Africa	2013	S. Langenhoven

[1] STE-U: Department of Plant Pathology, Stellenbosch University.

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Table 3. Genus- and species-specific primer sets used to identify the genera *Campylocarpon* and *Dactylonectria* and *Ilyonectria liriodendri*.

Primer	Detection	Sequence (5' – 3')	Tm (°C)	Gene amplified	Amplicon size	Reference
CamF	*Campylocarpon* spp.	AGAAAGGATTCCGCTCTGAGTAA	53.49	ITS	330-bp	Current study
CamR		TCCGGTGCGAGGTGCAG	54.29			Current study
YT2F	*Campylocarpon*, *Dactylonectria*,	GATGAAGAACGCAGCGAAAT	49.73	β-tubulin	250-bp	Tewoldemedhin *et al*., 2011
CylR	*Ilyonectria* spp.	TGTGCTACTACGCAGAGGAA	51.78			Dubrovsky and Fabritius, 2007
CyliF1	*Ilyonectria liriodendri*	CTCCTCTTCAACGATCCGACGTGCC	63.00	β-tubulin	192-bp	Mostert *et al*., 2010
CyliR1		GGGGCAGAGCAGATTTCG	56.20			Mostert *et al*., 2010

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Table 4. List of fungal isolates used in specificity testing of specific primers.

	Accession number				
Fungal species	**STE-U**	**Other**	**Location**	**Isolation date**	**Collector**
Campylocarpon fasciculare	8691	SL26-15	Nursery 1, Wellington, South Africa	2015	S. Langenhoven
Ca. pseudofasciculare	8694	SL88-15	Nursery 2, Wellington, South Africa	2015	S. Langenhoven
Dactylonectria alcacerensis	8710	SL74	Nursery 3, Wellington, South Africa	2013	S. Langenhoven
D. macrodidyma	8264	FH-C106	Farm 1, Paarl, South Africa	2011	F. Halleen
D. novozelandica	8709	SL73	Nursery 3, Wellington, South Africa	2013	S. Langenhoven
D. pauciseptata	8707	SL14-325	Nursery 4, Wellington, South Africa	2014	S. Langenhoven
D. torresensis	8704	SL159-15	Nursery 3, Wellington, South Africa	2015	S. Langenhoven
Ilyonectria liriodendri	8267	FH-C226	Nursery 2, Wellington, South Africa	2012	F. Halleen
Diplodia seriata	7035	–	Rawsonville, South Africa	2007	F. Halleen
Fusarium solani[1]	7214	–	Vyeboom, South Africa	2006	Y. Tewoldemedhin
Phaeoacremonium minimum	8272	–	Wellington, South Africa	2012	M. Baloyi
Phaeomoniella chlamydospora	8276	–	Farm 2, Paarl, South Africa	2002	L. Mostert
Phytophthora niederhauserii	6972	–	Ashton, South Africa	2009	C. Spies
Pythium irregulare	6799	–	Vredendal, South Africa	2006	C. Spies

[1] All the isolates were from *Vitis* spp. except for *F. solani* that was isolated from *Malus* sp.

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Table 5. Mean percentage mycelial growth inhibition of four species of black foot pathogens by volatile organic compounds produced by different *Trichoderma* isolates.

	Campylocarpon fasciculare			***Campylocarpon pseudofasciculare***			***Dactylonectria macrodidyma***			***Ilyonectria liriodendri***		
	8691	**8692**	**8693**	**8694**	**8280**	**8279**	**8264**	**8265**	**8702**	**8267**	**8266**	**8699**
T1	27.82ab[1]	33.80ab	23.23b-e	28.64a	15.75a	15.66a	55.27b	54.32a	66.94a	14.55a	15.77ab	21.89a
T2	23.87a-c	32.92ab	26.93bc	29.39a	11.84ab	10.41ab	37.69c	54.35a	57.98ab	-4.17b-d	10.57bc	14.46ab
T3	28.51a	33.87ab	30.81b	27.05a	11.28ab	12.10a	60.17ab	49.72a	40.97cd	5.68a-c	15.08ab	23.30a
T4	19.00b-d	23.53a-d	18.73c-e	21.31ab	3.62bc	2.57bc	60.68ab	53.59a	51.37bc	-10.95d	7.95bc	4.74bc
T5	24.71a-c	24.13a-c	22.10c-e	21.27ab	6.11bc	2.74bc	64.21a	58.31a	61.40ab	-10.74cd	7.81bc	3.59c
T6	6.28e	7.04e	9.03f	8.24c	-1.58c	-1.47c	8.31e	8.78c	21.76ef	-15.28d	4.38cd	-0.64c
T7	17.62cd	22.34b-d	24.69b-d	16.44bc	7.51a-c	9.72ab	33.08c	33.32b	34.16de	-7.24b-d	6.72c	17.29a
T8	28.59a	36.08a	39.61a	13.28bc	10.88ab	-2.49c	36.58c	37.58b	57.17ab	6.31ab	21.79a	23.85a
T9	12.64de	11.90c-e	18.58de	-2.56d	-1.15c	2.82bc	21.54d	14.10c	18.45f	-18.58d	-1.62d	-4.09c
T10	11.71de	10.60de	15.76ef	9.90c	6.38bc	3.90bc	18.36d	31.36b	30.53d-f	-5.18b-d	3.77cd	2.93c
LSD[2]	9.3835	13.298	8.3083	9.4297	9.2305	8.0472	8.1437	9.8068	14.061	16.545	8.0259	9.925

[1] Values within each column followed by the same do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 6. Mean percentage mycelial growth inhibition of four species of black foot pathogens by diffusible antibiotic compounds produced by different *Trichoderma* isolates.

	Campylocarpon fasciculare			***Campylocarpon pseudofasciculare***			***Dactylonectria macrodidyma***			***Ilyonectria liriodendri***		
	8691	**8692**	**8693**	**8694**	**8280**	**8279**	**8264**	**8265**	**8702**	**8267**	**8266**	**8699**
T1	47.98a[1]	53.82a	52.75a	46.07c	30.65b	36.38a	100.00a	100.00a	100.00a	35.92a	70.70a	38.29b
T2	16.41c	22.28bc	19.60b	35.73d	17.37c	18.66bc	46.09b	43.92b	47.08c	19.13bc	25.55b	16.38de
T3	30.46b	20.63b-d	18.21bc	16.40f	17.43c	13.64cd	28.99cd	35.69bc	82.56b	9.47d	28.68b	16.35de
T4	19.96c	19.93cd	13.02bc	7.93fg	10.50d	3.73d	24.99c-e	12.42ef	26.81d	10.03cd	15.27bc	8.40ef
T5	20.55c	14.58c-e	18.62bc	26.13e	14.39cd	13.22cd	34.50bc	21.90de	23.65de	24.16b	24.86b	13.82d-f
T6	0.90e	-6.28f	-14.07e	2.57g	8.20d	3.64d	15.11e	13.01ef	16.75ef	2.52d	-1.13c	4.74f
T7	3.60de	4.97ef	-2.83de	2.69g	9.02d	4.02d	18.17de	9.90f	10.13f	6.63d	3.13c	8.16ef
T8	35.37b	48.44a	39.66a	100.00a	33.06b	29.88ab	96.36a	100.00a	94.52a	37.99a	57.46a	65.51a
T9	39.58ab	33.84b	52.01a	76.71b	97.77a	25.17ab	99.07a	29.93cd	53.22c	19.55b	22.99b	22.51cd
T10	11.45cd	8.67de	4.34cd	14.50f	13.71cd	13.86cd	21.08de	39.61bc	20.05de	19.17bc	14.74bc	25.97c
LSD[2]	9.3704	13.332	14.688	8.6848	6.337	11.296	11.671	11.265	9.7408	9.4994	16.808	9.0823

[1] Values within each column followed by the same do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 7. Macroscopic interactions between duel-plated cultures of *Trichoderma* isolates and the respective black foot pathogens.

	Campylocarpon fasciculare			***Campylocarpon pseudofasciculare***			***Dactylonectria macrodidyma***			***Ilyonectria liriodendri***		
	8691	**8692**	**8693**	**8694**	**8280**	**8279**	**8264**	**8265**	**8702**	**8267**	**8266**	**8699**
T1	OGS[1]	OGS	OGS	OGS	SG	IZ	OGS	OGS	OGS	SG	SG	IZ
T2	OG	OG	OG	OG	PO	SG	PO	OG	OG	SG	PO	PO
T3	OGS	OGS	OGS	OGS	IZ	IZ	OGS	OGS	OGS	SG	IZ	SG
T4	OGS	OGS	OGS	OGS	IZ	SG	OGS	OGS	OGS	SG	IZ	SG
T5	OGS	OGS	OGS	OGS	SG	SG	OGS	OG	OGS	SG	IZ	SG
T6	POS	POS	POS	POS	IZ	IZ	OGS	POS	OGS	SG	IZ	SG
T7	OGS	OGS	OGS	OGS	OGS	OGS	OGS	POS	OGS	POS	OGS	POS
T8	OGS	OGS	OGS	OGS	SG	SG	OGS	OGS	OGS	SG	SG	SG
T9	OG	OG	OG	SG	SG	SG	OGS	OG	OGS	SG	IZ	SG
T10	OGS	OGS	OGS	OGS	IZ	IZ	OGS	OGS	OGS	SG	SG	SG

[1] PO = Partial overgrowth of the pathogen by *Trichoderma*; POS = Partial overgrowth of the pathogen by *Trichoderma* and sporulation thereof; OG = Overgrowth of the pathogen by *Trichoderma*; OGS = Overgrowth of the pathogen by *Trichoderma* and sporulation thereof; IZ = Inhibition zone formed between the two cultures; SG = Growth of both cultures stopped when in contact.

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Table 8. The microscopic interactions observed at 400× magnification for each *Trichoderma* isolate when co-inoculated with black foot pathogens.

***Trichoderma* isolate**	**Microscopic interaction[1]**
T1	HA, SM, HD[1]
T2	HA, HC, HD
T3	HA, HD
T4	HA, HC, SM
T5	HA, SM
T6	HA, HC, HD
T7	HA, HD
T8	HA, HD
T9	HD
T10	HA, HD

[1] HA = Adhesion of *Trichoderma*- to pathogen hyphae; HC = Coiling of *Trichoderma*- around pathogen hyphae; HD = Disintegration of pathogen hyphae; SM = Swelling and malformation of pathogen hyphae.

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Table 9. The severity of *Trichoderma* in dormant rootstock shoots at different depths following different periods of soaking in *Trichoderma atroviride* (T2) spore suspensions.

	***Trichoderma* severity following different periods of soaking (mean %)[1]**				
Depth in shoot	**1 min**	**10 min**	**60 min**	**120 min**	**Control**
2 cm	24.89[2] (-1.10ab)[3]	35.49 (-0.60a)	18.76 (-1.47abcde)	21.48 (-1.30abc)	0.50 (-5.30j)
4 cm	6.40 (-2.68fghi)	34.79 (-0.63a)	8.56 (-2.37efgh)	9.84 (-2.22defgh)	0.50 (-5.30j)
6 cm	16.59 (-1.62bcde)	13.04 (-1.90bcdef)	15.22 (-1.72bcde)	19.48 (-1.42abcd)	0.50 (-5.30j)
8 cm	11.73 (-2.02cdefg)	10.48 (-2.15cdefgh)	3.28 (-3.38i)	14.03 (-1.81bcdef)	0.50 (-5.30j)
10 cm	4.81 (-2.98hi)	6.70 (-2.63fghi)	5.37 (-2.87ghi)	20.77 (-1.34abcd)	0.50 (-5.30j)

[1] Values followed by the same letter do not differ significantly.

[2] Back transformed mean *Trichoderma* severity.

[3] Log transformed mean *Trichoderma* severity; LSD = 0.9105 (P = 0.05).

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Table 10. The severity of *Trichoderma* in the xylem and pith elements in the dormant rootstock shoot assay.

Tissue	**Mean *Trichoderma* severity (%)**[1]
Xylem	8.37[2] (-2.39a)[3]
Pith	5.82 (-2.78b)

[1] Values followed by the same letter do not differ significantly.

[2] Back transformed mean *Trichoderma* severity

[3] Log transformed mean *Trichoderma* severity; LSD = 0.276 (*P* = 0.05).

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Table 11. The incidence of *Trichoderma* in different tissue groups in the field trial evaluating different methods of application over two seasons.

Treatment	Incidence in 2016/17 (mean %)		Incidence in 2017/18 (mean %)	
	Basal end	**Roots**	**Basal end**	**Roots**
i. Coating of basal ends with dry product	50.40a[1]	32.53a	16.40bc	4.53bc
ii. Dry product coating followed by monthly soil drenches	39.20ab	26.40ab	28.00a	15.73a
iii. HWT at 50°C for 30 min and dry product coating	30.80bc	20.00b	20.40ab	12.00a
iv. Monthly soil drenches for 6 months	19.20cd	8.80c	9.60cd	7.47b
v. Soaking for 1 hr in conidial suspension	8.80de	5.87c	4.00d	2.40c
vi. HWT at 50°C for 30 min and soaking for 1 hr	9.60de	4.80c	–	–
vii. HWT at 50°C for 45 min and soaking for 1 hr	–	–	3.60d	2.12c
viii. HWT at 50°C for 30 min	–	–	1.20d	1.60c
ix. HWT at 50°C for 45 min	–	–	1.20d	1.33c
x. Untreated control	6.40e	5.87c	2.00d	1.33c
LSD[2]	12.347	8.9643	10.549	4.0774

[1] Values followed by the same letter do not differ significantly within a column.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 12. The percentage incidence of *Trichoderma* and BFD pathogens in different tissue types in the field trial evaluating different methods of application over two seasons.

		Incidence in 2016/17 (mean %)		Incidence in 2017/18 (mean %)	
Tissue		***Trichoderma***	**BFD pathogens**	***Trichoderma***	**BFD pathogens**
Base	Xylem	25.60a[1]	5.49c	9.69a	11.47c
	Pith	21.37b	2.97c	9.51a	6.58d
Root	Attachments	20.91b	17.83b	6.84b	16.62b
	Central roots	10.29c	20.11ab	3.20c	15.55b
	Root tips	13.49c	21.14a	6.13b	24.00a
LSD[2]		3.7691	3.0224	2.5400	3.5441

[1] Values within each column followed by the same letter do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 13. The percentage of certifiable vines determined as the total number of grafted cuttings planted in the field trial evaluating different *Trichoderma*-based products over both season.

	Certifiable vines (mean %)	
Product	**2016/17**	**2017/18**
Product 1	79.50ab[1]	82.25a
Product 2	72.00abcd	77.75ab
Product 3	81.75a	75.25abc
Product 4	69.50bcd	83.75a
Product 5	63.75d	68.25bc
Product 6	76.25abc	76.75ab
Product 7	66.50dc	62.75c
Product 8	–	65.25bc
Untreated control	73.25abcd	77.25ab
LSD[2]	11.487	12.737

[1] Values within each column followed by the same letter do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

Table 14. Mean root mass in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Product	**Average wet root mass (g)**
Product 1	23.97bc[1]
Product 2	27.37ab
Product 3	24.57bc
Product 4	26.00ab
Product 5	25.60abc
Product 6	20.98c
Product 7	28.53ab
Untreated control	29.50a

[1] Values followed by the same letter do not differ significantly (P = 0.05; LSD = 4.7947).

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Table 15. The incidence of *Trichoderma* in different tissue groups in the field trial evaluating different *Trichoderma*-based products over two seasons.

	Incidence in 2016/17 (mean %)		Incidence in 2017/18 (mean %)	
Product	**Basal end**	**Roots**	**Basal end**	**Roots**
Product 1	38.50b[1]	19.33bc	29.00abc	15.67abc
Product 2	40.50b	23.67b	26.50bc	12.00cd
Product 3	47.50ab	26.33b	40.50ab	22.00a
Product 4	5.00d	6.00d	1.5d	4.67de
Product 5	58.50a	37.67a	43.50a	20.33ab
Product 6	11.50cd	7.67d	17.00cd	5.67de
Product 7	22.00c	12.00cd	20.00c	12.00cd
Product 8	–	–	23.50c	13.67bc
Untreated control	6.00cd	4.00d	1.5d	4.00e
LSD[2]	16.114	10.424	16.175	7.3407

[1] Values within each column followed by the same letter do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 16. The incidence of BFD pathogens in the basal ends of the field trial evaluating different *Trichoderma*-based products over two seasons.

	BFD pathogen incidence in basal end (mean %)[1]	
Product	**2016/17 season**	**2017/18 season**
Product 1	1.50b[1]	4.50bc
Product 2	1.00b	4.00bc
Product 3	1.50b	5.00bc
Product 4	1.00b	11.00a
Product 5	2.00b	3.00bc
Product 6	2.50b	8.50ab
Product 7	2.00b	2.50c
Product 8	–	11.00a
Untreated control	6.50a	7.50abc
LSD[2]	3.0273	5.7491

[1] Values within each column followed by the same do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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Table 17. The percentage incidence of *Trichoderma* and BFD pathogens in different tissue types in the field trial evaluating different *Trichoderma*-based products over two seasons.

		Incidence in 2016/17 (mean %)		Incidence in 2017/18 (mean %)	
Tissue		***Trichoderma***	**BFD pathogens**	***Trichoderma***	**BFD pathogens**
Base	Xylem	29.25a[1]	3.13c	21.00ab	9.22c
	Pith	28.13ab	1.38c	24.11a	3.44d
Root	Attachments	25.00b	18.63a	18.33b	15.56b
	Central roots	13.88c	14.00b	7.22d	16.67b
	Root tips	12.38c	19.00a	11.11c	28.22a
LSD[2]		3.7728	3.2503	3.7768	3.5545

[1] Values within each column followed by the same letter do not differ significantly.

[2] Least significant differences of each column indicated in the bottom row (P = 0.05).

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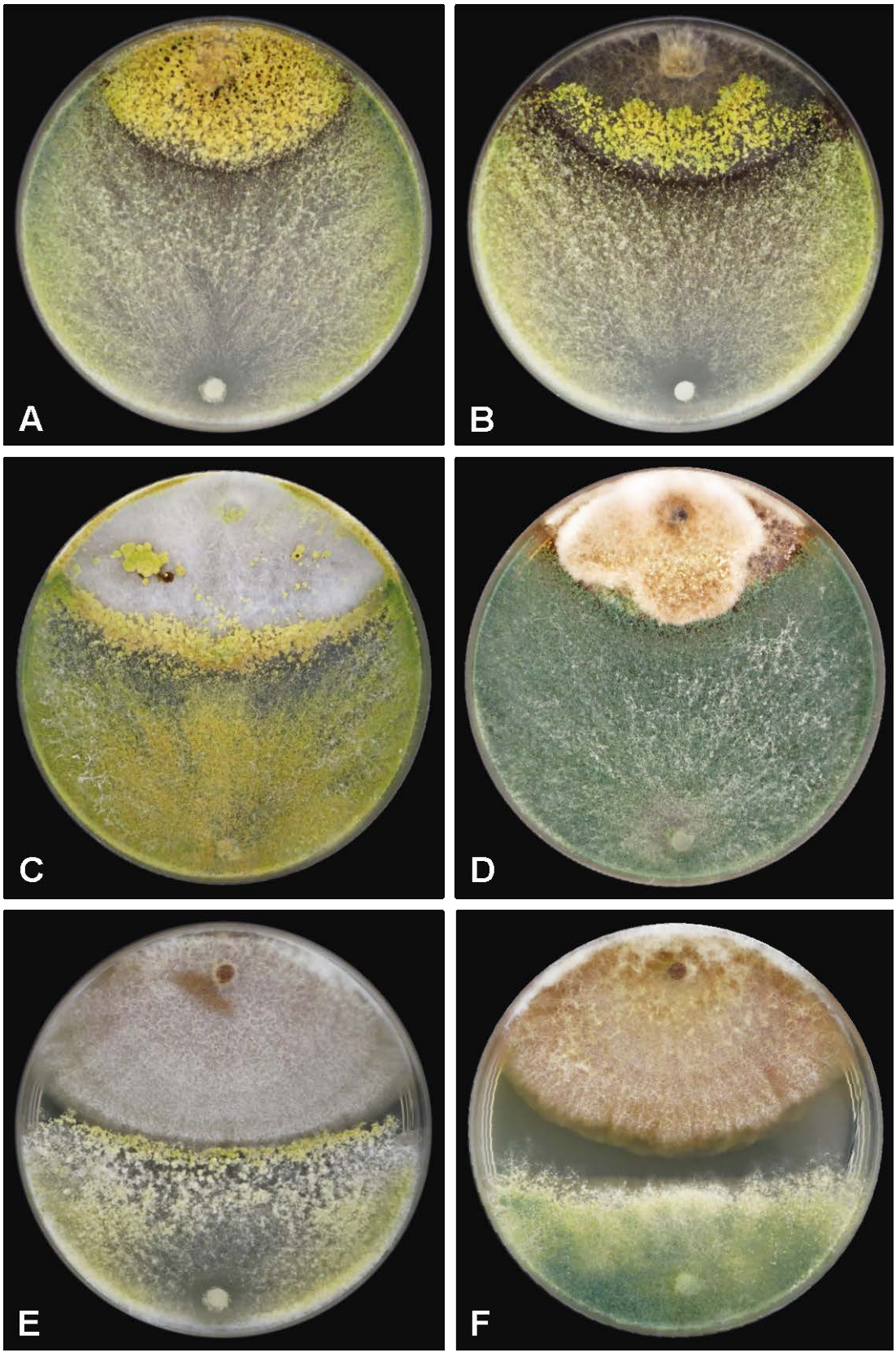

Figure 1. Macroscopic hyphal interactions observed on PDA medium. A) Overgrowth and sporulation of *T. atroviride* over *Ca. fasciculare*; B) partial overgrowth and sporulation of *T. fertile* over *Ca. fasciculare*; C) overgrowth with little to no sporulation of *T. harzianum* over *D. macrodidyma*; D) partial overgrowth with little to no sporulation of *T. fertile* over *Ca. pseudofasciculare*; E) arrested growth between *T. atroviride* and *I. liriodendri* and F) an inhibition zone between *T. fertile* and *Ca. pseudofasciculare.*

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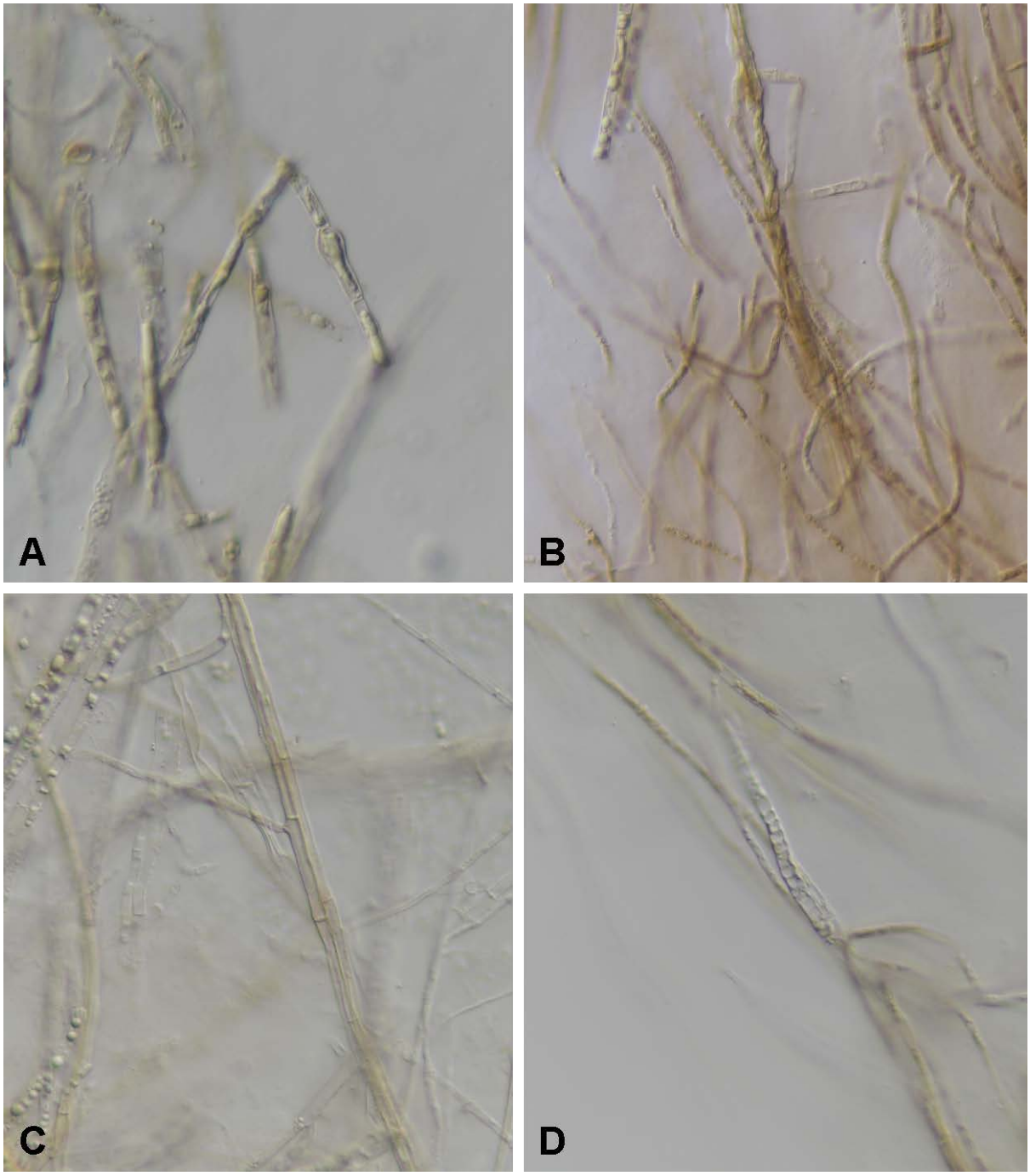

Figure 2. Microscopic interactions as observed at 400× magnification. A) Hyphal swelling of *Ca. fasciculare* by *T. atroviride*; B) coiling of *T. fertile* hyphae around hyphae of *Ca. fasciculare*; C) hyphal adhesion between *T. harzianum* and *Ca. fasciculare* and D) hyphal disintegration of *Ca. fasciculare* by *T. atroviride*.

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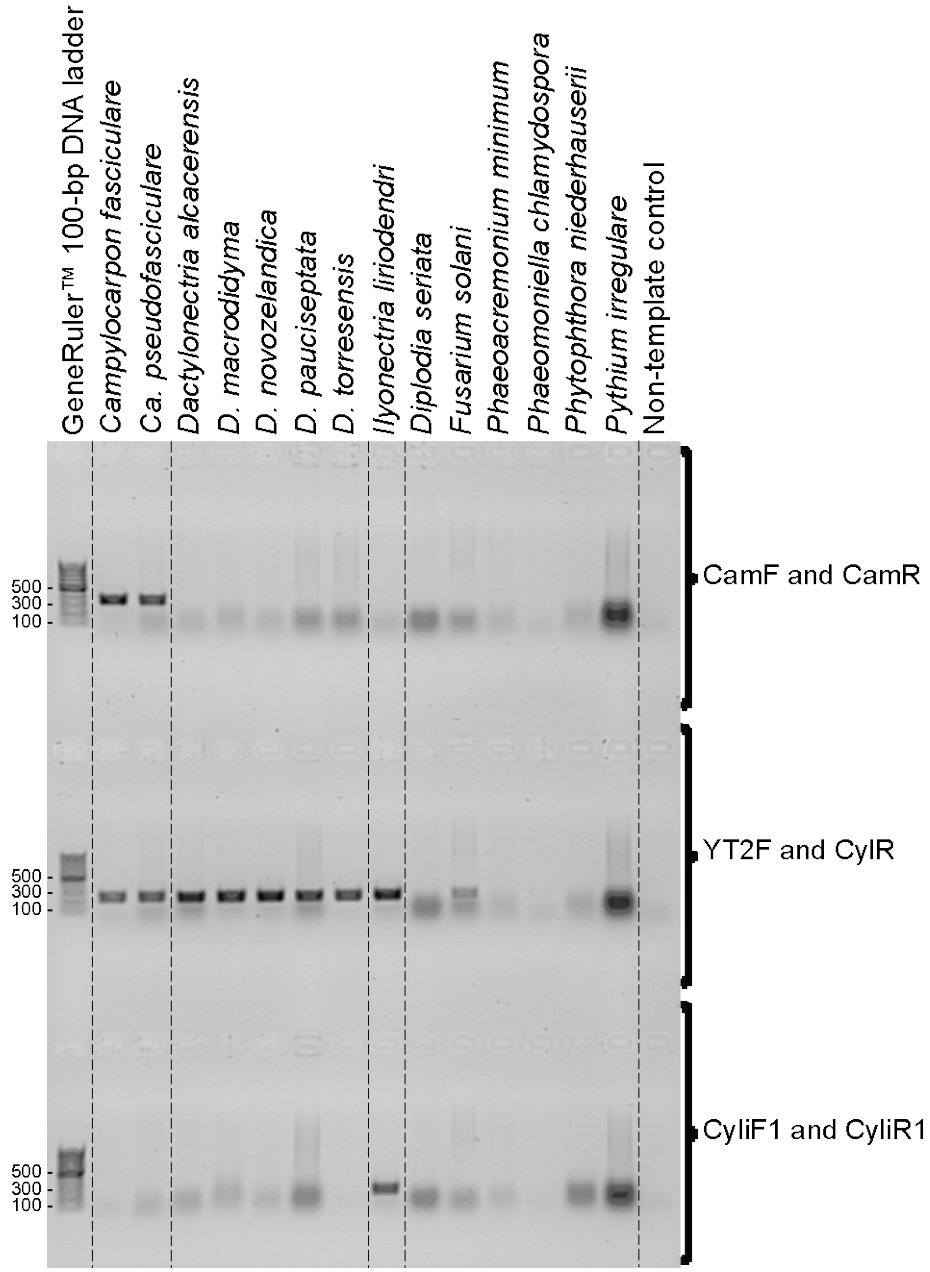

Figure 3. PCR amplification of 330-bp *Campylocarpon* species fragments using the primer set CamF/CamR (row 1), 250-bp *Dactylonectria* and *Ilyonectria* spp. fragments using the primer set YT2F/CylR (row 2) and a 192-bp *Ilyonectria liriodendri* fragment using the primer set CyliF1/CyliR1 (row 3). Lane 1 represents GeneRuler™ 100-bp DNA ladder, lane 2 and 3 *Campylocarpon* spp, 4 to 8 *Dactylonectria* spp., lane 9 *Ilyonectria liriodendri*, lane 10 to 15 negative controls and lane 16 a non-template control.

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APPENDIX A

Table 1. Analysis of variance for the *in vitro* screening of volatile organic compounds by *Trichoderma* against BFD pathogens.

Source of variation	Degrees of freedom	*F*-value	*P*-value
Pathogen	11	167.50	<0.0001
Trichoderma	9	77.07	<0.0001
Pathogen×Trichoderma	99	4.35	<0.0001

Table 2. Analysis of variance for the *in vitro* screening diffusible antifungal compounds by *Trichoderma* against BFD pathogens.

Source of variation	Degrees of freedom	*F*-value	*P*-value
Pathogen	11	112.60	<0.0001
Trichoderma	9	362.02	<0.0001
Pathogen×Trichoderma	99	12.80	<0.0001

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Table 3. Analysis of variance of the severity of *Trichoderma atroviride* in the dormant rootstock shoot assay.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Cultivar	4	2.38	0.0978
SoakingTime	4	12.95	<0.0001
SoakingTime×Cultivar	16	0.32	0.9843
Depth	4	9.11	<.0001
Depth×Cultivar	16	1.51	0.1272
Depth×SoakingTime	16	3.09	0.0008
Depth×SoakingTime×Cultivar	64	1.19	0.2526
Tissue	1	7.95	0.0061
Tissue×Cultivar	4	1.07	0.3781
Tissue×SoakingTime	4	1.17	0.3297
Tissue×SoakingTime×Cultivar	16	0.53	0.9242
Tissue×Depth	4	0.63	0.6450
Tissue×Depth×Cultivar	16	0.73	0.7563
Tissue×Depth×SoakingTime	16	0.49	0.9431
Tissue×Depth×SoakingTime×Cultivar	64	0.64	0.9648

Table 4. Analysis of variance of the *Trichoderma* incidence in different tissue groups in the field trial evaluating different methods of application in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	0.90	0.4788
Treatment	6	19.39	<0.0001
Tissuegroup	1	42.39	<0.0001
Treat×Tissuegroup	6	3.05	0.0200

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Table 5. Analysis of variance of the BFD incidence in different tissue groups in the field trial evaluating different methods of application in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	0.15	0.9610
Treatment	6	1.03	0.4295
Tissuegroup	1	368.55	<0.0001
Treat×Tissuegroup	6	1.56	0.1958

Table 6. Analysis of variance of the *Trichoderma* incidence in different tissue groups in the field trial evaluating different methods of application in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	1.66	0.1845
Treatment	8	11.32	<0.0001
Tissuegroup	1	13.04	0.0009
Treat×Tissuegroup	8	2.17	0.0535

Table 7. Analysis of variance of the BFD incidence in different tissue groups in the field trial evaluating different methods of application in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	2.25	0.0857
Treatment	8	0.54	0.8212
Tissuegroup	1	53.22	<0.0001
Treat×Tissuegroup	8	0.46	0.8727

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Table 8. Analysis of variance of the *Trichoderma* incidence in different tissue groups in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	1.59	0.2218
Treatment	7	15.04	<0.0001
Tissuegroup	1	66.43	<0.0001
Treat×Tissuegroup	7	5.07	0.0012

Table 9. Analysis of variance of the BFD incidence in different tissue groups in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.96	0.4302
Treatment	7	0.45	0.8591
Tissuegroup	1	150.04	<0.0001
Treat×Tissuegroup	7	0.64	0.7167

Table 10. Analysis of variance of the *Trichoderma* incidence in different tissue groups in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.94	0.4345
Treatment	8	8.04	<0.0001
Tissuegroup	1	48.95	<0.0001
Treat×Tissuegroup	8	3.89	0.0036

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Table 11. Analysis of variance of the BFD incidence in different tissue groups in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.13	0.9386
Treatment	8	1.36	0.2628
Tissuegroup	1	102.91	<0.0001
Treat×Tissuegroup	8	0.57	0.7915

Table 12. Analysis of variance of the *Trichoderma* incidence in the basal ends in the field trial evaluating different methods of application in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	0.65	0.6306
Treatment	6	16.24	<0.0001

Table 13. Analysis of variance of the *Trichoderma* incidence in the roots in the field trial evaluating different methods of application in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	1.02	0.4175
Treatment	6	13.63	<0.0001

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Table 14. Analysis of variance of the *Trichoderma* incidence in the basal ends in the field trial evaluating different methods of application in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	1.87	0.1404
Treatment	8	7.16	<0.0001

Table 15. Analysis of variance of the *Trichoderma* incidence in the roots in the field trial evaluating different methods of application in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	4	0.43	0.7860
Treatment	8	13.89	<0.0001

Table 16. Analysis of variance of certifiable vines in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.83	0.4903
Treatment	7	2.53	0.0469

Table 17. Analysis of variance of certifiable vines in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.96	0.4254
Treatment	8	2.83	0.0229

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Table 18. Analysis of variance of the mean wet root mass in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	5.88	0.0045
Treatment	7	2.78	0.0326

Table 19. Analysis of variance of the *Trichoderma* incidence in the basal ends in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	1.14	0.3572
Treatment	7	13.77	<0.0001

Table 20. Analysis of variance of the *Trichoderma* incidence in the roots in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	1.93	0.1562
Treatment	7	10.99	<0.0001

Table 21. Analysis of variance of the *Trichoderma* incidence in the basal ends in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	1.21	0.3276
Treatment	8	7.09	<0.0001

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Table 22. Analysis of variance of the Trichoderma incidence in the roots in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.41	0.7482
Treatment	8	6.78	<0.0001

Table 23. Analysis of variance of the BFD incidence in the basal ends in the field trial evaluating different *Trichoderma*-based products in the 2016/17 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.24	0.8703
Treatment	7	3.03	0.0228

Table 24. Analysis of variance of the BFD incidence in the basal ends in the field trial evaluating different *Trichoderma*-based products in the 2017/18 season.

Source of variation	Degrees of Freedom	*F*-value	*P*-value
Block	3	0.51	0.6819
Treatment	8	2.77	0.0253

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Table 25. BLAST results of isolates of which the PCR products were sequenced to confirm their identification.

Isolate number	Species identification	Reference strain number	Primer pair	Identity (%)	Length of query/ sequence	Qyery cover (%)[5]	Accession number (GenbBank ref)
715	*Campylocarpon fasciculare*	CBS 112600	CamF/CamR[1]	100	329	100	AY677298.1
591	*Ca. fasciculare*	CBS 112600	CamF/CamR	100	322	100	AY677298.1
387	*Ca. fasciculare*	CBS 112600	CamF/CamR	100	324	99	AY677298.1
374	*Ca. fasciculare*	CBS 113559	CamF/CamR	100	331	99	AY677303.1
306	*Ca. fasciculare*	CBS 112600	CamF/CamR	100	331	99	AY677298.1
301	*Ca. fasciculare*	CBS 113559	CamF/CamR	100	278	100	AY677303.1
41	*Ca. fasciculare*	CBS 113559	CamF/CamR	100	329	100	AY677303.1
318	*Ca. fasciculare*	CBS 112600	CamF/CamR	98	265	97	AY677298.1
1398	*Dactylonectria macrodidyma*/	CBS 112601	Yt2F/CylR[2]	100	231	100	MH862898.1
	Dactylonectria torresensis	CBS 129086		100	231	100	MH865183.1
1058	*D. macrodidyma*/	CBS 112601	Yt2F/CylR	100	231	99	MH862898.1
	D. torresensis	CBS 129086		100	231	99	MH865183.1
1013	*D. macrodidyma*/	CBS 112601	Yt2F/CylR	100	230	100	MH862898.1
	D. torresensis	CBS 129086		100	230	100	MH865183.1
512	*D. macrodidyma*/	CBS 112601	Yt2F/CylR	100	231	100	MH862898.1
	D. torresensis	CBS 129086		100	231	100	MH865183.1
267	*D. macrodidyma*/	CBS 112601	Yt2F/CylR	100	231	100	MH862898.1
	D. torresensis	CBS 129086		100	231	100	MH865183.1

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Table 25. Continued.

Isolate number	Species identification	Reference strain number	Primer pair	Identity (%)	Length of query/ sequence	Qyery cover (%)	Accession number (GenbBank ref)
220	*D. macrodidyma/*	CBS 112601	Yt2F/CylR	100	183	100	MH862898.1
	D. torresensis	CBS 129086		100	183	100	MH865183.1
104	*D. macrodidyma/*	CBS 112601	Yt2F/CylR	100	231	99	MH862898.1
	D. torresensis	CBS 129086		100	231	99	MH865183.1
78	*D. macrodidyma/*	CBS 112601	Yt2F/CylR	100	232	99	MH862898.1
	D. torresensis	CBS 129086		100	232	99	MH865183.1
308	*Ilyonectria liriodendri*	BF74	CyliF1/CyliR1[3]	100	197	100	KX778701.1
44	*I. liriodendri*	BF74	CyliF1/CyliR1	100	194	99	KX778701.1
1441	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R[4]	99	519	100	JF735502.1
1436	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	516	100	JF735502.1
1414	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	522	99	JF735502.1
1408	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	522	99	JF735502.1
1402	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	484	100	JF735502.1
1380	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	520	99	JF735502.1
1377	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	520	99	JF735502.1
1371	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	519	99	JF735502.1
1363	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	505	100	JF735502.1

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Table 25. Continued.

Isolate number	Species identification	Reference strain number	Primer pair	Identity (%)	Length of query/ sequence	Qyery cover (%)	Accession number (GenbBank ref)
1360	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	519	100	JF735502.1
1176	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	520	99	JF735502.1
1115	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	519	100	JF735502.1
1110	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	516	100	JF735502.1
822	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	521	99	JF735502.1
716	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	523	99	JF735502.1
696	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	525	98	JF735502.1
678	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	453	100	JF735502.1
347	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	517	100	JF735502.1
293	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	520	99	JF735502.1
247	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	518	100	JF735502.1
210	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	519	100	JF735502.1
162	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	510	100	JF735502.1
131	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	519	100	JF735502.1
87	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	521	99	JF735502.1
20	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	100	509	100	JF735502.1

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Table 25. Continued.

Isolate number	Species identification	Reference strain number	Primer pair	Identity (%)	Length of query/ sequence	Qyery cover (%)	Accession number (GenbBank ref)
958	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	390	100	JF735502.1
1063	*Ca. fasciculare*	CBS 112613	CYLH3-F/ CYLH3-R	99	482	100	JF735502.1
1136	*D. macrodidyma*	CBS 112605	CYLH3-F/ CYLH3-R	100	499	100	JF735646.1
132	*D. macrodidyma*	CBS 112594	CYLH3-F/ CYLH3-R	100	505	98	JF735643.1
448	*Dactylonectria novozelandica*	CBS 112608	CYLH3-F/ CYLH3-R	100	498	100	JF735632.1
125	*D. novozelandica*	CBS 112608	CYLH3-F/ CYLH3-R	99	497	100	JF735632.1
115	*D. novozelandica*	CBS 112608	CYLH3-F/ CYLH3-R	100	459	100	JF735632.1

[1] Internal transcribed spacer 1 and internal transcribed spacer 2 (ITS) sequenced for isolates that ampliefied with CamF/CamR.

[2] β-tubulin (btub) gene region sequenced for isolates that amplified with YT2F/CylR.

[3] β-tubulin (btub) gene region sequenced for isolates that amplified with CyliF1/CyliR1.

[4] Histone H3 gene region sequenced for isolates that did not amplify with the latter primer pairs.

[5] 0% gaps for all queries.